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Target and Non-target Techniques for the Quantitation of Drugs of Abuse, Identification of Transformation Products, and Characterization of Contaminants of Emergent Concern by High Resolution Mass Spectrometry

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

TARGET AND NON-TARGET TECHNIQUES FOR THE QUANTITATION OF
DRUGS OF ABUSE, IDENTIFICATION OF TRANSFORMATION PRODUCTS,
AND CHARACTERIZATION OF CONTAMINANTS OF EMERGENT CONCERN
BY HIGH RESOLUTION MASS SPECTROMETRY

A dissertation submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Nubia Vanesa Heuett

2015

To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Nubia Vanesa Heuett, and entitled Target and Non-target Techniques for the Quantitation of Drugs of Abuse, Identification of Transformation Products, and Characterization of Contaminants of Emergent Concern by High Resolution Mass Spectrometry, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2015

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DEDICATION

To my son Drakeston Heuett, my dad Dicler Sierra, my sister Cristy Sierra, my brother-in-law Miguel Parra, and Sarita. For their patience and support during this journey.

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I would like to give thanks to my major professor Dr. Piero R. Gardinali for all his great advise both professionally and personally, his flexibility in hard times, and for his much needed sarcasm. Also, special thanks to my committee members Dr. Jose Amirall, Dr. Anthony DeCaprio, Dr. John Landrum, and Dr. Berrin Tansel for their time, advice, and constructive criticism during our few meetings. I would also like to thank SERC, the chemistry department, and the FIU Graduate School (DYF) for funding my studies.

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ABSTRACT OF THE DISSERTATION

TARGET AND NON-TARGET TECHNIQUES FOR THE QUANTITATION OF
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Florida International University, 2015

Miami, Florida

Professor Piero R. Gardinali, Major Professor

Development and application of target and non-target techniques for routine analysis, identification of transformation products, and characterization of unknown compounds in water matrices using liquid-chromatography high-resolution mass spectrometry (HRMS) were explored in this dissertation. A novel analytical method based on online-SPE-LC-HRMS was developed for the detection of 18 drugs of abuse (DOAs) in raw sewage water from a college campus. Results showed the presence of 14 DOAs for which amphetamine and 11-nor-9-carboxy-THC (THC metabolite) were the most prevalent and had the highest potential consumption rates.

A second study dedicated to the identification of transformation products (TPs) generated from DOAs was conducted using a combination of HR-MS/MS and metabolic identification and structural elucidation software. Findings confirmed the presence of multiple phase I and II DOA TPs (n=35) in raw sewage influents. Concentrations of all TPs were estimated based on the parent DOAs response factors, and used to calculate the

percent mole fraction contributions of each TP to the parent concentrations. High abundance and frequency (compared to the parent drug) was determined for 9 of the TPs coming from drugs like oxycodone, morphine, codeine, methadone, LSD, cocaine, and MDEA.

Non-target analysis using HRMS was explored as a tool to characterize, and compare a series of interconnected water matrices along a river system. Several thousands of formulae were generated using automated heuristic rules from the full-scan acquisition at 140,000 resolution. Samples were part of a trajectory covering upstream, effluent, effluent mixing zone, downstream, drinking water intake, and treated drinking water locations. Graphical representations of the data were used to evaluate commonalities among the system. Using this approach, a total of 64 recalcitrant components were identified throughout the samples downstream of the effluent release point. Using a combination of MS/MS and computer-aid software techniques 4 out of the 64 compounds were tentatively confirmed. In addition, comparison of drinking water intake and finalized treated drinking water sites showed the presence of 1,152 chemical entities that were common to both locations; and 1,857 that were unique to the treated drinking water. Therefore, this non-target technique could be used to identify the potential formation of treatment byproducts.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL NAME
6-AMO	6-Acetyl Morphine
ADHD	Attention Deficit Hyperactivity Disorder
AGC	Automated Gain Control
ALS	Amphetamine-like-stimulants
AM	Amphetamine
BE	Benzoyllecgonine
BFRs	Brominated Flame Retardants
CCV	Continuing Calibration Verification
CE	Cocaethylene
CO	Cocaine
COD	Codeine
DBE	Double Bond Equivalence
DBPs	Disinfection Byproducts
DI	Deionized
DOAs	Drugs of Abuse
DS	Downstream
DWI	Drinking Water Intake
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EFF	Effluent
EMZ	Effluent Mixing Zone
ESI	Electrospray Ionization

FISh	Fragment Ion Search
FIU	Florida International University
FTICR	Fourier Transform Ion Cyclotron Resonance
HCD	High Collision Dissociation
HE	Heroin
HESI	Heated Electrospray Ionization
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IT	Injection Time
KMD	Kendrick Mass Defect
LC	Liquid Chromatography
LFB	Laboratory Fortified Blank
LFB-DUP	Laboratory Fortified Blank Duplicate
LFM	Laboratory Fortified Matrix
LSD	Lysergic Acid Diethylamide
m/z	Mass-to-charge ration
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-methylenedioxy- <i>N</i> -ethyl-amphetamine
MDL	Method Detection Limit
MDMA	3,4-methylenedioxy-methamphetamine
ME	Methadone

Mf	Mole fraction
MO	Morphine
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MXC	Mixed Mode Cation Exchange
NCE	Normalized Collision Energy
NOM	Natural Organic Matter
OXY	Oxycodone
PAHs	Polycyclic Aromatic Hydrocarbons
PET	Polyethylene Terephthalate
PFCs	Perfluorinated Compounds
PPCPs	Pharmaceuticals and Personal Care Products
ppm	parts per million
ppt	parts per trillion
QA/QC	Quality Assurance/Quality Control
QqQ	Triple Quadrupole
qTOF	Quadrupole Time of Flight
RDBE	Ring Double Bond Equivalence
RSD	Relative Standard Deviation
rt	Retention Time
SERC	South East Environmental Research Center
SMX	Sulfamethoxazole
SPE	Solid Phase Extraction

SPM	Suspended Particulate Matter
SRM	Selected Reaction Monitoring
TDW	Treated Drinking Water
THC	Tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-delta-9-tetrahydrocannabinol
TPs	Transformation Products
TSQ	Triple Stage Quadrupole
UK	United Kingdom
UNODC	United Nations Office on Drugs and Crime
UP	Upstream
US-EPA	United States Environmental Protection Agency
UV	Ultra Violet
WWTP	Wastewater Treatment Plant

CHAPTER 1

Introduction

The interdisciplinary nature of the field of high-resolution mass spectrometry has substantially broadened the possibilities in the environmental, forensic, and clinical fields with numerous advances each of the areas or a combination of them. Through multiple studies reported to date, it is now known of the presence of several hundreds of microconstituents ranging from pesticides, drugs of abuse (DOAs), pharmaceuticals and personal care products (PPCPs) in water and soil matrices, food products, and biological samples. While a considerable amount of information regarding the occurrence, fate, transport, toxicity, and consumption of a great variety of analytes is available; there are still many scientific challenges to overcome as human practices continue to expand the list of contaminants of emergent concern.

1.1 Analytical methods

1.1.1 Pre-concentration

Pre-concentration of aqueous samples has typically been accomplished with the use of solid phase extraction (SPE) (offline and online) because of the large selection of sorbent materials to accommodate compounds with different polarities and physico-chemical characteristics (Gilart et al. 2014). Two of the most popular types of cartridges include the polymeric HLB cartridge, which contain functionalized porous polymers capable of extracting a wide range of polar and nonpolar components (Petrović et al. 2010); and mixed-mode cartridges such as cation-exchange and anion-exchange, which have been used to increase selectivity and capacity of extraction through ion-exchange mechanisms (Fontanals et al. 2013). Studies published by Gros et al., and Baker et al., have demonstrated superior recoveries for pharmaceuticals and illicit drugs using

polymeric HLB and mixed-mode (MCX) cartridges respectively (Gros et al. 2006; Baker and Kasprzyk-hordern 2011).

1.1.2 Liquid chromatography

Liquid chromatography-mass spectrometry (LC-MS) has for the past few decades been the gold standard for the investigation of organic compounds in different types of complex matrices. Chromatographic methods have evolved to ultra-fast separation techniques as a result of advances in particle size and types and structures of packing materials (Baker and Kasprzyk-hordern 2011). These advances have provided advantages in terms of sensitivity, resolution of analytes, and sample throughput. For instance, the development of multi-residue methods for the simultaneous detection of multiple classes of compounds have been reported for pharmaceuticals and drugs of abuse (DOAs) with overall good separation and sensitivity (Gros et al. 2006; Kasprzyk-hordern et al. 2008; Loos et al. 2009; Baker and Kasprzyk-hordern 2011; Gago-Ferrero et al. 2015).

1.1.3 Mass spectrometry

1.1.3.1 Low-resolution MS

Tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) instruments has been an ideal detection technique for the target analysis of known compounds. It offers great sensitivity and selectivity in the selected reaction monitoring (SRM) mode, making it an attractive technique for the quantitation and identification of a considerable number of compounds (Petrović et al. 2010). Trace level determinations of numerous classes of emergent contaminants have been published in the literature using triple quadrupole instruments (Postigo et al. 2008; Bijlsma et al. 2009; Castiglioni et al. 2011; Ibáñez et al.

2012; Batchu et al. 2013; Gorga et al. 2013; Idder et al. 2013; Panditi et al. 2013). However, one of the major pitfalls of this technique is the inability to detect or screen for compounds not compiled in the SRM list, even if their abundance in the sample is high.

1.1.3.2 High-resolution MS

High-resolution instruments are capable of simultaneously analyzing large number of low and high abundant compounds for characterization, screening, identification and even quantitation of known and unknown compounds, with minimal optimization compared to low-resolution quadrupole instruments (Krauss et al. 2010; Díaz et al. 2012; Hopfgartner et al. 2012; Xian et al. 2012; Bijlsma et al. 2013b). These capabilities are attributed to the instruments high resolving power, which allows them to discriminate signals observed in a mass spectrum from ions of similar mass to charge ratio (m/z) (Hernández et al. 2012; Ojanperä et al. 2012). Fourier transform ion cyclotron (FTICR) and Orbitrap instruments are capable of reaching resolutions of up to 1,000,000 and 240,000 respectively. High-resolution mass spectrometry (HRMS) has been gaining momentum in the field of environmental science. Several applications for target analysis, suspect screening, metabolite identification, and non-target screening reported in the literature (Bijlsma et al. 2011; Díaz et al. 2012; Chiaia-Hernandez et al. 2013; Moschet et al. 2013; Hug et al. 2014; Li et al. 2014; Vergeynst et al. 2014), have demonstrated the enormous potential of this powerful technique.

1.2 Target analysis

Identification and quantitation of compounds has for many decades been with the use of reference standards and their labeled analogs; and with the instrumental power of triple

quadrupole instruments. While this approach has been implemented for routine analysis of many different types of microconstituents including PPCPs, DOAs, and pesticides, certain limitations have become evident. The requirement to two SRM transitions restricts the number of analytes in a run, as a result of short acquisition times and peak resolution (Krauss et al. 2010). In addition, some low molecular weight ions only provide one transition with low signal intensity, or isobaric interferences may show the same transitions as the analyte of interest (Krauss et al. 2010). High-resolution mass spectrometry resolves these issues with the use of full-scan for detection of thousands of compounds, and data-dependent and target MS/MS acquisition modes for confirmation.

1.2.1 Occurrence

Target analytical methods have been used to detect the occurrence of a large number of compounds in water and solid matrices. Excellent reviews have been published throughout the years on the subject of contaminants of emergent concern, providing a great wealth of information on the occurrence of many of these compounds (Petrovic et al. 2008; Barceló et al. 2012; Richardson and Ternes 2014). Some of these compounds include perfluorinated compounds (PFCs), Pharmaceuticals and hormones, disinfection byproducts, pesticides, artificial sweeteners, and drugs of abuse (DOAs).

1.2.1.1 Wastewater

Environmental occurrence of DOAs have been reported in wastewater, surface water, ground water, and air (Zuccato et al. 2005; Postigo et al. 2009, 2010; Jurado et al. 2012; Mastroianni et al. 2013). Drugs of abuse (DOAs) reached wastewater treatment plants (WWTPs) soon after they are excreted or disposed of. In here, a combination of the

parent DOA and its metabolite(s) can survive treatment and be release surface waters, soil, and sediments. In wastewaters (influent and effluent), prevalence of DOAs range in ppt and ppb levels for cocaine (benzoylecgonine, 7.500 ng/L) (Huerta-Fontela et al. 2008a), opioids (codeine, 32,295 ng/L) (Kasprzyk-hordern et al. 2008), cannabinoids (THC-COOH, 402 ng/L) (Boleda et al. 2009), and amphetamines (MDMA, 15,380 ng/L) (Bijlsma et al. 2009).

1.2.1.2 Surface and ground water

While concentration levels of DOAs in surface waters are significantly lower (low ppt) than those in wastewaters, several of these types of drugs are still present in the environment. Drugs like cocaine, morphine, methadone, THC, amphetamine, methamphetamine, MDA, MDMA, and some of their metabolites are found ubiquitously in countries like Spain, Italy, and Switzerland where most research has been reported (Zuccato et al. 2005; Huerta-Fontela et al. 2008b; González-Mariño et al. 2010; Postigo et al. 2010; Bueno et al. 2012; Valcárcel et al. 2012). In addition, Jurado et al., found low ppt levels of MDMA (3.9 ng/L), morphine (1.4 ng/L), methadone (7.4 ng/L), cocaine (3.8 ng/L), and a few of their metabolic products in ground water (Jurado et al. 2012). These findings are very important since drinking water treatment plants rely on surface and ground water as source for drinking water production.

1.2.1.3 Drinking water

Two reports published by Boleda et al., and Valcárcel et al. studied the presence of DOAs in drinking water throughout several countries in Europe, Latin America, and Japan (Boleda et al. 2011; Valcárcel et al. 2012). Their findings demonstrated the

persistence of cocaine (0.1 ng/L to 2.11 ng/L), benzoylecgonine (0.2 ng/L to 4.5 ng/L), cocaethylene (0.2 ng/L), methadone (0.1 ng/L to 0.99 ng/L), and EDDP (0.1 ng/L to 0.4 ng/L) after drinking water treatment (Boleda et al. 2011; Valcárcel et al. 2012); which confirms that drinking water facilities require better treatment processes to deal with these types of compounds.

1.2.2 Consumption

Drugs of abuse concentration values obtained from wastewater analysis can easily be used to estimate consumption levels in communities. Wastewater flows are utilized to determine daily mass loads of parent drugs and/or metabolites (these values are usually normalized to 1,000 inhabitants) (Zuccato et al. 2005). In addition, excretions rates, metabolite/parent molar ratios, and known doses are then factored into the analysis to ultimately determine the number of daily doses/day/1000 inhabitants for each drug (Zuccato et al. 2005). This approach has been very useful in determining real time prevalence of drug use in a community, as well as rates and patterns of consumption (Nuijs et al. 2011).

Drug consumptions have been estimated in small settings such as a music festival and a prison, in which the major drugs consumed were cannabis (70 daily doses/day/1000 people) and methadone (156 daily doses/day/1000 people) for the festival and the prison, respectively (Postigo et al. 2011; Lai et al. 2013). Regional estimates of drug use have also been determine for cocaine, opiates, cannabis, and amphetamine-like-stimulants (ALS), in order to asses drug prevalence across different areas within a region (Postigo et

al. 2010), and to identify weekly, monthly, and even yearly patterns of consumption (Zuccato et al. 2008, 2011; Karolak et al. 2010; Kasprzyk-Hordern and Baker 2012).

1.3 Identification of transformation products (TPs)

Following drug consumption, drugs can undergo phase I and phase II metabolic processes in the body generating excretion products which may contain portions of the parent drug and some of the metabolic products (Celiz et al. 2009). Following excretion, these microconstituents are exposed to different types of treatments and environmental conditions, and can suffer additional degradation (Pérez and Barceló 2007). As a result, the absence or reduction of drug (illicit and prescription) concentrations in environmental analyses can erroneously provide false negatives on the actual conditions of aquatic ecosystems.

Nowadays, with the use of high-resolution instruments, it is possible to screen and identify transformation products that were not previously known to be present in the environment (Bijlsma et al. 2013a). Methods have been developed to determine transformation products from pharmaceuticals and DOAs. For instance, several transformation products (TPs) from unapproved analogs of the erectile dysfunction drug sildenafil were identified and confirmed in controlled laboratory studies, and two of the TPs were found in environmental samples (Aceña et al. 2014). In addition, our group recently reported the identification and quantitation of two phase II metabolic products of the antibiotic sulfamethoxazole (SMX) in reclaimed water (Wang and Gardinali 2014). The two metabolites represented 54% of the source of SMX in reclaimed water suggesting the importance of measuring TPs in the environment (Wang and Gardinali

2014). Other studies have been performed to investigate the degradation products of cocaine and benzoylecgonine in aquatic environments (Bijlsma et al. 2013a); and to identify pharmaceutical TPs from oxidation and disinfection processes in drinking water treatment (Postigo and Richardson 2014).

1.4 Non-target analysis

To date, the list of organic pollutants, both regulated and unregulated, comprises chemicals such as pharmaceuticals and personal care products (PPCPs), hormones, pesticides, drugs of abuse (DOAs), artificial sweeteners, nanomaterials, disinfection by products (DBPs), perfluorinated compounds (PFCs), polycyclic aromatic hydrocarbons (PAHs), brominated flame retardants (BFRs), phthalates, sunscreens/UV filters, and many more that are being introduced and transformed in the environment (Huerta-Fontela et al. 2008a; Barceló et al. 2012; Zareitalabad et al. 2013; Richardson and Ternes 2014). Since it is not possible to develop a method to monitor such a large number of known and unknown compounds, non-target techniques are used to mine accurate mass data even after being acquired.

Multiple approaches for non-target analysis have been reported in the literature showing commonalities in the workflows used (García-Reyes et al. 2007; Díaz et al. 2012; Chiaia-Hernandez et al. 2014; Hug et al. 2014). The initial step in non-target analysis is the detection of peaks by mass filtering from the chromatographic run (Krauss et al. 2010). Since high-resolution instruments operating in full scan mode generate massive amounts of data, it is wise (if not necessary) to perform a blank subtraction to remove background ions (Moschet et al. 2013). At this point, stepwise filtering of data is

applied starting from generation of a peak list, followed by determination and confirmation of formulas (Hug et al. 2014). The latter step follows a series of heuristic rules developed to remove erroneous formula assignments, and reduce the number of possible elemental compositions (Kind and Fiehn 2007).

While a significant number of masses have been filtered throughout these steps, there is still a considerable amount of data. To deal with the dilemma, the following approaches have been used: database search to exclude formulas with no hits (Hug et al. 2014); retention time (rt) filtering, which excludes formulas that do not match the predicted rt (Krauss et al. 2010; Díaz et al. 2012); and data comparisons through graphical interpretations, Venn diagrams, multivariate, and cluster analysis (Müller et al. 2011; Tseng et al. 2013; Vergeynst et al. 2015).

The last step in non-target analysis is confirmation using MS/MS data, and a combination of *in-silico* fragmentation, structural elucidation, and database search for the predicted structures (Krauss et al. 2010; Hug et al. 2014; Bletsou et al. 2015). It is important to keep in mind that for non-target analysis to be possible, high-resolution and mass accuracy are a must. In addition one of the key elements in this process is automation through different algorithms and databases for it to be efficient.

1.5 Hypothesis

Application of target and non-target techniques using the capabilities of high resolution mass spectrometry can be used to understand human activities and habits through assessment of drug consumption, metabolite identification, and characterization of contaminants of emergent concern in water matrices.

1.6 Objectives

To develop an analytical method based on high-resolution mass spectrometry capable of detecting ppt levels of drugs of abuse, and to implement the technique in the communal assessment of consumption.

To develop a workflow using HR-MS/MS for the identification of DOA transformation products in raw sewage influents, to further determine their abundance and assess their prevalence.

To characterize different types of interconnected water matrices using non-target high-resolution mass spectrometry for classification and comparison of compound diversity among the water matrices, to further identify and understand the composition of recalcitrant chemicals.

1.7 References

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CHAPTER 2

Analysis of drugs of abuse by online SPE-LC high-resolution mass spectrometry:

Communal assessment of consumption

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2.1 Abstract

An online SPE-LC-HRMS method was developed to monitor the consumption of 18 drugs of abuse (DOAs) including amphetamines, opioids, cocaine, cannabinoids, lysergics, and their corresponding metabolites in a well characterized college campus setting via wastewater analysis. Filtered and diluted (10X) sewage water samples (5 mL inj.) were automatically pre-concentrated and analyzed in 15 min using a Thermo EQUAN MAX online SPE system equipped with a HyperSep™ Retain PEP (20 × 2.1 mm × 12 µm) SPE column and a Hypersil Gold™ aQ (150 × 2.1 mm × 3 µm) analytical column. A Q Exactive™ Hybrid Quadrupole-Orbitrap HRMS was used in full scan mode (R=140,000) for positive identification, and quantitation of target compounds. Method detection limits for all analytes ranged between 0.6 to 1.7 ng/L in sewage. A total of 14 DOAs were detected from two different locations (dorms and main college campus) within one-year period. Most frequently detected drugs throughout the entire study were amphetamine (> 96%) and THC's metabolite 11-nor-9-carboxy-Δ-9-THC (> 100%) with maximum concentrations of 5,956 and 2,413 ng/L respectively. Daily doses per 1000 people were determined in order to assess consumption of THC, amphetamine, heroin and cocaine, in both dorms and main campus.

2.2 Introduction

Every year, the United Nations Office on Drugs and Crime (UNODC) generates a comprehensive report on the latest developments in drug markets, covering production, trafficking, consumption, and health consequences (UNODC 2014). In the year 2012, the US alone reached its highest levels of illicit drug use in the past 10 years (16% of the

population 12 years and older), with consumption being the greatest among those in their late teens or early twenties, as noted in the World Drug Report 2014 (UNODC 2014). These Fig., however, are not evaluated fast enough to provide real time understanding of the situation for law enforcement officials to take appropriate timely actions.

Multiple studies have reported wastewater-derived Fig. for the consumption of drugs of abuse (illicit and prescription drugs), as they provide a nonintrusive way to quickly assess social trends, usage patterns, and drug prevalence at the community level (Castiglioni et al. 2006; Zuccato et al. 2008; Chiaia and Banta-green 2008; Huerta-Fontela et al. 2008; Bartelt-hunt et al. 2009; Boleda et al. 2009; Postigo et al. 2010; Baker and Kasprzyk-hordern 2011; Nuijs et al. 2011; Bijlsma et al. 2012; Kasprzyk-Hordern and Baker 2012). This in contrast to the more conservative, time-consuming, and intrusive approaches of social surveys, drug seizures, crime statistics, and medical examiner reports (Nuijs et al. 2009d; Baker and Kasprzyk-hordern 2011; Burgard et al. 2013).

Besides the social, health, and economic implications illicit drugs pose, there is also the impact that these drugs could inflict over aquatic environments. For instance, Parolini et al., reported that exposure to 0.5 µg/L and 1 µg/L of cocaine (benzoylecgonine) caused oxidative stress in freshwater bivalve *Dreissena polymorpha*, due to imbalances of the enzyme defense chain (Parolini et al. 2013). Similarly to pharmaceuticals, illicit drugs are metabolized in the body and excreted in their unchanged form or as their metabolite(s) reaching water streams (Hummel et al. 2006; Castiglioni et al. 2011; González-Mariño et al. 2012). Moreover, inadequate human disposal of these drugs increases their load and frequency of exposure to the environment, posing possible detrimental effects due to their

unknown persistence and ecotoxicity (González-Mariño et al. 2012; Jurado et al. 2012; Valcárcel et al. 2012; Pal et al. 2013). This group of emerging contaminants as initially recognized by Petrovic and Richardson are typically assessed via analysis of effluent sewage and surface waters; and include (but are not limited to) parent and metabolites of cocaine, amphetamine like stimulants (ALS), cannabinoids, and opiates (Petrovic et al. 2008; Richardson 2008).

Solid phase extraction (SPE) has been the technique of choice for pre-concentration of illicit drugs in off-line applications. However, offline SPE is time consuming and requires multiple manual sample preparation steps decreasing sample throughput. Despite associated problems such as the need of careful method development for carryover control, online SPE has been gaining popularity for large environmental monitoring efforts as it provides minimal preparation steps, reduced reporting times and lower solvent usage relative to offline techniques (Batchu et al. 2013; Gorga et al. 2013; Panditi et al. 2013; Wang and Gardinali 2013; Ramirez et al. 2014). Although studies using online SPE have been reported in the analysis of illicit drugs in sewage matrices, cannabinoids (one of the most consumed drugs) are only included in two of these methods which still required a separate pre-concentration protocol or suffered from poor sensitivity (Postigo et al. 2008; Fontanals et al. 2013; Ostman et al. 2013; Repice et al. 2013).

From the initial report of analysis of illicit drugs in sewage waters documented by Zuccato et al. in 2005, most analyses of DOAs have been performed using liquid chromatography coupled to MS or MS/MS detection, the latter typically involving triple quadrupole instruments operating in selected reaction monitoring (SRM) mode (Zuccato

et al. 2005; Boleda et al. 2007; Castiglioni et al. 2008; Postigo et al. 2010; Fontanals et al. 2011, 2013; Hernández et al. 2011; Vazquez-Roig et al. 2013). Nowadays, high-resolution instruments such as the quadrupole time of flight (Q-TOF) or the Orbitrap are routinely used in urine analysis as screening tools for doping (Li et al. 2013; Musenga and Cowan 2013; Sundström et al. 2013). However, few methods have been implemented for the analysis of illicit drugs in wastewaters (Bijlsma et al. 2012; González-Mariño et al. 2012; Fedorova et al. 2013) despite clear advantages offered by full-scan HRMS such as enabling the screening of previously-acquired data for drugs of abuse that are identified after initial data processing has occurred.

Considering the fact that drug consumption is the highest among high school/college age students (U.S. Department of Health and Human Services 2013), the objective of the present study is twofold a) to develop and validate a fully automated method using online SPE-HPLC high resolution mass spectrometry for the detection of 18 drugs of abuse including some of their known metabolites in a controlled college campus setting; and b) to assess and quantify potential consumption of prescription and/or illicit drugs within the service population.

2.3 Experimental

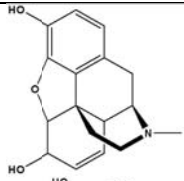
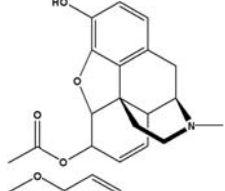
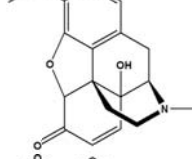
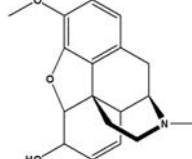
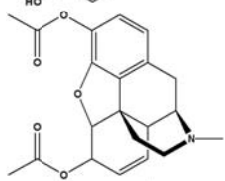
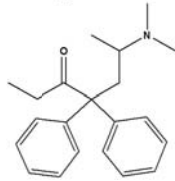
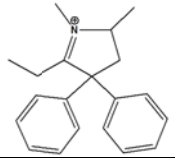
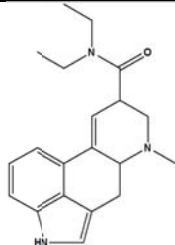
2.3.1 Reagents and chemicals

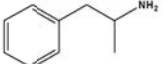
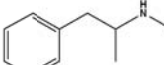
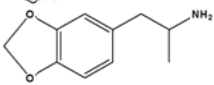
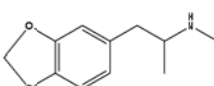
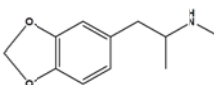
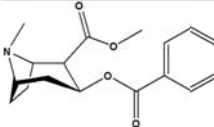
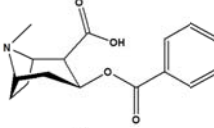
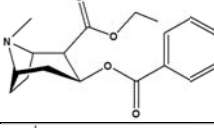
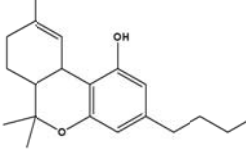
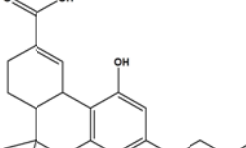
A total of 18 drugs of abuse and some of their metabolites belonging to five different chemical classes were used in this study: amphetamine-like compounds (ALCs): amphetamine (AM), methamphetamine (MA), MDA, MDEA, MDMA; cannabinoids: Δ -9-THC (THC), 11-nor-9-carboxy- Δ -9-THC (THC-COOH); cocaine: cocaine (CO),

benzoylecgonine (BE), cocaethylene (CE); opioids: morphine (MO), 6-acetylmorphine (6-AMO), codeine (COD), oxycodone (OXY), heroin (HE), methadone (ME), EDDP; and Lysergic acid diethylamine (LSD). All standards and their deuterated analogs were purchased as certified solutions in methanol or acetonitrile from Cerilliant (Round Rock, TX, USA). Table 2.1 shows a list of analytes used in this study, including their structure, elemental formula, exact mass, mass error (ppm), pKa, log Kow, and deuterated analogs used for quantitation.

Individual stock solutions for both target and deuterated compounds were prepared in methanol at concentrations of 5 and 10 µg/mL. Working standard and internal standard solutions were prepared in methanol at a concentration of 20 ng/mL, and an additional working standard solution of 5 ng/mL was made for preparation of low calibration levels (5–50 ng/L). All standards and prepared solutions were stored in the dark at -20 °C. Optima LC/MS grade methanol, water, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used for standard preparation and HPLC mobile phase. Optima LC/MS grade formic acid and ammonium formate were also purchased from Fisher Scientific and used for modifier mobile phase preparation (1% formic acid and 100 mM ammonium formate).

Table 2.1. Structures, elemental formula, exact mass, mass error, pKa, Log Kow, and internal standard used for quantitation of target analytes.

Class	Analyte	Structure	Formula	Exact Mass	Mass Error (ppm)	pK _a *	Log Kow**	Int. Std.
Opioids	Morphine (MO)		C ₁₇ H ₁₉ NO ₃	286.1438	-2.4	10.26 9.12	0.89	MO-D6
	6-Acetylmorphine (6-AMO)		C ₁₉ H ₂₁ NO ₄	328.1543	-3.0	10.19 9.08	0.53	MO-D6
	Oxycodone (OXY)		C ₁₈ H ₂₁ NO ₄	316.1543	-1.9	13.56 8.21	0.66	OXY-D6
	Codeine (COD)		C ₁₈ H ₂₁ NO ₃	300.1594	-2.7	13.78 9.19	1.19	COD-D6
	Heroin (HE)		C ₂₁ H ₂₃ NO ₅	370.16490	-2.4	9.1	1.58	MO-D6
	Methadone (ME)		C ₂₁ H ₂₇ NO	310.2165	-2.9	9.12	3.93	ME-D9
	2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)		C ₂₀ H ₂₃ N	278.1903	-3.2	9.64	4.94	ME-D9
Lysergic acid	Lysergic acid diethylamide (LSD)		C ₂₀ H ₂₅ N ₃ O	324.2070	-3.1	7.98	2.95	LSD-D3

Class	Analyte	Structure	Formula	Exact Mass	Mass Error (ppm)	pK _a *	Log Kow**	Int. Std.
Amphetamine-like compounds (ALCs)	Amphetamine (AM)		C ₉ H ₁₃ N	136.1121	-0.7	10.1	1.76	AM-D5
	Methamphetamine (MA)		C ₁₀ H ₁₅ N	150.1277	-1.3	10.21	2.07	MA-D14
	3,4-Methylenedioxyamphetamine (MDA)		C ₁₀ H ₁₃ NO ₂	180.1019	-2.2	10.01	1.64	MDA-D5
	3,4-Methylenedioxy-N-methylamphetamine (MDMA)		C ₁₁ H ₁₅ NO ₂	194.1176	-1.0	10.14	2.28	MDMA-D5
	3,4-Methylenedioxy-N-ethylamphetamine (MDEA)		C ₁₂ H ₁₇ NO ₂	208.1332	-1.4	10.22	2.77	MDEA-D6
Cocainics	Cocaine (CO)		C ₁₇ H ₂₁ NO ₄	304.1543	0.3	8.85	2.30	CO-D3
	Benzoylcegonine (BE)		C ₁₆ H ₁₉ NO ₄	290.1387	-2.1	3.15 9.54	-1.32	BE-D3
	Cocaethylene (CE)		C ₁₈ H ₂₃ NO ₄	318.1700	-3.5	8.77	2.66	CO-D3
Cannabinoids	Δ-9-Tetrahydrocannabinol (THC)		C ₂₁ H ₃₀ O ₂	315.2319	-3.8	10.6	6.97	THC-D3
	11-nor-9-carboxy-Δ-9-tetrahydrocannabinol (THC-COOH)		C ₂₁ H ₂₈ O ₄	345.2060	-0.3	4.21	6.36	THC-COOH-D3

*Values obtained from DrugBank version 4.1 (Open Data Drug & Drug Target Database). ** Values were obtained from ChemSpider Database.

2.3.2 Sample collection and preparation

Samples were collected in a college campus in the city of Miami, FL USA. An automated ISCO 3700 sampler purchased from Teledyne ISCO (Lincoln, NE) was equipped with twelve 500 mL wide mouth glass bottles lined with 2 Mil White Block polyethylene reclosable bags purchased from Uline (Pleasant Prairie, WI, USA). The sampler was deployed in a pump station servicing 560 people from a campus dormitory. Monitoring was carried out during the 2012 – 2013 academic year collecting samples for twelve days at the beginning of September, 2012; nine days during the second and third week of December, 2012; and seventeen days between February and March. These times represent the beginning, end, and middle of a semester. A second set of samples was also collected from the main pump station servicing 9,456 people at the main university campus at the end the semester (April 1st to 21st, 2013) every day for a period of 3 weeks. The automated sampler was programmed to collect samples (200 mL) every four hours. Wastewater influent samples were vacuum filtered using a coarse 1 μm GFC filter followed by a 0.5 μm PreSep Prefilter glass fiber filter purchased from Fisher Scientific (Fair Lawn, NJ, USA). Filtered samples were then stored in clear polyethylene terephthalate (PET) bottles (SKS Science, Watervliet, NY, USA) in the dark at -20 °C until analysis time (1 day to 3 months).

On the day of analysis, samples were allowed to reach room temperature and shaken for about 10 seconds. A 1-milliliter subsample of pre-filtered sewage influent sample was diluted to 10 mL with LC/MS grade water, and fortified with the appropriate internal standards (200 ng/L). QA/QC included the preparation of blanks, laboratory fortified blanks (LFBs), laboratory fortified blank duplicate (LFB-DUP), laboratory fortified

matrix (LFM) samples, duplicate samples, and a seven-point calibration curve (5 ng/L – 500 ng/L).

2.3.3 Online SPE pre-concentration and HPLC separation

A Thermo EQUAN MAX online SPE system (Thermo Scientific, Waltham, MA, USA) was employed in this study. The EQUAN system consisted of two six-port rotatory valves (valve A and valve B), an AccelaTM 600 loading pump, an AccelaTM 1250 analytical pump, and a HTC-PALTM autosampler. Filtered and diluted samples, quality controls, and calibration solutions were loaded at 1 mL/min onto a 5 mL stainless steel loop connected to valve A. The loading pump, also connected to valve A, transferred the sample from the sample loop to a pre-conditioned HyperSepTM Retain PEP SPE (20 x 2.1 mm, 12 µm) column, which was connected to valve B. A three-minute washing step with LC/MS water was used to remove inorganics. Simultaneous to this process, the analytical pump connected to valve B was used to precondition the analytical column. After the washing step, valve B was then switched to connect the loading column to a Hypersil GoldTM aQ (150 × 2.1 mm, 3 µm) analytical column protected by a Hypersil GoldTM aQ guard column (10 x 2.1 mm, 3 µm). The analytical pump provided the gradient flow and composition for chromatographic separation of analytes using water, methanol, and 1 % formic acid/100 mM ammonium formate buffer as mobile phases. While elution and separation of analytes took place in valve B, valve A switched to rinse and fill the sample loop with methanol and then turned again to allow for the next sample in queue to be injected. Finally, valve B switched one last time to connect the loading pump to the SPE column, for cleaning (acetonitrile/methanol) and conditioning (water). The total run time

was achieved in 15 min, and at this point, the system was ready to start the process with the preloaded sample. The detailed gradient programs for the loading and analytical pumps are shown in table 2.2.

Table 2.2. Analytical pump and SPE pump gradient programs. Mobile phases used: water (A), methanol (B), acetonitrile (C), and 1% formic acid/100 mM ammonium formate buffer (D).

Analytical Pump						SPE Pump						
Time	A	B	D	Flow (µl/min)		Time	A	B	C	D	Flow (µl/min)	
Conditioning	0	0	97	3	500	0	100				1000	Condition / SPE / Wash
	1.5	0	97	3	500							
						2.5	99	1			1000	
						3	94	3		3	1000	
						3.5	94	3		3	1000	
	4.5	94	3	3	500							
Gradient Separation						5		100			1000	Elution
						8		100			1000	
						8.1		100			2000	
	8.3	94	3	3	500	9.16		100			2000	Idle
	8.8	37	60	3	500	9.17		100			1000	
	9.3	22	75	3	500							
	9.8	12	85	3	500	10		100			0.1	Conditioning
	10.3	7	90	3	500							
	10.8	3	94	3	500							
	11.3	1	96	3	500							Conditioning
	11.8		97	3	500							
	12		97	3	500							
	12.5		97	3	600							
						12.9		100			0.1	Conditioning
						13			100		2000	
						13.5			100		2000	
						14.2						
						14.5	100				2000	Conditioning
	15		97	3	700	15	100				2000	

2.3.4 Mass spectrometry

Detection of analytes was performed using a Q Exactive™ hybrid Quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization source (HESI-II) operated in the positive ion mode. Source parameters were the following: capillary temperature 300 °C, S-lens voltage 50 V, and source voltage 3kV. Ultrapure nitrogen was used as ion source gas and collision gas, set at the following arbitrary units: sheath gas 35, aux gas 30, and sweep gas 5. The Orbitrap analyzer was operated in full scan mode at a resolving power of 140,000, with a scan range from 100-500 m/z. Automated gain control (AGC) was set to 3×10^6 , with a maximum injection time (IT) of 400 ms. Xcalibur 2.1 software was used for instrument control and data acquisition (Thermo Fisher Scientific, San Jose, CA, USA). External mass calibration was performed once a week using Pierce LTQ Velos ESI Positive Calibration Solutions (Thermo Scientific, Rockford, IL) for which mass accuracy was always achieved with ± 0.3 ppm. Positive identification of analytes was based on the accurate mass of the analyte with less than ± 5 ppm error, retention time comparison to that of its labelled analog within ± 0.03 min, and isotopic distribution match between the measured and expected patterns of the calculated elemental composition (≥ 90 % fit threshold, $< \pm 5$ ppm) (Bijlsma et al. 2013b; Moschet et al. 2013). An example of positive identification for amphetamine is shown in Fig. 2.1 of the electronic supplementary material.

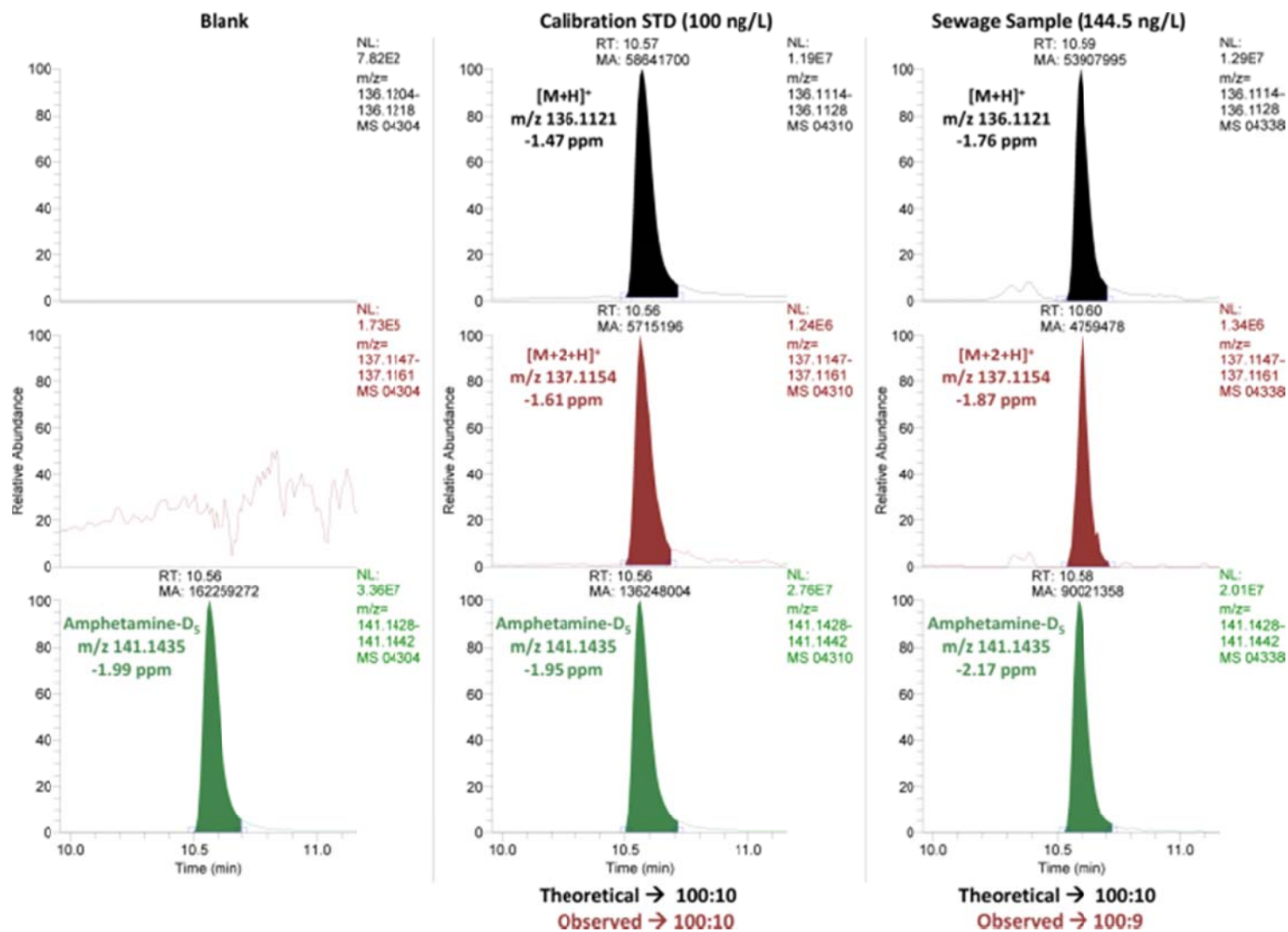


Fig. 2.1. Positive identification criteria for amphetamine in sewage water sample and its comparison to a calibration standard, and a blank.

2.4 Results and discussion

2.4.1 Online SPE optimization

Sorbent selection for multi-residue analysis of wastewaters has been previously reviewed in the literature (Petrović et al. 2010). Hydrophilic-lipophilic balance stationary phases have shown good performance for the simultaneous pre-concentration of target analytes with a broad spectrum of polarities, as it is the case of illicit drugs (Zuccato and Castiglioni 2009; Petrović et al. 2010); for this reason the above mentioned sorbent was selected for online SPE.

Online SPE recoveries of analytes were determined in DI water and sewage influent in order to test the extraction efficiency of the online SPE method. A direct injection method was developed using the same analytical gradient as the online SPE method, but taking into account the 8-minute period in which extraction/cleanup takes place (see table 2.3).

Table 2.3. Analytical pump gradient for the direct injection method. Mobile phases used: LC-MS grade water (A), methanol (B), and 1% formic acid/100mM ammonium formate buffer (D).

Time	A	B	D	Flow (µl/min)
0	94	3	3	500
0.3	94	3	3	500
0.8	37	60	3	500
1.3	22	75	3	500
1.8	12	85	3	500
2.3	7	90	3	500
2.8	3	94	3	500
3.3	1	96	3	500
3.8	0	97	3	500
4	0	97	3	500
4.5	0	97	3	600
7	0	97	3	700
8.5	0	97	3	500
13	94	3	3	500

For direct injection, standard solutions of composition similar to that of the starting gradient conditions (94 %: water, 4 %: Methanol, 2 %: water/1 % formic acid/100 mM ammonium formate) were prepared at the target concentration of 10,000 ng/L. 50 μ L of this mixture were injected (in triplicates) yielding a 0.5 ng on column equivalent for each compound. In addition, triplicate spiked solutions (100 ng/L) in DI water and diluted (1:10) sewage water, were prepared and 5 mL injections were made, also accounting for 0.5 ng on column for each compound. SPE recoveries are shown in table 2.4. High online SPE recoveries were obtained for most analytes (on average 90 % \pm 20 % in DI water and 67 % \pm 6 % in influent sewage water). Those averages exclude Δ -9-THC and morphine which presented relatively low SPE recoveries (41 \pm 3 % and 39 \pm 3 % in deionized water, respectively). As can be seen in table 2.4, excellent relative recoveries are obtained despite of the observed SPE recovery decrease with the presence of the matrix, highlighting the importance of the use of isotopic analogs to enable correct quantitation. The stability of the online SPE procedure was constantly evaluated in terms of signal intensity. As can be seen in Fig. 2.2 (supplementary information) the peak areas of most isotopically labelled analogs remained stable (within one order of magnitude) throughout an analytical sequence (68 injection, 17 hours of continuous operation). This observation indicates that the online SPE is able to remove most of inorganic species that could cause a signal decrease in the ESI source and also that the column is able to hold its extraction capabilities during long sequences.

Table 2.4. Quality control parameter of analytical method: linearity (R²), precision (%RSD), SPE recoveries, quantitation recoveries, and method detection limits (MDLs) in HPLC water and influent raw sewage water.

Limits (MDLs) in H ₂ O water and Influent raw sewage water:																
DI Water							Influent Sewage Water 1:10 Dilution									
Class	Analyte	R ²	Interday % RSD (n=5)	SPE Rec. (%) (n=3)		Relative. Rec. (%) (n=5)*		Intraday % RSD (n=7)	Interday % RSD (n=5)	SPE Rec. (%) (n=3)		Relative. Rec. (%) (n=5)*		Relative. Rec. (%) (n=7)**	MDLs (ng/L) (n=7)	
Amphetamine-like compounds (ALCs)	AM	0.9999	4	78	± 6	99	± 4	0.7	3	60	± 1	104	± 3	119	± 1	1.3
	MA	0.9996	2	84	± 5	99	± 3	0.8	5	59	± 1	101	± 5	139	± 1	1.7
	MDA	0.9999	2	105	± 8	98	± 2	0.4	11	65	± 1	117	± 12	101	± 0	0.6
	MDMA	0.9997	5	92	± 2	100	± 5	0.7	5	62	± 1	101	± 5	95	± 1	1.0
	MDEA	0.9999	2	91	± 6	100	± 2	0.4	5	58	± 1	104	± 5	105	± 0	0.7
Cocainics	CO	0.9998	10	88	± 5	96	± 9	0.4	11	65	± 1	100	± 11	141	± 1	1.0
	BE	0.9999	2	101	± 4	101	± 2	0.3	4	68	± 1	99	± 4	127	± 0	0.7
	CE	0.9996	4	87	± 3	95	± 3	0.4	5	66	± 2	114	± 5	143	± 1	0.8
Cannabinoids	THC	0.9998	4	41	± 3	96	± 4	0.3	3	36	± 2	97	± 3	131	± 0	0.6
	THC-COOH	0.9997	3	102	± 6	98	± 5	0.7	6	81	± 1	122	± 4	119	± 1	1.3
Opioids	MO	0.9997	3	39	± 3	97	± 3	0.5	6	70	± 2	98	± 5	147	± 1	1.1
	6-AMO	0.9998	1	87	± 4	99	± 1	0.5	5	66	± 2	93	± 4	90	± 0	0.7
	OXY	0.9998	3	85	± 4	98	± 3	0.4	6	70	± 2	104	± 7	111	± 0	0.7
	COD	0.9999	3	84	± 6	98	± 4	0.5	5	70	± 2	103	± 5	163	± 1	1.2
	HE	0.9964	5	93	± 5	107	± 5	0.7	5	66	± 2	95	± 4	83	± 1	0.9
	ME	0.9999	2	91	± 5	101	± 2	0.4	4	79	± 2	103	± 4	101	± 0	0.7
	EDDP	0.9997	2	87	± 4	101	± 3	0.4	4	76	± 1	108	± 5	129	± 0	0.8
Lysergic acid	LSD	0.9954	4	78	± 2	91	± 3	0.5	7	51	± 2	89	± 6	133	± 1	1.1

* Quantitation recoveries at 100 ng/L ** Quantitation recoveries at 5 ng/L

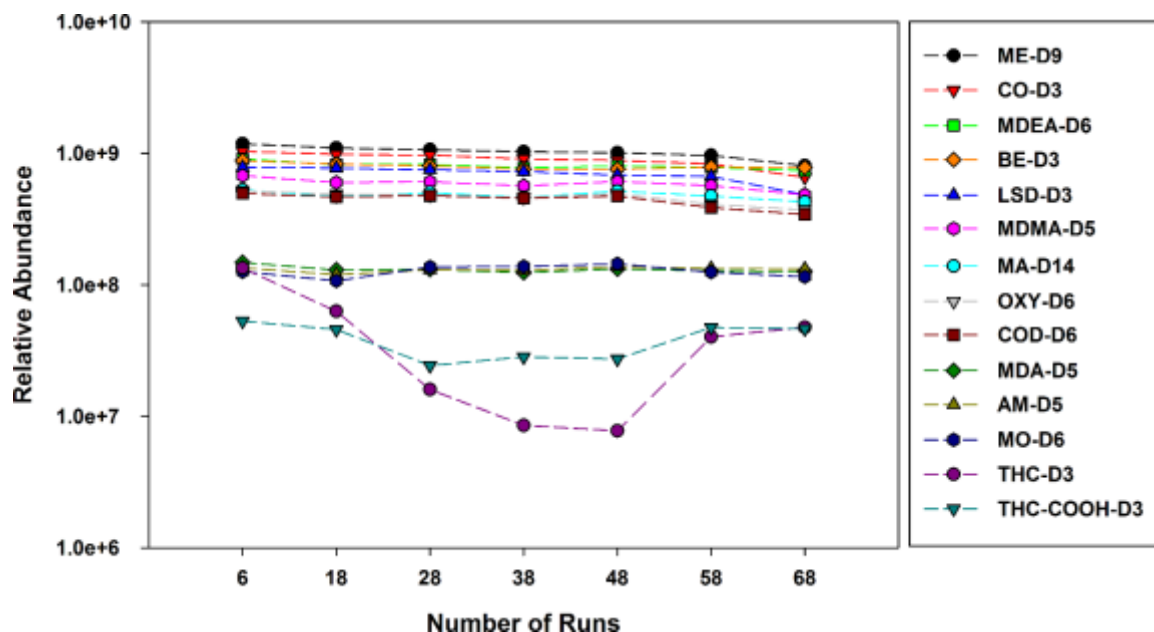


Fig. 2.2. Stability of the online SPE procedure based on signal intensity of labelled analogs from CCV solutions (100 ng/L) throughout an analytical sequence (68 injections, 17 hour of continuous operation).

2.4.2 Liquid chromatography optimization

Four different chromatographic columns and three different mobile phase compositions were tested in order to obtain good peak shape for all analytes.

Initially, a Hypersil Gold PFP (50 x 2.1 mm) particle size (1.9 μm) was used; this column provided adequate separation for most compounds but poor peak shape for cannabinoids, which was not fixed by mobile phase or flow rate optimizations. In addition, an interfering signal was observed co-eluting with THC-COOH but absent from the THC-COOH-D₃ chromatogram, which suggested the presence of a possible isobaric interference that required further optimization of the chromatographic separation. A Kinetex 2.6 μm HILIC 100A (150 x 2.10 mm) was also tested to inverse elution order and improve cannabinoids peak shape. However, a total retention loss of these compounds was observed, and therefore this approach was abandoned. Next, a highly

end-capped C₁₈ stationary phase (Hypersil Gold aQ, 50 x 2.1 mm, particle size 1.9 µm) was tested resulting in better peak shape for all the analytes but without resolution of the THC-COOH isobaric interference regardless of gradient optimization. Finally, using a longer column (Hypersil Gold aQ, 150 x 2.1 mm particle size 3.0 µm) provided adequate chromatographic separation of THC-COOH and its isobaric interference with excellent peak shape for every analyte, and therefore this column was selected for this study (see Fig. 2.3).

Mobile phase composition is another important factor affecting peak shape and signal intensity. An initial gradient using methanol and 0.1 % formic acid in water was tested. This mobile phase composition showed increasingly higher peak tailing at higher retention times. Two actions were taken in order to resolve this issue: the modifier phase (1 % formic acid/ 100 mM ammonium formate in water) was kept constant throughout the program and flow rate was ramped from 500 to 700 µL/min (table 2.2 provides details on the gradient compositions and flow rates).

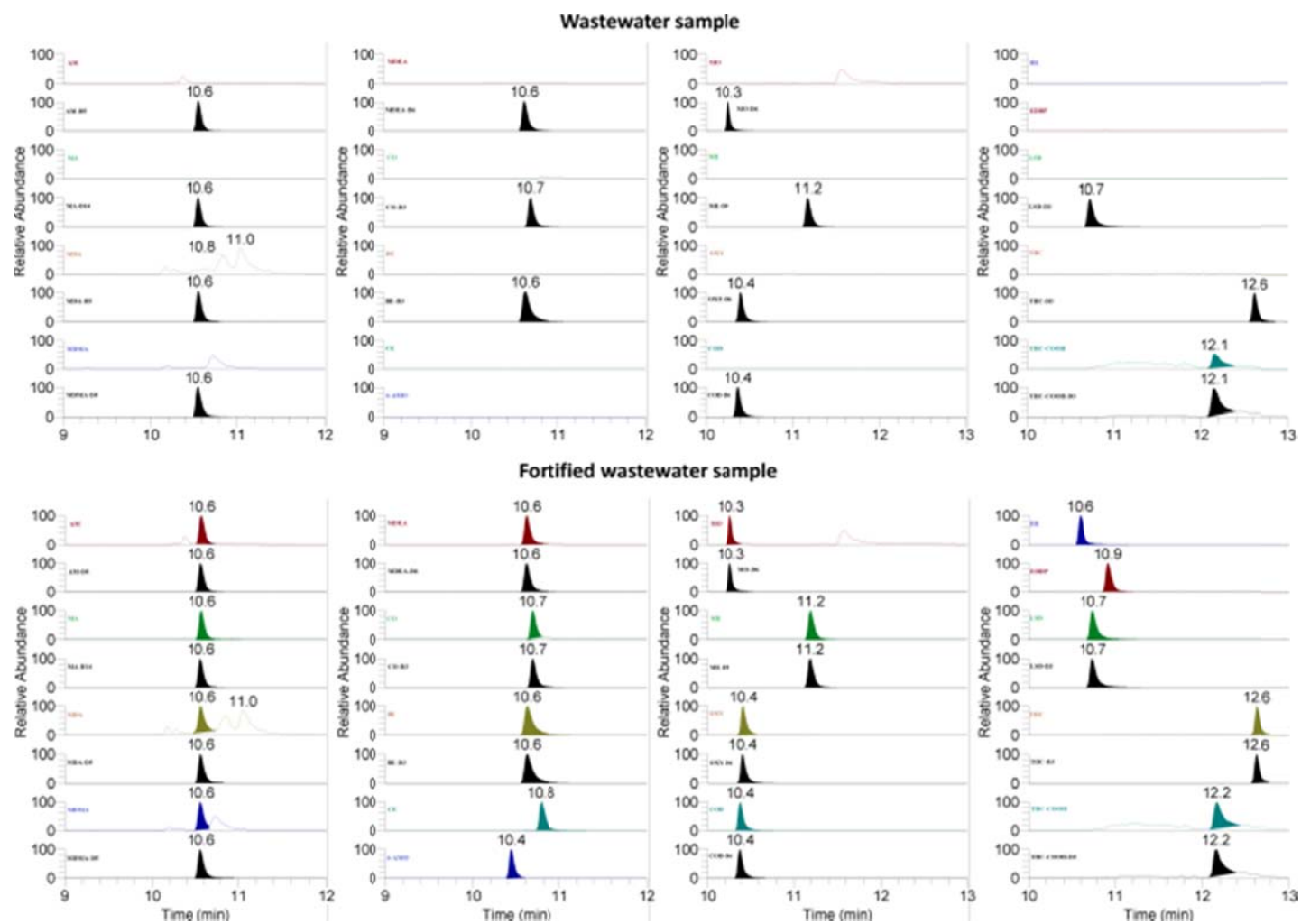


Fig. 2.3. Online-SPE-HPLC-HRMS chromatograms of wastewater (top) and fortified (100 ng/L) wastewater (bottom) samples. Including their deuterated analogs.

2.4.3 Method validation

2.4.3.1 Calibration

DI water calibration curves were obtained from the area ratio of each analyte to its deuterated analog as a function of the analyte concentration. For analytes without an analog, an internal standard of similar chemical structure or retention time was used. Linearity was observed for all analytes ($R^2 > 0.995$; 5 to 500 ng/L) (see table 2.2). Every ten runs a continuing calibration verification (CCV) was run to assess its accuracy (± 20 %) by injecting a fortified DI water solution (100 ng/L). Sewage samples free of target analytes were not a viable option in this study, therefore, calibration curves in this study were prepared in DI water.

2.4.3.2 Quality control samples

Blanks were run with each set of samples to assess possible contamination and background levels. Two laboratory fortified blanks (LFB and LFB-DUP) (100 ng/L) and a laboratory fortified matrix (LFM) sample (1:10, 100 ng/L) were also included with each batch as a measure of accuracy and precision of the analytical procedure, and to determine accuracy of the method in the presence of the matrix. High relative recoveries based on deuterated analogs were observed for all compounds in both LFB (99 ± 7 %) and LFM (103 ± 25 %) (See table 2.2).

2.4.3.3 Method detection limits

MDLs were calculated by multiplying the standard deviation of seven replicate 1 mL

spiked influent sewage samples (5 ng/L; 1:10 dilution) multiplied by the Student t value ($t = 3.143$, six degrees of freedom) according to the procedures outlined by the US-EPA (US EPA 2010). Since sewage samples may contain the target analytes, the same non-spiked sample was also run and the concentration of any analyte present was subtracted from the spiked sample. MDLs were similar or in some cases lower than other available methods which require larger sample volumes (Boleda et al. 2007; Baker and Kasprzyk-hordern 2011). In addition, reproducibility of the method was assessed from the seven replicate samples with RSDs ranging from 0.3 to 0.8 %. Analyte recoveries throughout the seven replicates ranged from 83 % (HE) to 163 % (COD) (see table 2.2).

2.4.3.4 Matrix effects

Matrix effects of the method were evaluated by comparing the area of a spiked sewage sample to the area of spiked DI water, both fortified at the same level (250 ng/L). Areas from any analytes present in the non-spiked sewage sample were subtracted from the areas in the spiked sample before comparison to spiked DI water areas. Fig. 2.4 shows a decrease in signal ranging from 12 % (Δ -9-THC) to 38 % (MDA). These results are in agreement to those reported in the literature. However, morphine presented a signal increase of 80 ± 7 % ($n=3$) in the presence of the matrix (see Fig. 2.4), which was also observed on the signal of the deuterated analog morphine-D6 (88 ± 6 %, $n=3$). These results are contrasting with previous studies that reported 10-80 % suppression on this compound (Postigo et al. 2008; Nuijs et al. 2009d; González-Mariño et al. 2012; Bijlsma et al. 2013b; Ostman et al. 2013).

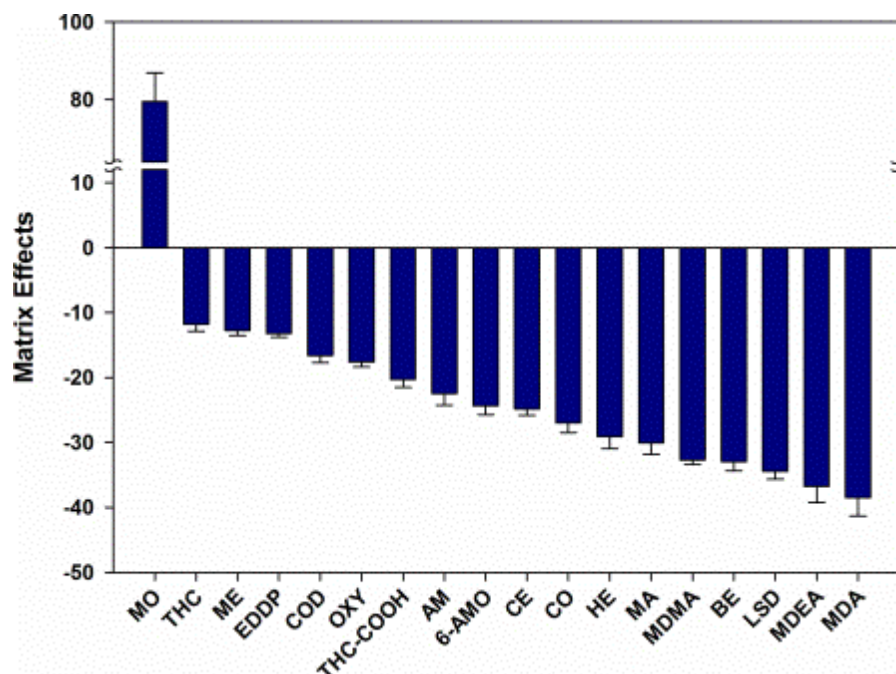


Fig. 2.4. Matrix effects of the method on analytes in sewage samples compared to HPLC water.

2.4.4 Sample preparation

Sample preparation steps are limited to filtration, addition of internal standards, and dilution. A recovery test was performed to determine the effect of filtration on the analytes. 100-mL triplicate DI water samples were spiked at 250 ng/L. A total of 10 mL of each sample were fortified with internal standards and injected. The remaining 90 mL were passed through the 1.0 and 0.5 μm glass fiber filters and 10 mL filtered aliquots were fortified with internal standards and analyzed. Recoveries were on average 89 ± 21 % for all compounds with the exception of Δ -9-THC, which presented a 42 ± 6 % recovery (see Fig. 2.5). However, this observation did not significantly affect the calculation of consumption trends for that illicit drug since the concentration of its main metabolite (THC-COOH) is often the predominant parameter used for that purpose.

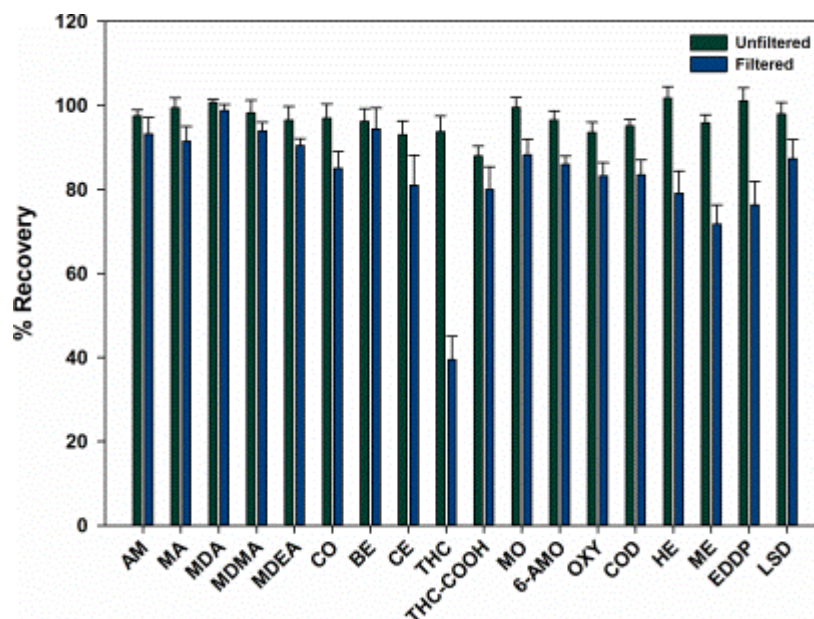


Fig. 2.5. Effects of filtration on illicit drug analytes. Analyte recovery comparison for filtered and unfiltered samples.

2.4.5 Storage stability of samples

One common difficulty in DOA analysis is that analytes in sewage samples could be subjected to bacterial degradation and can potentially undergo transformations; therefore, a stability test was performed to determine the effect of the storage conditions. A set of six raw sewage samples was collected for this experiment. Four of the samples were fortified with illicit standards at a 250-ng/L level. A field blank and a fortified field blank (250 ng/L) were also used in this test. All samples were filtered and a 1 mL aliquot, of each, was used in sample preparation and analyzed, corresponding to initial point (T0). The remaining volume of the samples was stored in the dark at -20 °C. Samples were further analyzed after 3, 7, 17, 27, and 123 days (see Fig. 2.6). Labelled standards were added to each sample prior to analysis. All amphetamine-like compounds remained stable throughout the 123-day period, with overall recovery losses or gains of less than 17 % in relation to T0. Cocaine, however, showed a 29 % loss at seven days, and a 97 % loss

from T0 was calculated at the end of the 123 days. In similar manner, cocaethylene also showed losses (65 %) in relation to T0. It is known for cocaine and cocaethylene to undergo transformation to benzoylecgonine, and as it is seen from Fig. 2.6, there is an increase in % recovery of benzoylecgonine from T0 to T123 (Brzezinski et al. 1997; Castiglioni et al. 2008; Bijlsma et al. 2013a).

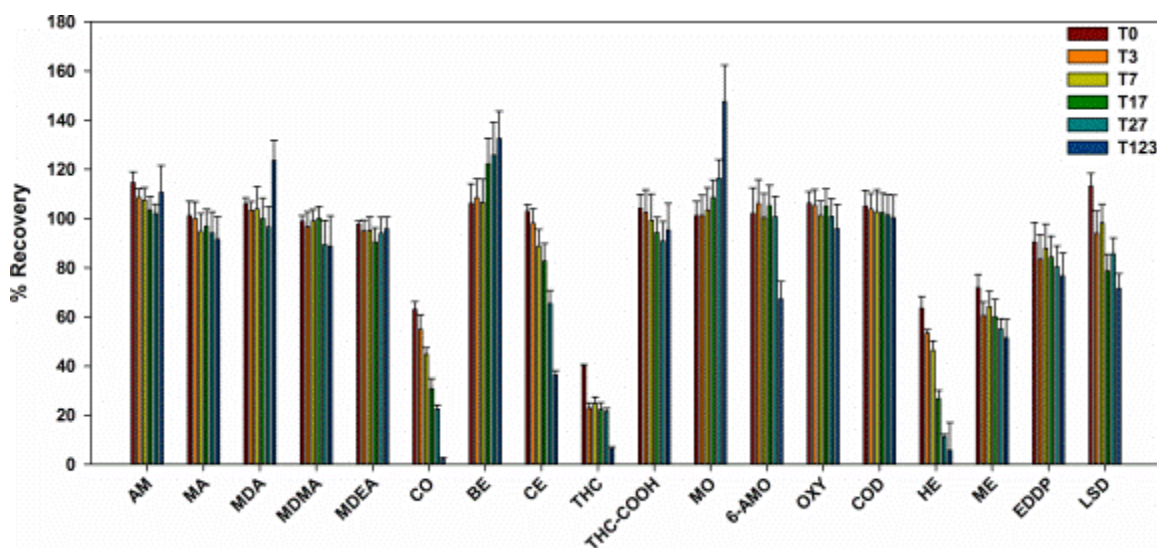


Fig. 2.6. Storage stability of drugs of abuse at -20 °C, during 123-day period.

In the case of opioids, % recoveries decrease/increase up to 23 % in the first month (T27) in relation to T0, with the exception of heroin. At the end of the 4-month study (T123) 6-acetylmorphine and methadone concentrations decreased by more than 25 % (34 % and 28 % recovery from T0 respectively). Similarly to cocaine, heroin showed 27 % recovery losses on the first week (T0 – T7), and 91 % decrease in recovery from T0 to T123. Analysis of wastewater is typically focused on morphine, a common residue to some opioids including heroin, which concords with the observed loss of heroin and increase of morphine (Castiglioni et al. 2008).

Table 2.5. Storage stability test. Results expressed in terms of % recovery (relative to fortification level, 250 ng/L) and % deviation for all analytes (relative to T0) after 3, 7, 17, 27 and 123 days. Test was performed in four independently stored aliquots of a single sewage sample.

Analyte	% Recovery						% Deviation					% Deviation				
	T0	T3	T7	T17	T27	T123	T0- T3	T0 - T7	T0 - T17	T0 - T27	T0 - T123	T0 - T3	T3 - T7	T7 - T17	T17 - T27	T27 - T123
AM	115	109	108	103	102	111	-5	-6	-10	-11	-4	-5	-1	-4	-1	8
MDA	106	103	104	100	97	124	-3	-2	-6	-9	17	-3	1	-4	-3	28
MDEA	98	95	95	90	94	96	-3	-2	-7	-4	-2	-3	0	-5	4	2
MDMA	99	97	99	100	89	89	-2	0	1	-10	-10	-2	3	1	-11	-1
MA	101	100	95	97	94	92	-1	-6	-4	-7	-10	-1	-5	2	-3	-3
BE	106	108	107	122	126	133	2	0	15	18	25	2	-2	15	3	5
CE	103	98	89	83	65	36	-5	-14	-20	-37	-65	-5	-10	-7	-21	-44
CO	63	55	45	31	22	2	-13	-29	-51	-64	-97	-13	-18	-31	-27	-91
6-AMO	102	106	100	105	101	67	4	-2	3	-1	-34	4	-5	5	-4	-33
COD	105	104	103	102	101	100	-1	-2	-3	-3	-4	-1	-1	0	-1	-1
ME	72	60	64	60	55	51	-16	-11	-16	-23	-28	-16	6	-6	-8	-7
EDDP	90	83	88	84	80	77	-8	-3	-7	-11	-15	-8	5	-4	-5	-5
HE	63	53	46	27	11	6	-16	-27	-58	-82	-91	-16	-13	-42	-58	-49
MO	101	101	104	109	116	148	0	2	7	15	46	0	2	5	7	27
OXY	106	105	101	105	101	96	-1	-5	-1	-5	-10	-1	-4	4	-4	-5
THC-COOH	104	102	99	94	91	95	-2	-5	-10	-13	-9	-2	-3	-5	-4	5
THC	40	23	25	22	22	6	-43	-38	-44	-46	-84	-43	8	-10	-3	-71
LSD	113	94	98	79	86	72	-17	-13	-31	-24	-37	-17	4	-20	9	-16

Almost half (46 %) of Δ -9-THC was lost during the first month (T0 – T27), and by the end of the study only 6 % from the initial concentration (250 ng/L) was recovered. In contrast, THC-COOH remained stable throughout the four-month study (up to 9 % loss from T0 – T123). This may suggest the potential presence of other type of transformation products (Heuett et al. 2014). The reported results in this study are in agreement with previous studies on storage stability of illicit drugs and their metabolites (Chiaia and Banta-green 2008; González-Mariño et al. 2010). Details on % recovery and % deviation values are shown in table 2.5.

2.4.6 Occurrence of illicit drugs and metabolites in sewage water

A total of 14 drugs of abuse were detected during the period of this study. Concentrations of the parent drugs and metabolites found in sewage waters for both locations (dorms and college campus) were plotted as box plots and shown in Fig. 2.7A and B. The frequency of detection is shown in parenthesis above the box plot. The boundaries of box plot cover 25th–75th percentile, the centerline indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles. THC-COOH (99 %), AM (95 %), and Δ -9-THC (44 %) were the most frequently detected compounds in the campus dorms, with median concentration of 420 ng/L, 320 ng/L, and 46 ng/L respectively (See Fig. 2.7A). This makes amphetamine and THC the two most potentially consumed drugs in the campus dorms. The remaining eleven parent drugs and metabolites were found in less than 15 % of the samples, with concentrations ranging from 4.4 ng/L (BE) to 980.7 ng/L (COD). Heroin, LSD, MDEA, and 6-acetylmorphine metabolite were not detected in any of the dorm samples. In

similar manner, THC-COOH (100 %) and AM (99 %) were also detected in high frequency in the main college campus pump station, with median concentrations of 464 ng/L and 1098 ng/L respectively (See Fig. 2.7B). In addition, compounds like benzoylecgonine, morphine, and codeine were also found in more than 50% of the samples. Cocaine, EDDP, heroin, LSD, MDA, MDEA, Methadone, and Δ -9-THC were not detected in any of this group of samples. Concentrations found throughout the course of this study were for the most part highly variable. Levels (ng/L) for all compounds were in the same range as those published in other countries with the exception of THC-COOH (30 – 2413 ng/L), which displayed higher levels than those commonly reported in the literature (see table 2.6) (Castiglioni et al. 2006; Huerta-Fontela et al. 2008; Boleda et al. 2009; Kasprzyk-hordern et al. 2009b; Postigo et al. 2010; Terzic et al. 2010; Baker and Kasprzyk-hordern 2011; Bijlsma et al. 2012). Concentration ranges and frequency of detection for all analytes are found in table 2.7.

Table 2.6. Concentrations of most frequently (> 50 %) detected illicit drugs (ng/L) in influent raw sewage and a comparison with previous studies.

Analyte	Netherlands ^a	Italy ^b	Switzerland ^b	Spain ^{c,d,e}	Croatia ^f	UK ^{g,h}	This study
Amphetamine	<4 - 682	14.7	<LOQ	3 - 688	2.6 - 31	292 - 12020	30 - 5956
Benzoylecgonine	<2 - 2306	1132	100 - 547	545 - 3790	89 - 325	126 - 2114	4 - 1214
THC-COOH	<7 - 375	62.7	7.2 - 91.2	23.5 - 402	21 - 128	-	30 - 2413
Morphine	-	83.3	55.4 - 204.4	25.5 - 278	160 - 476	819.2	21 - 491
Codeine	73 - 894	-	-	5.7 - 120	159 - 364	1732 - 32295	14 - 981

a. Bijlsma et al. 2012, b. Castiglioni et al. 2006, c. Huerta-Fontela et al. 2008, d. Postigo et al. 2010, e. Boleda et al. 2009, f. Terzic et al. 2010, g. Kasprzyk-hordern et al. 2009, h. Baker and Kasprzyk-hordern 2011.

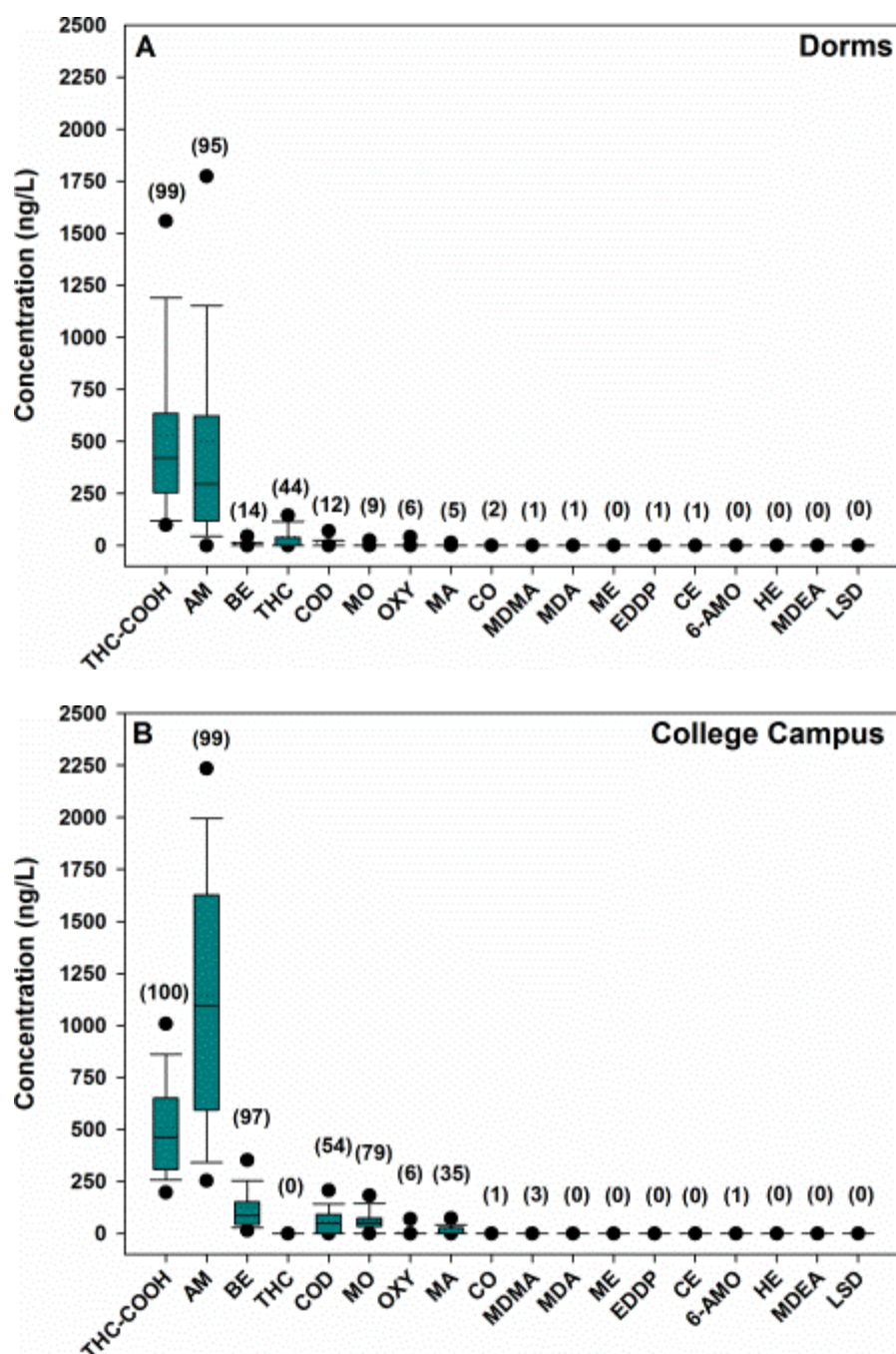


Fig. 2.7. Concentrations (ng/L) and frequency of detections (shown in parenthesis) of drugs of abuse in influent sewage samples from college dorms (4A Dorms) and main college campus (4B College Campus). The boundaries of box plot cover 25th-75th percentile, the center line indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles. Dotted line indicates the mean of the sample population.

Table 2.7. Concentrations and frequency of detections for parent drugs and metabolites found in sewage waters for both locations (dorms and college campus).

Analyte	Dorms Sewage Water (n=149)				College Campus Sewage Water (n=96)			
	Freq. (%)	Median (ng/L)	Min. (ng/L)	Max. (ng/L)	Freq. (%)	Median (ng/L)	Min. (ng/L)	Max. (ng/L)
THC-COOH	99	420	30	2413	100	464	152	1373
6-AMO	-	-	-	-	1	44	44	44
AM	95	320	30	5956	99	1098	195	3017
BE	14	25	4	350	97	87	13	1214
CE	1	77	77	77	-	-	-	-
CO	2	93	24	184	1	40	40	40
COD	12	46	14	981	54	91	43	575
EDDP	1	30	30	30	-	-	-	-
HE	-	-	-	-	-	-	-	-
LSD	-	-	-	-	-	-	-	-
MDA	1	348	348	348	-	-	-	-
MDEA	-	-	-	-	-	-	-	-
MDMA	1	30	30	30	3	81	79	108
ME	-	-	-	-	-	-	-	-
MA	5	44	26	56	35	32	20	783
MO	9	25	21	217	79	59	23	491
OXY	6	43	40	66	6	79	60	169
THC	44	45	22	2070	-	-	-	-

2.4.7 Estimation of drug consumption

Doses were back-calculated for THC, amphetamine, cocaine, and heroin in both dorms and main campus (see Fig. 2.8A and B). For THC, cocaine, and heroin, doses were produced using their major metabolites THC-COOH, benzoylecgonine, and morphine respectively using previously reported calculations (Zuccato et al. 2008; Postigo et al. 2011). Even though 6-acetylmorphine is an exclusive metabolic product of heroin, its excretion rate is low which may explain why it was only detected once in this study. Amphetamine is excreted mostly intact and therefore it was used for the calculations. Measured concentrations (ng/L) for THC-COOH, BE, MO, and AM were multiplied by daily flow rates (L/day) to obtain mass loads (mg/day) for the student population. Mass loads were normalized on a 1000 people, and a correction factor accounting for parent/drug residue molar ratio and excretion rate of the metabolite or parent compound was applied (Zuccato et al. 2008). Two recent publications by Baker et al. and Jones et al. proposed and implemented a more formal statistical framework to better account for sources of uncertainty. In addition to the back-calculation parameters listed above, analyte stability in wastewater and sorption to suspended particulate matter (SPM) was also accounted for (Baker et al. 2014; Jones et al. 2014). For this study, these additional variables (Analyte stability in wastewater and sorption to SPM) were not accounted for in our sampling process, therefore, the approach first described by Zuccato et al. was implemented here. Correction factor parameters, and theoretical daily doses used in this study are found in table 2.8.

Table 2.8. Analytes, correction factor parameters, and theoretical daily doses used for back-calculation estimates.

Analyte	Metabolite used for calculation	Molar mass ratio (Metabolite/Parent)	Excretion rate (%)	Daily Dose (mg)
AM	-	1	30	30
THC	THC-COOH	0.91	0.6	125
CO	BE	1.05	45	100
HE	MO	1.29	42.5	100

Zuccato et al. 2008

Even though campus population could reach as many as 34,187 people, due to the largely commute dependent and transient nature of the student population, it is difficult to estimate a typical population size. Therefore, for this study we have calculated possible minimum and maximum consumption levels using both the total campus population (35,187 people) and the maximum number of daily occupied parking spaces (9,456). The rationale behind the parking spaces is based on a 2009 FIU's climate action plan report, in which it was estimated that more than 97 % of the student, faculty and staff population commuted by personal vehicle distances of approximately 15 miles per trip (FIU President's Climate Commitment Task Force 2009).

Fig. 2.8 shows box plots of consumption levels for THC, amphetamine, cocaine, and heroin in the dorms (2.8A) and the main campus (2.8B). THC average dose levels in the dorms were higher (233 ± 199 doses/day/1000 people) than those in the main campus and those reported in the literature (4 – 61 doses/day/1000 people) (Zuccato et al. 2008; Boleda et al. 2009; Postigo et al. 2010). THC intake within the main campus ranged between 12.7 and 46 daily doses/1000 people. These values were based on a 125 mg daily dose of marijuana (Zuccato et al. 2008). Moreover, Average levels of amphetamine among the student population in the dorms (22 ± 31 daily doses/1000 people) were within

the range of those reported by Huerta-Fontela et al. (2.5 doses/day/1000 people), Zuccato et al. (2.6 doses/day/1000 people), Postigo et al. (9.2 daily doses/1000 people), and Kasprzyk et al. (83.3 daily doses/1000 people) (Huerta-Fontela et al. 2008; Zuccato et al. 2008; Kasprzyk-hordern et al. 2009a; Postigo et al. 2010). In addition, consumption of amphetamine within the main campus was in the range of 2.7 and 9.8 doses/day/1000 people, which is much lower than those from the dorms, yet still within the range of those reported in the literature as mentioned previously. Dose levels were based on a 30 mg daily intake of amphetamine (Zuccato et al. 2008).

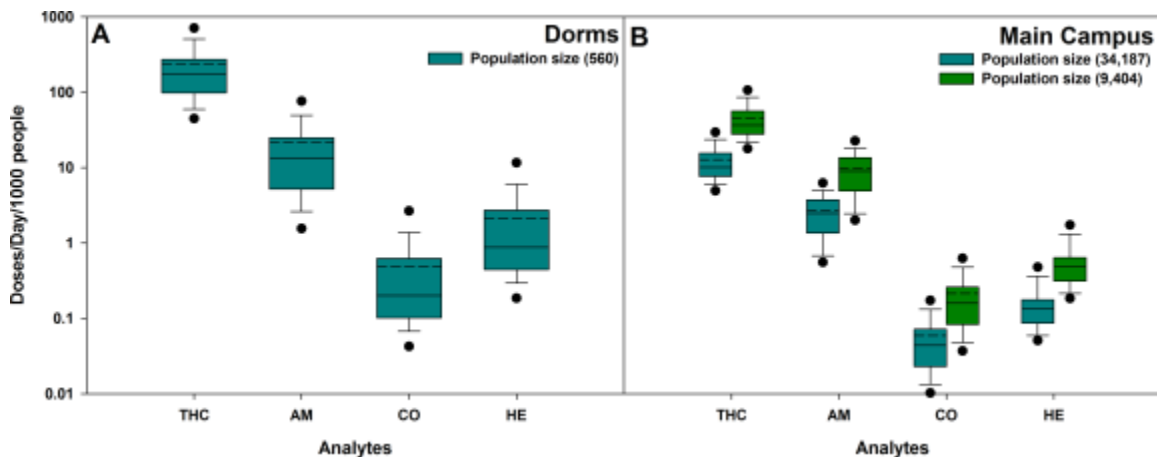


Fig. 2.8. Daily dose consumption (doses/day/1000 people) estimates for THC, amphetamine, heroin, and cocaine in both dorms (5A) and main campus (5B). Campus estimates were determined using both population sizes.

Average heroin intake in the dorms was similar to reported values by Zuccato (2.3 doses/day/1000 people) (Zuccato et al. 2008). Main campus heroin intake, on the other hand, was very minor with average levels ranging from 0.18 and 0.64 daily doses/1000 people depending on the population size used to calculate these values. As mentioned before morphine (target residue used for back-calculation purposes) could be a transformation product for other opiates including morphine itself. This introduces even

greater uncertainty of encountering any heroin consumption in the dorms and/or campus. With regards to cocaine, even though benzoylecgonine (metabolite used for cocaine consumption determination) was found in 97 % of the samples in the main college campus, the magnitude of its levels is rather low (0.1 – 0.2 doses/day/1000 people) suggesting consumption by only a minor fraction of the student population, or possible degradation in the samples. Low consumption levels of cocaine were also observed in the dorms with maximum observed levels of 2.8 doses/day/1000 people (dorms). All observed cocaine consumption levels were lower than mean values reported in Belgium, Ireland, Italy, Spain, Switzerland, and UK (Bones et al. 2007; Zuccato et al. 2008; Kasprzyk-hordern et al. 2009a; Nuijs et al. 2009a, 2009b, 2009c; Postigo et al. 2010). Dose determination for heroin and cocaine was based on single daily doses equivalent to 30 mg and 100 mg respectively (Zuccato et al. 2008).

2.4.8 Amphetamine and marijuana consumption variations

Consumption levels of samples collected at the beginning, middle, and end of a semester (2012-2013 academic year) in one of the college dorms were compared to determine potential variations throughout the semester. Adderall, an Attention Deficit Hyperactivity Disorder (ADHD) drug composed of a mixture of amphetamine salts, is known to be frequently used by students without prescription for performance and cognitive enhancement purposes (Burgard et al. 2013). In recent years, a study done at a college campus in Oregon demonstrated an increase in amphetamine levels during periods of high stress levels such as midterms and finals week, with a 760 % consumption increase (compared to base-line levels) during finals week (end of the

semester) (Burgard et al. 2013). In contrast to the previously mentioned study, Fig. 2.9A shows that the average amphetamine dose consumption remained relatively constant at the beginning, middle and end of the semester, yet larger variability was seen at the end of the semester. On the contrary, higher average doses of cannabinoids were observed at the end of the semester, suggesting larger consumption of marijuana during this period (see Fig. 2.9B). In general, consumption of marijuana was always greater than amphetamine throughout the semester.

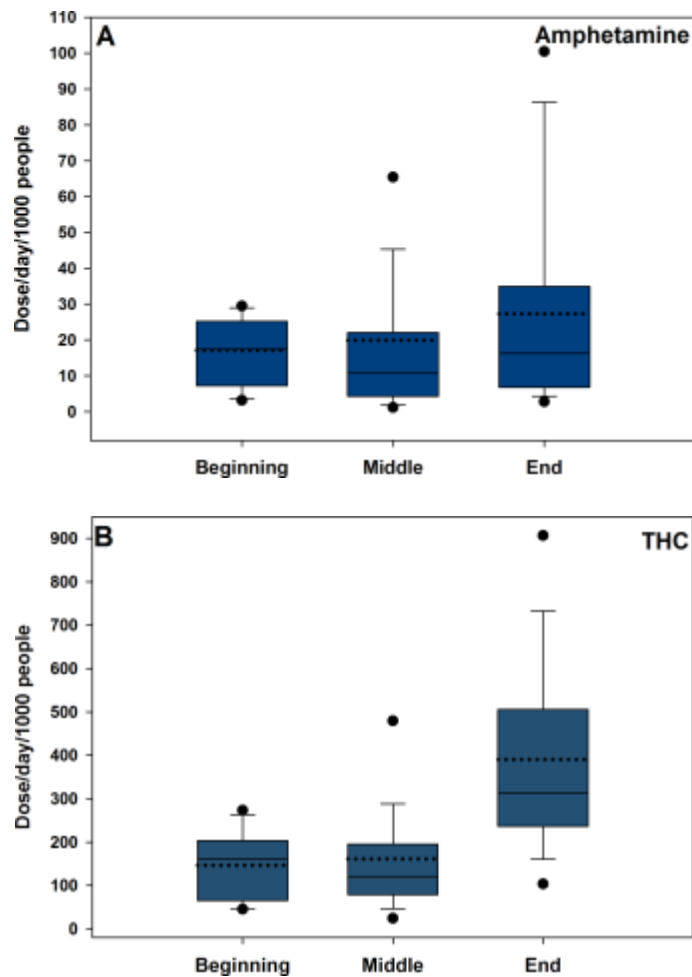


Fig. 2.9. Drug consumption in the dorms throughout the semester for amphetamine (6A) and THC (6B).

The boundaries of box plot cover 25th-75th percentile, the center line indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles. Dotted line indicates the mean of the sample population.

Daily variations of amphetamine and cannabinoids were assessed in the main campus during the last three weeks of classes. Fig. 2.10A and B show the average daily doses of amphetamine and marijuana (respectively) consumed during this period. A statistically significant change in consumption levels between the weekdays and the weekends for both amphetamine ($p < 0.001$) and marijuana ($p < 0.001$) was observed, with higher doses taken during the week. These findings are consistent with the number of students attending classes during the weekdays versus the weekends.

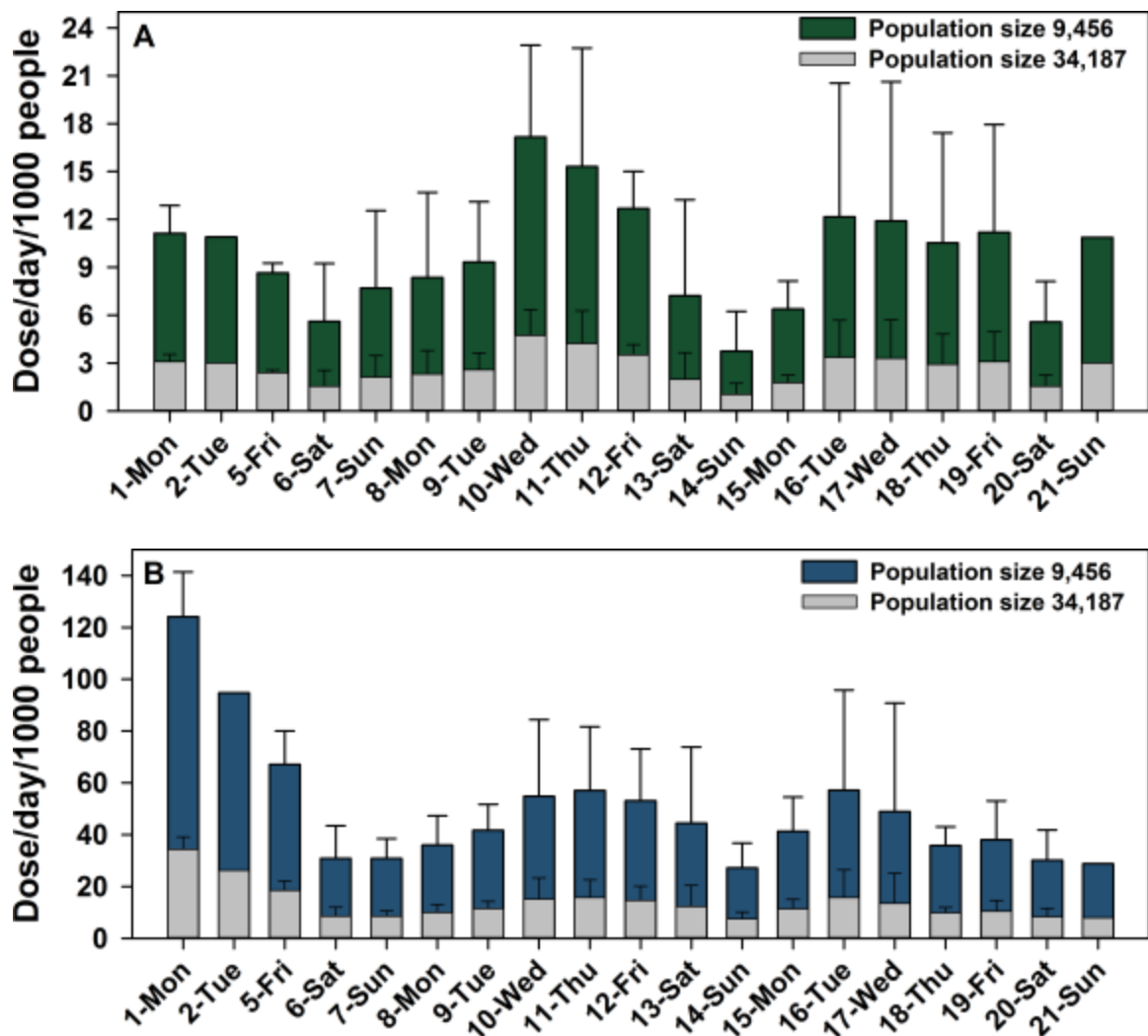


Fig. 2.10. Daily variations of amphetamine (7A) and cannabinoid (7B) consumption during the last three weeks of classes in the main campus. Campus estimates were determined using both population sizes.

2.5 Conclusion

This manuscript reports a novel online-SPE-LC-HRMS method, developed for the analysis of 18 drugs of abuse and their metabolites in sewage water matrixes. The method consisted of three very simple steps (filtration, 10X dilution, and fortification) prior to automated injection (5 mL), pre-concentration, and clean-up of samples. Method validation parameters such as SPE recoveries, matrix effects, linearity, reproducibility, and method detection limits were successfully assessed and optimized. The validated method was applied to real samples collected from two different locations at a college campus (college dormitory and campus-wide). Average concentrations for college students residing in the dorms ranged from 30 ng/L to 537 ng/L for EDDP/MDMA and THC-COOH respectively. Detections in the main campus ranged from 40 ng/L to 1,159 ng/L for cocaine and amphetamine. Measured concentration values for amphetamine, THC-COOH, benzoylecgonine, and morphine were used for mass loads and daily dose consumption determination of amphetamine, THC, cocaine, and heroin respectively. Results suggest greater consumption of these drugs in the dorms than the main campus, with a peak in THC intake at the end of the semester in the dorm population. In addition, similar daily trends are seen for both amphetamine and THC in the main campus (higher consumption during the weekdays and the weekends).

2.6 Acknowledgements

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CHAPTER 3

Understanding the magnitude of emergent contaminant releases through target screening and metabolite identification using high resolution mass spectrometry:

Illicit drugs in raw sewage influents

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3.1 Abstract

A QExactive Orbitrap was used for the identification of phase I and II transformation products (TPs) of illicit drugs in raw sewage influents. Two operating modes, (targeted MS² and Data-dependent screening) were used for data acquisition. Even though, data-dependent scan is a faster route towards the potential identification of metabolites, it suffered from its limitation to provide enough data points across the chromatographic peak during the MS² cycle in contrast to targeted MS². Therefore, the later technique was implemented as the method of choice in this study for the positive confirmation and quantitation of TPs (n = 54). The vast majority of the identified TPs were products of phase I transformation reactions, with the latter being more prevalent in the nature. Estimated mole fractions showed that for a large number of the analytes, TPs must also be monitored in order to fully understand their environmental fate and calculate potential consumption.

3.2 Introduction

The prevalence of illicit drug consumption continues to be a growing problem worldwide. In US alone, it was estimated that 23.9 million Americans aged 12 or older used illicit drug in 2012 [1]. These figures do not even consider the abuse of common prescription drugs such as Adderall (amphetamine) and Oxycontin (oxycodone) [2–4]. Upon consumption, drugs of abuse (DOA; illicit and prescription) are subjected to phase I and II metabolic transformations in the body. Phase I reactions involve hydrolysis, reduction, and oxidation, making their products slightly more hydrophilic than the parent by exposing or introducing functional groups such as –OH, –NH₂, –SH, or –COOH. In

addition, Phase II reactions include glucuronidation, sulfation, acetylation, methylation, glutathione conjugation, and conjugation with amino acids such as glycine, taurine, and glutamic acid [5,6], also increasing the polarity of their transformation products (TPs)[7]. A combination of phase I and phase II TPs plus their unchanged parents are excreted via urine and/or faeces. Multiple studies have demonstrated that once these excretion products reach wastewater treatment plants, not all of the compounds can be efficiently removed prior to the release of effluent waters into aquatic environments[8–11]. Even treatment plants equipped with what could be classified as advanced treatment are not designed to eliminate these compounds from their effluents. Emission of these compounds will likely continue until new materials and or technologies are developed for their removal. Occurrence of DOAs in sewage influents, effluents, as well as surface waters, have previously been reported with concentrations reaching up to 27,500 ng/L [12]. Moreover, analysis of raw sewage water from small settings such as a college campus can provide further information on metabolic transformation products since the residence time of these compounds in the actual sewage is minimal compared to that of influent waters arriving at WWTPs where additional TPs can be produced by microbial activity during transit. For instance, amphetamine concentrations of 2,100 ng/L were reported in a college campus in Oregon, when comparing this value to those reported by Chiaia from influent sewage waters throughout the US (550 ng/L), a loss of the parent compound is noted confirming the possibility of external transformations [2]. External transformations include photodegradation, hydrolysis, chemical oxidation and biodegradation, and could also be observed in aquatic environments, increasing the possibility of finding new metabolites that were not previously taken into account.

Assessment on the occurrence of illicit drugs have been for the most part monitored using the unchanged form of the drug (parent), and in some cases by the evaluation of very few well known metabolites [10,13–17]. Furthermore, it is sometimes critical to monitor metabolites since many compounds are excreted as glucuronide or sulfate conjugates and are likely to be transformed back into the parent molecule from adducts during treatment process [15,18]. As an example, deconjugation of one of the phase II metabolites of morphine and heroin (morphine-3 β -D-glucuronide) is converted back into the parent molecule (morphine) once it reaches aquatic environments [19,20]. As in the case with antibiotics, lack of availability of metabolite standards in the market, diminishes the ability for identification of metabolites and other transformation products [21]. In any way, absence of the parent drug does not necessarily indicate lack of consumption, efficiency of treatment plants, or analytical issues with the method of detection; it could very well mean that there is simply not enough information on metabolic transformations to confirm their presence.

Robust and sensitive analytical tools are required to identify and confirm the low abundant metabolites (parts per trillion level) in the complex matrices. To date, high resolution mass spectrometric instruments such as the quadrupole-time-of-flight (qTOF), Fourier transform-ion cyclotron resonance (FT-ICR), and the Orbitrap have become the ideal tools for the identification of metabolites and transformation products, thanks to their high resolving power (30,000–1,000,000), high mass accuracy (<5 ppm), linear dynamic range, and sensitivity [5,22–24]. The Orbitrap is, however, gaining popularity for the identification of metabolites due to its sensitivity, large dynamic range and its

ability to use an external calibration as opposed to an internal calibration to maintain high accuracy (as in the qTOF) [23,25,26].

In the recent years there have been some reports in the identification of new TPs for cocaine and codeine utilizing high resolution instruments as the ones mention above [27,28] (Bijlsma 2013, Wick 2011). Bijlsma's group focused on the degradation products of cocaine and its major metabolite (benzoylecgonine) resulting from in lab controlled degradation experiments (e.g. chlorination, hydrolysis, and photo-degradation. Four newly identified TPs were reported in this study, three of them were isomers. However, target screening of these TPs in influent, effluent, and surface waters was carried out using a TSQ (QqQ) mass spectrometer in SRM. The downside of this approach is that it increases the possibility for neglecting potential TPs that can arise from matrix interactions. In addition, a similar screening method to the one reported in this manuscript have been perform in the area of pesticides, validating the importance of combined target analysis and target screening for the efficient identification and monitoring of TPs [29].

Data acquisition is an important aspect in the process of TP identification when using an Orbitrap, since intensity threshold parameters and limited data points across the chromatographic peak can potentially omit useful data or, on the contrary, acquire unreliable data. Therefore, the objective of the present study is a threefold: (1) to develop and compare two workflows based on two different types of acquisition modes (targeted-MS² and data-dependent) to identify the most complete and reliable manner of data collection; (2) to implement these workflows in the identification of illicit drug metabolic contaminants of emerging concern; and (3) to further determine their abundances relative

to their parent compounds from % mole fraction determinations, and their prevalence as a way to demonstrate the importance of their assessment.

3.3 Experimental

3.3.1 Reagents and chemicals

Illicit drug and metabolite standards as well as some of their deuterated analogs used in the analytical method cocaine (CO, cocaine-d3), benzoylecgonine (BE, benzoylecgonine-d3), cocaethylene (CE, cocaine-d3), codeine (COD, codeine-d6), morphine (MO, morphine-d6), 6-acetylmorphine (6-AMO, morphine-d6), oxycodone (OXY, oxycodone-d6), methadone (ME, methadone-d9), EDDP (methadone-d9), heroin (HE, morphine-d6), LSD (LSD-d3), Δ -9-THC (THC, Δ -9-THC-d3), 11-nor-9-carboxy- Δ -9-THC (THC-COOH, 11-nor-9-carboxy- Δ -9-THC-d3), amphetamine (AM, amphetamine-d5), MDA (MDA-D5), MDEA (MDEA-D6), MDMA (MDMA-D5), and, methamphetamine (MA, Methamphetamine-d14). However, 6-AMO and HE were never detected, nor any possible TPs, therefore they were left out of the rest of the study. Standards and deuterated analogues were purchased from Cerilliant (Round Rock, TX, USA). Standard stock solutions containing target analytes or deuterated compounds were prepared at a concentration of 20 μ g/L in methanol and stored in the dark at -20°C. Optima LC/MS grade methanol, water, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used for standard preparation and HPLC mobile phases. Optima LC/MS grade formic acid was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used to prepare 0.1% solutions for modifier mobile phase. 1.0 and 0.45 micrometer PreSep Prefilter, glass filters were also purchased from Fisher Scientific.

3.3.2 Sample collection and preparation

A total of 12 200 mL grab influent raw sewage water samples were collected during the month of April 2013 from the main lift station at a college campus using an automated ISCO sampler. Samples were transported to the laboratory and were filtered using a 1.0 micron PreSep Prefilter glass filter followed by 0.45 μm PreSep Prefilter glass filter. Samples were stored in clear polyethylene terephthalate (PET) bottles in the dark at -20°C until the time of analysis. Filtration of the sample is essential to remove most of the matrix components including bacteria. Therefore, very minimal or no biological activity, or photodegradation should be observed in the dark and at -20°C [30,31]. On the day of analysis raw sewage water samples were allowed to thaw at room temperature and shaken for about 10 seconds. Samples were diluted ten times (10x) in deionized water (DI) water, and fortified with the appropriate internal standards (200 ng/L). A seven-point calibration curve (5 ng/L- 500 ng/L) containing illicit standards and their deuterated analogues was prepared using DI water.

3.3.3 Pre-concentration and chromatographic separation of analytes

Pre-concentration of samples was achieved using an EQuan online solid phase extraction (SPE) system from Thermo Scientific (Waltham, MA, USA). The on-line EQuan SPE procedure has been explained in detail elsewhere [32,33]. In short, the pre-concentration and analytical separation of analytes were performed using a Thermo EQuan Max online-SPE system equipped with a Hypersep Retain PEP column (20 x 2.1 mm x 12 μm) and Hypersil Gold PFP (50 x 2.1 mm x 1.9 μm), respectively, both purchased from the same manufacturer (Thermo Scientific, USA). Methanol and 0.1%

formic acid were used in gradient elution mode for separation of analytes at 1000 $\mu\text{L}/\text{min}$ and 200 $\mu\text{L}/\text{min}$ for pre-concentration and analytical separation, respectively [34]. Sample volume selected was 5 mL, and a three minute washing step was performed to remove most of the matrix components. This small volume is a compromise between method sensitivity and matrix effects. Larger sample volumes can increase ion suppression due to matrix effects, affecting the overall sensitivity of the method. Moreover, to account for possible ion suppression due to interactions with matrix components, isotopically label internal standards were used. Future studies should, however, be conducted to fully understand the magnitude of the effect the sample matrix imposes on the TPs. Figures of merit for the method including linearity, precision (%RSD), relative recoveries, and method detection limits (MDLs) are shown in table 3.1.

Table 3.1. Quality control parameter of analytical method: linearity (R²), precision (%RSD), relative recoveries (spike recoveries), and method detection limits (MDLs) in HPLC water and influent raw sewage water.

ANALYTE	LINEARITY R ² (n=8)	HPLC WATER			SEWAGE WATER		
		% RSD (n=3)	RELATIVE RECOVERIE S (%) (n=3)	MDLs (ng/L) (n=7)	% RSD (n=3)	RELATIVE RECOVERIE S (%) (n=3)	MDLs (ng/L) (n=7)
AMPHETAMINE	0.9995	1.92	102	0.87	3.03	98	9.83
(-)-11-NOR-CARBOXY-THC	0.9965	1.33	102	1.81	3.08	89	-- ^d
6-ACETYLMORPHINE ^b	0.9993	5.93	103	1.59	1.76	76	70.9
BENZOYLECGONINE	0.9999	0.50	100	0.48	5.01	100	4.74
COCAETHYLENE ^a	0.9985	1.04	98	1.18	0.62	121	24.71
COCAINE	0.9995	1.50	101	0.62	0.90	115	12.77
CODEINE	0.9986	2.03	98	0.60	1.44	103	23.44
DELTA-9-THC	0.9986	2.45	86	0.79	11.59	108	-- ^d
DL-METHADONE	0.9996	1.07	102	0.76	1.54	102	35.74
EDDP ^c	0.9996	1.33	101	0.64	1.20	107	18.19
HEROIN ^b	0.9987	4.66	110	1.92	4.48	60	18.57
LSD	0.9989	3.23	96	0.71	0.75	95	61.8
MDA	0.9998	2.16	100	0.79	3.52	118	19.45
MDEA	0.9998	0.69	102	0.43	3.50	114	4.00
MDMA	0.9989	1.63	101	0.68	3.34	111	10.50
METHAMPHETAMINE	0.9998	2.65	100	0.76	1.07	112	3.39
MORPHINE	0.9999	1.50	100	0.83	0.97	102	61.52
OXYCODONE	0.9998	1.77	98	1.28	3.93	113	4.53

^a Cocaine internal standard was used for quantitation

^b Morphine internal standard was used for quantitation

^c Methadone internal standard was used for quantitation

^d Could not be calculated due to high background

3.3.4 Mass spectrometric Analysis

Detection of analytes was performed in positive ionization mode on a QExactive Orbitrap high resolution mass spectrometer equipped with heated electrospray ionization source (HESI-II) (Thermo Scientific, NJ, USA). Data was acquired using two different pathways explained in the schematic presented in Fig. 3.1. In the first pathway, samples were initially run in full scan mode (m/z 100 – m/z 500) at a resolving power of 70,000 and quantitation of known target analytes was performed using TraceFinder 3.0 processing software (Thermo Scientific, NJ, USA). In addition, raw data was also processed using a metabolite finding software (MetWorks 1.3 from Thermo Scientific, NJ, USA). The software created a list of possible phase I and II reactions and their corresponding exact mass difference expected in the products (Table 3.2). Given the target analyte molecular formula, polarity, a minimal signal to noise ratio (>3) threshold and the desired mass accuracy (< 5 ppm), the software identified potential phase I and II TPs by comparing raw files from a sample and a standard (control). Full scan raw data files were searched (in Xcalibur software) for these exact masses and an elemental composition was obtained based on the observed mass. In the case where multiple elemental compositions were possible for an observed mass, mass error (in ppm) and knowledge obtained on the type of transformation in MetWorks software was used for the final structure selection. Targeted MS/MS was then performed on the identified TPs at 35, 000 resolution and at varying levels of collision energies (10, 25 and 35) to obtain the best fragmentation patterns. At this point, the potentially identified molecule was fragmented *in-silico* using spectral interpretation software (Mass Frontier from Thermo

Scientific, NJ, USA) to confirm the identity of the TPs using the accurate mass MS/MS data.

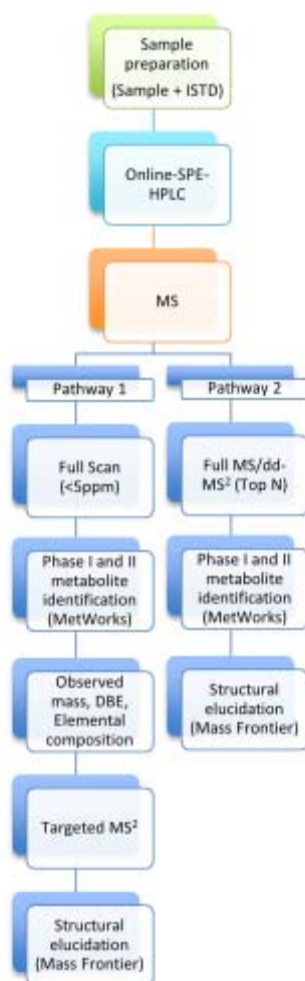


Fig. 3.1. Metabolite identification using two different operational modes in a Q Exactive Orbitrap.

In the second pathway, data was acquired in data dependent mode which includes a full scan (m/z 100 – m/z 500) at a resolving power of 70,000 followed by a MS/MS data dependent scan event (at 35,000 resolution) triggered by the set intensity threshold (AGC target 1×10^5) for the 3 most abundant precursor ions. Normalized collision energy (NCE) was set constant to 35 for MS/MS all selected ions. The full scan data was processed in MetWorks in the same way as explained before. For the identified TPs,

acquired MS/MS data was compared with the theoretical spectra generated from the assigned chemical structure by the fragment ion search (FISh) feature in Mass Frontier software, similar to above. Similarities between the spectra, DBE along with isotope patterns were considered to help in the structural assignment of TPs. Detailed operating conditions of both the acquisition modes (targeted MS/MS and data dependent scan) are listed in Table 3.3.

Table 3.2. Phase I and phase II transformations and their exact masses.

Modification^a	Phase type	Exact mass
Debenzylation	I	-90.047
Decarboxylation	I	-43.9898
Hydroxymethylene Loss	I	-30.0106
Nitro Reduction	I	-29.9742
Deethylation	I	-28.0313
Alcohol Dehydration	I	-18.0106
Sulfoxide to Thioether	I	-15.9949
Isopropyl to Acid	I	-14.052
Demethylation	I	-14.0157
Desaturation	I	-2.0157
Oxidation and Demethylation	I	-1.9793
Saturation	I	2.0157
Hydroxylation and Desaturation	I	13.9793
Aromatic Hydroxylation	I	15.9585
Hydrolysis	I	18.0106
Demethylation to Carboxylic Acid	I	29.9742
Alkenes to Dihydrodiols	I	34.0055
Methylation	II	14.0157
Hydroxylation and Methylation	II	30.0106
Acetylation	II	42.0106
Glycine Conjugation	II	57.0215
Taurine Conjugation	II	107.0041
Glutathion Conjugation	II	305.0682
Sulfation	II	79.9568

^a Suggested modification by MetWorks metabolic profiling software

Table 3.3. Source, Full scan, and MS/MS operating conditions.

PARAMETER		SETTING
Source		^a HESI II
Capillary temperature (°C)		300
S-lens voltage		50
Source voltage (kV)		3
FULL-MS PARAMETERS		
Mass range (m/z)		100 - 500
Resolution settings		70,000
AGC Target		3 x 10 ⁶
Max injection time (ms)		200
MS/MS PARAMETERS	Targeted-MS/MS	dd-MS/MS
HCD		
Resolution settings	35,000	35,000
AGC Target	2 x 10 ⁴	1 x 10 ⁵
Max injection time (ms)	40	100
Isolation window (m/z)	2	2
Collision energy NCE	10, 25, 35	35
Intensity threshold	--	1 x 10 ⁵ (10.0% underfill)
Loop count	--	3

^aHESI: heated electrospray ionization

3.3.5 Concentration and mole fractions of TPs

Trace Finder 3.0 (Thermo Scientific, NJ, USA) was used to calculate the concentration of all target analytes. Because of lack of available authentic standards for TPs and because of their structural similarity with the parent molecule, a relative response factor obtained for the parent DOAs was used to determine metabolite concentrations. Mole fractions of the parent DOAs and their TPs were calculated by taking the ratio of measured concentration to the molar masses. The mole fraction of each TP was computed for every sample and these values were used to compute standard deviation and average mole fraction.

3.4 Results and discussion

3.4.1 Transformation products of DOAs

Initially 16 target analytes (DOAs) including 12 parent compounds and 4 of their metabolites were used to further identify 54 TPs in raw sewage influents. From the 12 parents, 45 TPs were identified. 7 of them were target analytes (parents and metabolites) leaving 38 remaining TPs. Out of the 38, 19 of them had an initial positive match (based on m/z) with other compounds. After further MS/MS analysis, it was determined that only 9 of them had a positive spectral match with other compounds (ephedrine, ethylamphetamine, methylone, and norbenzoylecgonine) from TraceFinder database and/or had been previously been reported [27,35,36] (see table 3.4). Up to this point 29 TPs remained. 2 of the 29 TPs were formed from different parents; therefore, from 12 initial parents positive identification was obtained for 27 TPs. From the group of the 4 target metabolites, 9 TPs were originally identified. However, one of them resulted being benzoylecgonine (BE) which had formed from cocaethylene (CE). 3 of the remaining 8 had an initial positive match with other compounds. Nevertheless, none of them had a positive MS/MS spectral match. Hence, 8 TPs were identified from the 4 target metabolites. In the end and to our knowledge, a total of 35 TPs (never monitored in the environment) formed from 16 target analytes (12 parents and 4 metabolites) have been potentially identified. The overall description of the process is shown in Fig. 3.2.

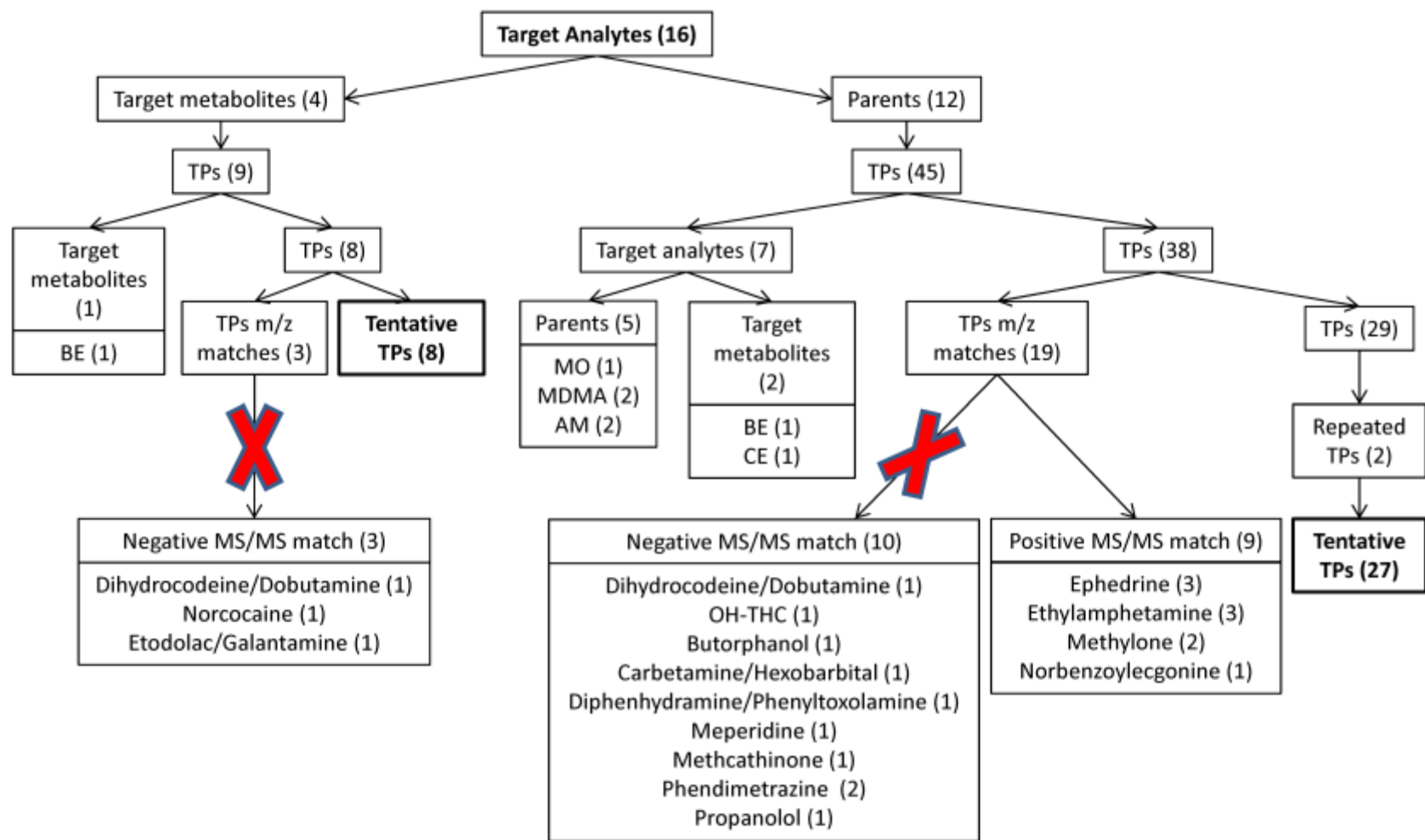


Fig. 3.2. Diagram distribution of 54 tentatively identified TPs divided in two main categories. TPs formed from parent DOAs (45), and TPs formed from target DOA metabolites (9). Parenthesis indicated number of TPs. X indicates TPs that had the same m/z as other compounds, but no spectral match.

Table 3.4. MS/MS fragmentation for 9 TPs that had a positive spectral match with ephedrine, ethylamphetamine, methyloine, and norbenzoylecgonine.

NAME	ELEMENTAL FORMULA	EXACT MASS (m/z)	OBSERVED MASS (m/z)	RETENTION TIME	DBE	MASS ACCURACY (ppm)	COMPOUND MATCH	REPORTED MASS (m/z)
AM-4, MA-1, MDMA-3	C ₁₀ H ₁₆ NO	166.1226	166.1223	11.55	3.5	-1.8	Ephedrine	166.1232
	C ₇ H ₇	91.0542	91.0548		4.5	6.8		91.0550
	C ₉ H ₇	115.0542	115.0549		6.5	5.8		115.0547
	C ₉ H ₁₁ N	133.0886	133.0888		5.0	1.3		133.0891
	C ₁₀ H ₁₄ N	148.1120	148.1123		4.5	0.8		148.1126
	C ₉ H ₉	117.0699	117.0703		5.5	3.4		-
MA-4, MDEA-4, MDMA-5	C ₁₁ H ₁₈ N	164.1434	164.1431	12.03	3.5	-1.8	Ethylamphetamine	164.1434
	C ₇ H ₇	91.0542	91.0547		4.5	5.5		91.0542
	C ₉ H ₁₁	119.0855	119.0858		4.5	2.0		119.0856
	C ₈ H ₉	105.0699	105.0703		4.5	4.0		-
	C ₈ H ₁₁ O	123.0804	123.0807		3.5	2.1		-
	C ₁₀ H ₁₁ O	147.0804	147.0805		5.5	0.4		-
MDEA-6, MDMA-4	C ₁₁ H ₁₄ NO ₃	208.0968	208.0969	11.72	5.5	-0.2	Methyloine	208.0968
	C ₁₀ H ₁₀ NO	160.0757	160.0758		6.5	0.7		160.0756
	C ₁₁ H ₁₂ NO ₂	190.0862	190.0863		6.5	0.2		190.0861
CO-2	C ₁₅ H ₁₈ NO ₄	276.1230	276.1226	11.90	7.5	-1.4	Norbenzoylecgonine	276.1229
	C ₈ H ₁₂ NO ₂	154.0862	154.0864		3.5	0.6		154.0853
	C ₈ H ₁₀ NO	136.0757	136.0758		4.5	1.8		136.0744
	C ₇ H ₅ O	105.0335	105.0338		5.5	2.9		105.0332
	C ₄ H ₁₂ NO ₂	106.0863	106.0867		-0.5	3.9		-

Table 3.5. Elemental composition, retention time, mass accuracy and the acquisition mode of identified TPs. Names in bold represent the parent molecule.

Name	Metabolite type	Elemental formula	Exact mass (m/z)	Observed mass (m/z)	RT	DBE	Mass accuracy (ppm)	MS/MS
AM		C ₉ H ₁₄ N	136.1121	136.1120	11.43	3.5	-0.7	t-MS ²
AM-1 ^b	I	C ₈ H ₁₂ N	122.0964	122.0965	11.51	3.5	0.8	t-MS ²
AM-2 ^b	I	C ₉ H ₁₆ NO ₂	170.1176	170.1173	11.87	2.5	-1.8	t-MS ²
AM-3 ^b	I	C ₉ H ₁₆ NO	154.1226	154.1223	12.12	2.5	-1.9	t-MS ²
AM-4 (MA-1, MDMA-3) ^a	II	C ₁₀ H ₁₆ NO	166.1226	166.1223	11.55	3.5	-1.8	t-MS ²
AM-5 ^b	II	C ₁₉ H ₂₉ N ₄ O ₆ S	441.1802	441.1810	12.05	7.5	1.8	t-MS ²
AM-6 ^b	I	C ₈ H ₁₂ NO	138.0913	138.0911	12.08	3.5	-1.4	t-MS ²
AM-7 ^b	I	C ₇ H ₁₀ NO	124.0757	124.0758	11.38	3.5	0.8	t-MS ²
MDA		C ₁₀ H ₁₄ NO ₂	180.1019	180.1015	11.82	4.5	-2.2	t-MS ²
MDA-1 (AM, MA-2) ^a	I	C ₉ H ₁₄ N	136.1121	136.1120	11.43	3.5	-0.7	t-MS ² /DD
MDA-2 ^b	I	C ₁₀ H ₁₄ NO	164.1070	164.1065	12.15	4.5	-3.0	t-MS ²
MDA-3 ^b	I	C ₁₀ H ₁₄ NO ₄	212.0917	212.0926	13.06	4.5	4.2	t-MS ²
MDA-4 (MDMA, MDEA-2) ^a	II	C ₁₁ H ₁₆ NO ₂	194.1176	194.1174	11.92	4.5	-1.0	t-MS ² /DD
MDA-5 ^b	II	C ₁₂ H ₁₇ N ₂ O ₃	237.1234	237.1230	11.51	5.5	-1.7	t-MS ²
MDA-6 ^b (MDMA-1, COD-3) ^a	II	C ₁₁ H ₁₆ NO ₃	210.1125	210.1122	11.45	4.5	-1.4	t-MS ²
MDA-7 ^b	I	C ₁₀ H ₁₀ NO ₂	176.0706	176.0704	11.44	6.5	-1.1	t-MS ²
MDA-8 ^b	I	C ₉ H ₁₂ NO ₃	182.0812	182.0808	11.68	4.5	-2.2	t-MS ²
MA		C ₁₀ H ₁₆ N	150.1277	150.1275	11.80	3.5	-1.3	t-MS ²
MA-1 (AM-4, MDMA-3) ^a	I	C ₁₀ H ₁₆ NO	166.1226	166.1223	11.56	3.5	-1.8	t-MS ² /DD
MA-2 (AM, MDA-1) ^a	I	C ₉ H ₁₄ N	136.1121	136.1120	11.40	3.5	-0.7	t-MS ² /DD
MA-3 ^b	I	C ₁₀ H ₁₄ N	148.1121	148.1118	11.50	4.5	-2.0	t-MS ² /DD
MA-4 (MDEA-4, MDMA-5) ^a	II	C ₁₁ H ₁₈ N	164.1434	164.1431	12.03	3.5	-1.8	t-MS ²
MA-5 ^b (MDEA-5) ^a	II	C ₁₂ H ₁₈ NO	192.1383	192.1378	12.30	4.5	-2.6	t-MS ²
MDEA		C ₁₂ H ₁₈ NO ₂	208.1332	208.1329	11.97	4.5	-1.4	t-MS ²
MDEA-1 ^b	I	C ₁₂ H ₁₈ NO ₃	224.1281	224.1278	11.50	4.5	-1.3	t-MS ²
MDEA-2 (MDMA, MDA-4) ^a	I	C ₁₁ H ₁₆ NO ₂	194.1176	194.1174	11.92	4.5	-1.0	t-MS ² /DD
MDEA-3 ^b	I	C ₁₂ H ₂₀ N	178.1590	178.1587	12.10	3.5	-1.7	t-MS ²
MDEA-4 (MA-4, MDMA-5) ^a	I	C ₁₁ H ₁₈ N	164.1434	164.1431	12.01	3.5	-1.8	t-MS ² /DD
MDEA-5 ^b (MA-5) ^a	I	C ₁₂ H ₁₈ NO	192.1383	192.1378	12.30	4.5	-2.6	t-MS ²
MDEA-6 (MDMA-4) ^a	I	C ₁₁ H ₁₄ NO ₃	208.0968	208.0968	11.72	5.5	0.0	t-MS ² /DD
MDEA-7 ^b	II	C ₁₄ H ₂₁ N ₂ O ₃	265.1547	265.1545	11.68	5.5	-0.8	DD
MDMA		C ₁₁ H ₁₆ NO ₂	194.1176	194.1174	11.90	4.5	-1.0	t-MS ²
MDMA-1 ^b (MDA-6, COD-3) ^a	I	C ₁₁ H ₁₆ NO ₃	210.1125	210.1122	11.40	4.5	-1.4	t-MS ² /DD
MDMA-2 ^b	I	C ₁₁ H ₁₄ NO ₂	192.1019	192.1015	11.60	5.5	-2.1	t-MS ² /DD
MDMA-3 (AM-4, MA-1) ^a	I	C ₁₀ H ₁₆ NO	166.1226	166.1223	11.56	3.5	-1.8	t-MS ² /DD
MDMA-4 (MDEA-6) ^a	I	C ₁₁ H ₁₄ NO ₃	208.0968	208.0964	11.69	5.5	-1.9	t-MS ² /DD
MDMA-5 (MA-4, MDEA-4) ^a	I	C ₁₁ H ₁₈ N	164.1434	164.1433	12.05	3.5	-0.6	t-MS ² /DD
CO		C ₁₇ H ₂₂ NO ₄	304.1543	304.1544	12.16	7.5	0.3	t-MS ²
CO-1 ^b	I	C ₁₆ H ₂₂ NO ₂	260.1645	260.1640	12.70	6.5	-1.9	t-MS ² /DD
CO-2	I	C ₁₅ H ₁₈ NO ₄	276.1230	276.1226	11.90	7.5	-1.4	t-MS ²
BE		C ₁₆ H ₂₀ NO ₄	290.1387	290.1381	11.96	7.5	-2.1	t-MS ² /DD
BE-1 ^b	I	C ₁₅ H ₂₀ NO ₂	246.1489	246.1483	12.60	6.5	-2.4	t-MS ² /DD
BE-2 ^b	I	C ₁₆ H ₂₂ NO ₅	308.1492	308.1481	11.80	6.5	-3.6	t-MS ²
BE-3 ^b	I	C ₁₆ H ₁₈ NO ₄	288.1230	288.1226	11.40	8.5	-1.4	t-MS ²
CE		C ₁₈ H ₂₄ NO ₄	318.1700	318.1689	12.38	7.5	-3.5	t-MS ²
CE-1 (BE) ^a	I	C ₁₆ H ₂₀ NO ₄	290.1387	290.1381	11.96	7.5	-2.1	t-MS ²
CE-2 ^b	I	C ₁₈ H ₂₆ NO ₆	352.1755	352.1743	11.80	6.5	-3.4	t-MS ²
CE-3	I	C ₁₇ H ₂₂ NO ₃	288.1594	288.1588	12.50	7.5	-2.1	t-MS ²
CE-4 ^b (COD-2) ^a	I	C ₁₈ H ₂₄ NO ₃	302.1751	302.1743	12.40	7.5	-2.6	t-MS ²
OXY		C ₁₈ H ₂₂ NO ₄	316.1543	316.1537	11.67	8.5	-1.9	t-MS ²
OXY-1 ^b	I	C ₁₅ H ₂₂ NO ₂	248.1670	248.1674	12.70	5.5	1.6	t-MS ²
COD		C ₁₈ H ₂₂ NO ₃	300.1594	300.1586	11.50	8.5	-2.7	t-MS ²

Name	Metabolite type	Elemental formula	Exact mass (m/z)	Observed mass (m/z)	RT	DBE	Mass accuracy (ppm)	MS/MS
COD-1 (MO) ^a	I	C ₁₇ H ₂₀ NO ₃	286.1438	286.1431	11.24	8.5	-2.4	t-MS ²
COD-2 ^b (CE-4) ^a	I	C ₁₈ H ₂₄ NO ₃	302.1751	302.1744	12.40	7.5	-2.3	t-MS ² /DD
COD-3 ^b (MDA-6, MDMA-1) ^a	I	C ₁₁ H ₁₆ NO ₃	210.1125	210.1122	11.45	4.5	-1.4	t-MS ² /DD
MO		C ₁₇ H ₂₀ NO ₃	286.1438	286.1431	11.26	8.5	-2.4	t-MS ²
MO-1 ^b	I	C ₁₇ H ₂₂ NO	256.1696	256.1690	12.50	7.5	-2.3	t-MS ²
ME		C ₂₁ H ₂₈ NO	310.2165	310.2156	13.21	8.5	-2.9	t-MS ²
ME-1 ^b	I	C ₂₁ H ₃₀ NO ₂	328.2271	328.2283	12.20	7.5	3.7	t-MS ²
ME-2 ^b	II	C ₂₃ H ₃₃ N ₂ O ₃ S	417.2206	417.2206	11.90	8.5	0.0	t-MS ²
EDDP		C ₂₀ H ₂₄ N	278.1903	278.1894	12.80	9.5	-3.2	t-MS ²
EDDP-1 ^b	I	C ₂₀ H ₂₂ NO ₂	308.1645	308.1643	12.40	10.5	-0.6	t-MS ²
LSD		C ₂₀ H ₂₆ N ₃ O	324.2070	324.2060	12.20	9.5	-3.1	t-MS ²
LSD-1 ^b	I	C ₂₀ H ₂₄ N ₃ O	322.1914	322.1913	11.60	10.5	-0.3	t-MS ² /DD
LSD-2 ^b	I	C ₂₀ H ₂₄ N ₃	306.1965	306.1962	11.50	10.5	-1.0	t-MS ² /DD
Δ-9-THC		C ₂₁ H ₃₁ O ₂	315.2319	315.2307	13.50	6.5	-3.8	t-MS ²
THC-1 ^b	I	C ₁₉ H ₂₅ O ₃	301.1798	301.1790	12.20	7.5	-2.7	t-MS ² /DD
THC-2 ^b	I	C ₂₁ H ₃₁ O ₃	331.2268	331.2259	12.40	6.5	-2.7	DD
THC-COOH		C ₂₁ H ₂₉ O ₄	345.2060	345.2059	13.18	7.5	-0.3	DD
THC-COOH-1 ^b	I	C ₁₇ H ₂₁ O ₅	305.1384	305.1379	11.49	7.5	-1.6	DD

^a TPs that are present in more than one group. ^b New tentatively identified TPs in raw influent sewage water.

Table 3.5 shows the elemental composition, retention time, mass accuracy and the acquisition mode for all of the identified DOAs and their TPs. The majority of the identified metabolites (45 out of 54) were phase I transformations, which could happen both inside human body and also in the environment. For all the TPs, the mass error between the observed mass and exact mass was less than 4.2 ppm. Observed phase II transformations included acetyl, glycine, glutathione and taurine conjugations. Acetyl conjugates of sulfomethoxazole have also been reported for reclaimed waters from South Florida WWTP [37].

3.4.2 Structural elucidation of transformation products

Amphetamine was chosen as the target analyte to show the steps in structure elucidation using pathway 1 shown in Fig. 3.1. In short, raw sewage water samples were run in full scan mode at high resolution and processed in the MetWorks software, which identified 7 (5-phase I and 2-phase II) possible TPs of amphetamine. A sample

chromatogram of amphetamine and one of its phase II metabolite (AM-2, m/z 170.1176) is shown in Fig. 3.3. Then its observed mass, corresponding elemental composition, DBE and mass error was recorded in Xcalibur software. Using this information and the type of transformation, a preliminary structure was proposed. Next, targeted MS/MS was performed for m/z 170.1176 with varying collision energies. The MS/MS spectra showed m/z 138.0915, 110.0604, 95.0860 and 85.0653 as abundant fragments (Fig. 3.4). The Fragment ion search (FISh) algorithm in Mass Frontier software was run *in-silico* with the proposed structure. As a result, all the fragments observed in the MS/MS spectra were explained and a mechanism of formation of the TP from the parent molecule could be elaborated. As shown in the Fig. 3.4, the mass error for all the fragments was less than 0.8 ppm validating the structural assignment for the transformation product m/z 170.1176.

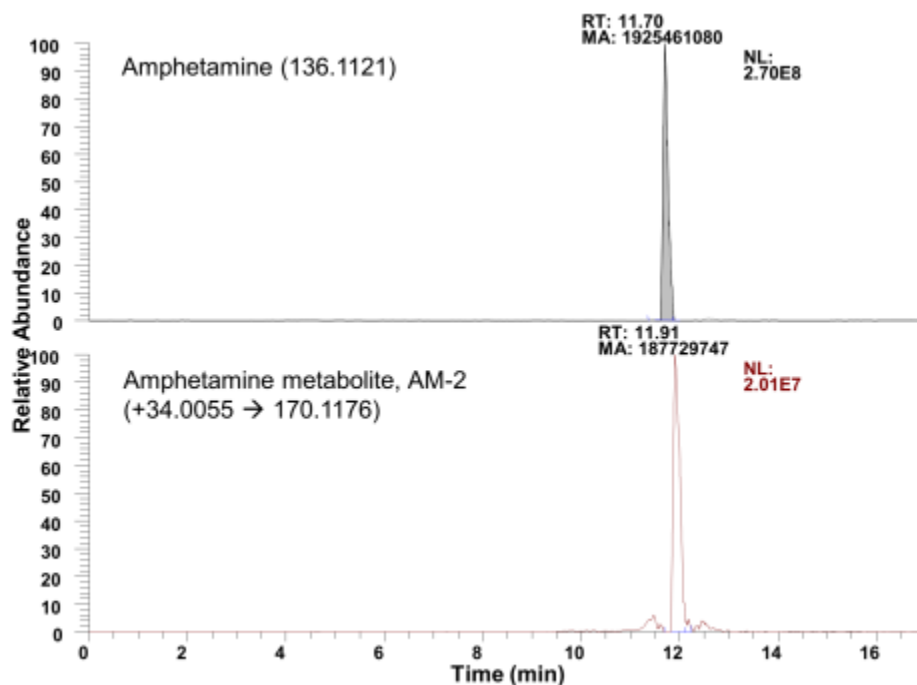


Fig. 3.3. LC-Q Exactive Orbitrap extracted ion chromatograms (XICs) of amphetamine (m/z 136.1121) and its metabolite (m/z 170.1176) in a sewage water sample.

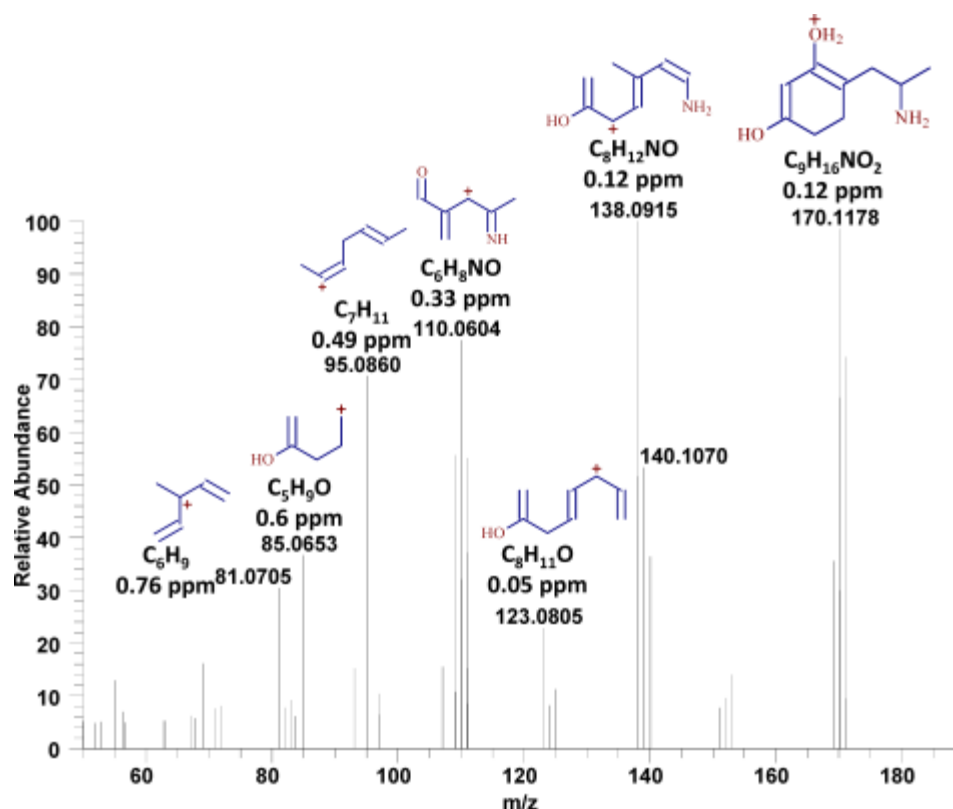


Fig. 3.4. Targeted MS2 spectra of amphetamine metabolite, m/z 170.1176.

The structure proposed for another metabolite, m/z 166.1226 identified as a TP from amphetamine (AM-4), methamphetamine (MA-1), and, MDMA (MDMA-3), matches with a known compound; ephedrine. Moreover, the abundant fragments observed in MS/MS spectra, m/z 148.1121, m/z 133.0887 and 117.0701 also match with those generated by the FISh search for ephedrine, with mass errors less than 0.2 ppm and with those reported by Bijlsma group [22]. In addition, ephedrine reference standard was obtained, and MS/MS fragmentation was performed for validation purposes (Fig. 3.5). It is important to keep in mind that since ephedrine and MA-4 share the spectral information, it is not possible to identify the exact source of the signal. MS/MS spectral confirmation against reference standards was also done for n-ethylamphetamine and norcocaine (See appendices 1 and 2).

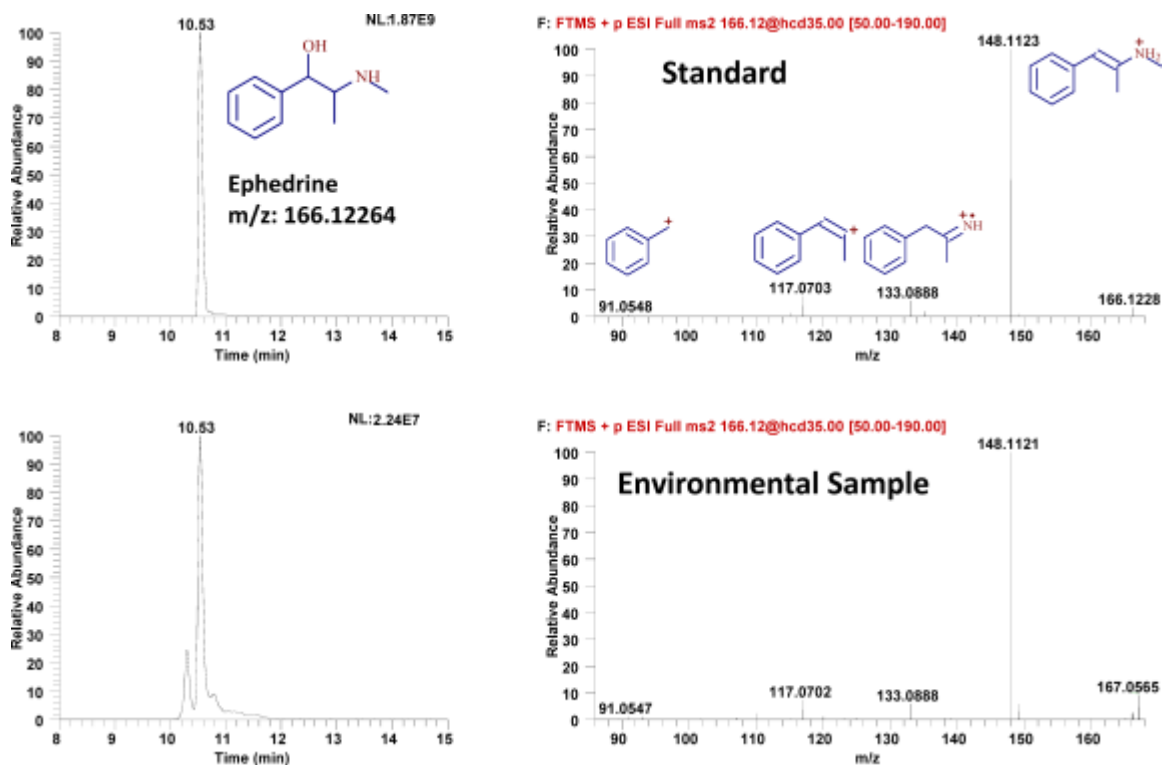


Fig. 3.5. Targeted MS2 spectra of amphetamine metabolite, m/z 166.1226.

MetWorks also identified an acetylation conjugate of amphetamine at m/z 178.1226. However, a sympathomimetic drug, phenmetrazine has the same exact mass but these two molecules are structurally very different. The MS/MS of m/z 178.1226 yielded two major fragments at m/z 146.0601 and m/z 119.0494, which could only be explained with confidence (mass error < 0.3 ppm) using phenmetrazine and therefore the acetyl conjugate was not included in the list of metabolites for amphetamine.

Methamphetamine (MA) produced a total of 5 TPs (MA-1 – MA-5), 3 phase-I and 2 phase-II. Fig. 3.6 demonstrates tentative chemical structures, formulas, and possible pathways of TP formation. Briefly, MA-1 (ephedrine) is shown a hydroxylation product of methamphetamine. MA-1 undergoes further dehydroxylation forming MA-3. MA-2

(amphetamine), as it has previously been demonstrated, is a demethylation product of methamphetamine [13]. The remaining phase II metabolites shown, come from methylation and acetylation processes. All these examples prove that tandem mass spectrometry data in combination with high resolution accurate mass measurements is valuable in producing accurate structural assignment of unknown metabolites. MS/MS spectral elucidation for all target analytes (parents and metabolites) and some of the identified TPs, showing tentative structure, chemical formulas, and mass error (ppm); and MS/MS spectra for all 54 TPs is provided in appendix 3.

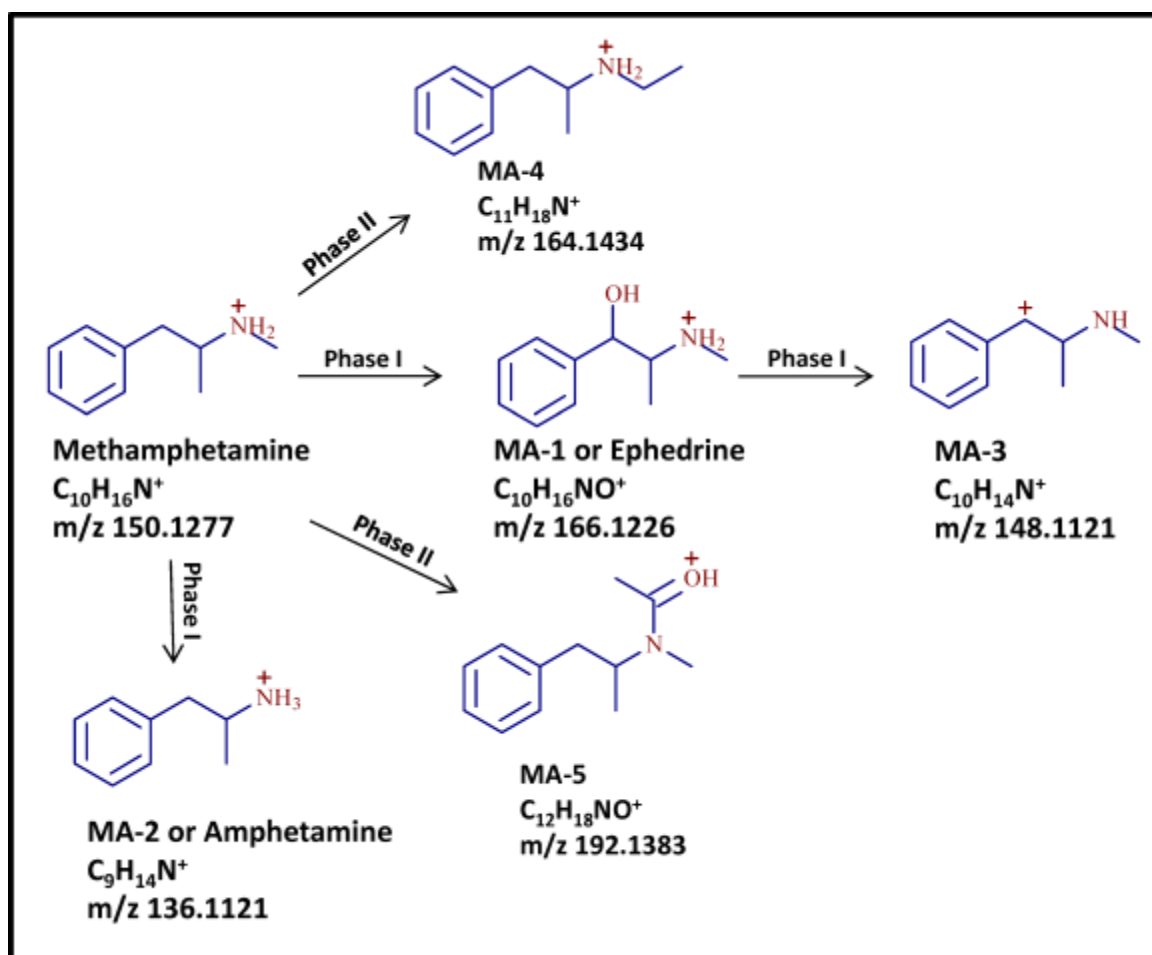


Fig. 3.6. Methamphetamine's tentative transformation pathway of five identified TPs, including proposed structure, chemical formula, accurate mass (m/z), and type of transformation.

Other researchers from our group used pathway 2 (Fig. 3.1) for the identification of metabolites of antibiotics in reclaimed waters. Acetyl-sulfamethoxazole and desmethyl anhydroerythromycin was found as the major metabolite and theoretical fragments generated by FISh (from Mass Frontier) provided a good correlation with the observed MS/MS spectra from data dependent scan [38]. Because the success of pathway 2 may be related to analyte concentration and chromatographic separation a comparison was conducted using the same samples.

3.4.3 Comparison of data acquisition modes

The two acquisition modes used for generating the data in the present study are targeted MS/MS (pathway 1) and data dependent scan (pathway 2). Identical samples were run in both the modes for comparison. The pathway 1 is longer and needs two injections per sample, one for full scan and the other for MS/MS whereas the pathway 2 is completely automated and requires only one run. An initial full scan performed in both pathways under same settings yielded ~ 50 transformation products (in MetWorks), most of them being similar. However, it was only possible to “shortlist” half of the TPs identified from the data dependent acquisition due to the small number of successful MS/MS scans across the chromatographic peaks (Fig. 3.7). Since MS/MS event is intensity threshold driven in data dependent scan, one must be very careful in choosing the detection thresholds and isolation-time events. One approach is to set a low threshold value (2×10^4) to allow the detection of low abundant TPs; however, this approach leads to the collection of unnecessary background ions. The other approach is to set a high

threshold value (5×10^6) to remove the majority of the background noise by acquiring less MS/MS spectra at the expense of losing a large array of meaningful TPs [39].

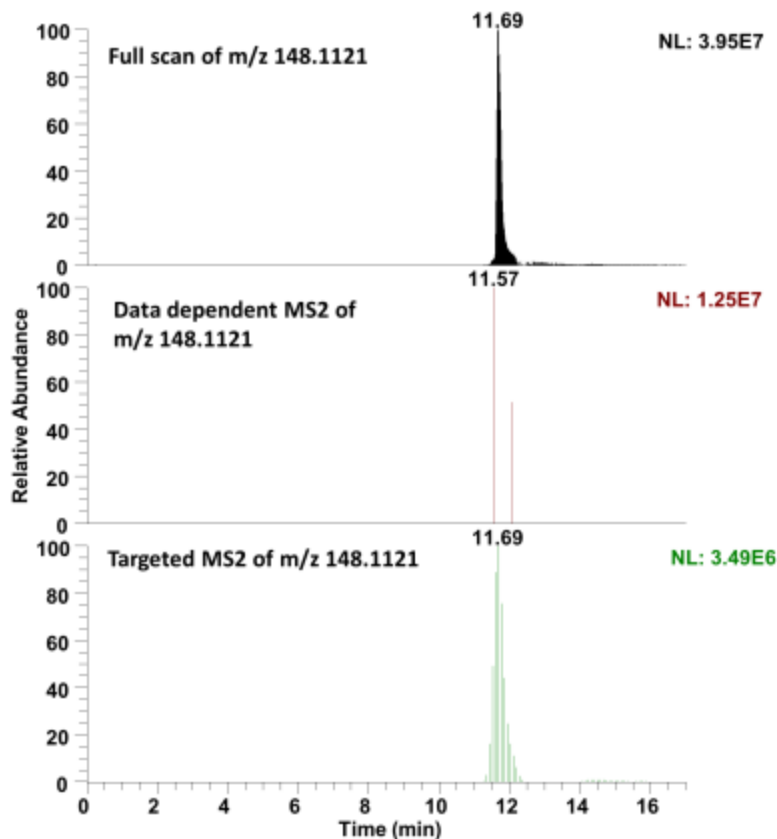


Fig. 3.7. Comparison of number of scans obtained in full scan, targeted MS2 and data dependent MS2 for one of the most abundant TP of Methamphetamine, MA-3 (m/z 148.1121).

One drawback of FTMS instruments such as the Orbitrap is that quality of MS/MS data gets compromised as the acquisition time decreases, which is likely the limitation for data dependent scans at low concentrations. However, this scan mode is still a powerful tool to acquire the data provided the intensity of the TPs is high enough and the chromatographic resolution is good. Moreover, one of the main advantages of data-dependent acquisition mode is the ability to obtain MS/MS fragmentation for all precursors above the specified threshold, facilitating the search for any other possible

analytes and transformation products of interest (full scan) plus their fragment ions without the need to run again. Some examples of this advantage include caffeine (m/z: 195.0877, 0.01 ppm), the aromatic hydroxylation product of sulfadimidine (m/z: 295.0860, 0.28 ppm), and the aromatic hydroxylation product of propyphenazone (m/z: 247.1438, 1.19ppm), which were identified at a later time but were not part of this study. For low-abundance TPs, data acquisition using pathway 1 is the preferred method of choice and was used in the present study to report mole fractions of TPs shown in Table 3.6.

Table 3.6. Percent frequency of detection and mole fraction of the identified TPs.

NAME	FREQUENCY OF DETECTION (n = 12)	% MOLE FRACTION	SD
EDDP^a	0	0.0	0.0
EDDP-1	58	58	51
LSD	0	0.0	0.0
LSD-1	100	42	0.5
LSD-2	100	58	0.5
ME	0	0.0	0.0
ME-1	100	81	20
ME-2	58	19	20
EDDP ^a	0	0.0	0.0
OXY	0	0.0	0.0
OXY-1	100	100	0.0
Δ-9-THC	0	0.0	0.0
THC-1	25	25	45
CE^a	8	0.4	1.5
CE-1 (BE) ^b	92	6.0	4.7
CE-2	58	3.3	3.0
CE-3	100	28	15
CE-4	100	62	16
MA	25	0.5	0.9
MA-1 (AM-4, MDMA-3) ^b	42	3.1	4.1
MA-2 (AM, MDA-1) ^b	100	27	10
MA-3	100	21	15
MA-4 (MDEA-4, MDMA-5) ^b	92	4.9	3.7
MA-5 (MDEA-5) ^b	100	43	18
MDEA	42	1.1	1.6
MDEA-1	58	1.3	1.5
MDEA-2 (MDMA, MDA-4) ^b	100	15	6.8
MDEA-3	25	0.5	0.9
MDEA-4 (MA-4, MDMA-5) ^b	92	9.2	7.8
MDEA-5 (MA-5) ^b	100	67	13
MDEA-6 (MDMA-4) ^b	25	5.4	11
MO	17	1.7	4.0

NAME	FREQUENCY OF DETECTION (n = 12)	% MOLE FRACTION	SD
MO-1	100	98	4.0
COD	17	2.4	6.4
COD-1 (MO) ^b	17	2.2	5.7
COD-2 (CE-4) ^b	100	89	17
COD-3 (MDA-6, MDMA-1) ^b	17	6.5	15
MDA	42	4.8	6.0
MDA-1 (AM, MA-2) ^b	100	25	13
MDA-2	50	4.9	5.3
MDA-3	100	6.7	3.4
MDA-4 (MDMA, MDEA-2) ^b	100	7.2	2.6
MDA-5	92	7.5	3.0
MDA-6 (MDMA-1, COD-3) ^b	17	3.5	8.7
MDA-7	67	14	12
MDA-8	92	26	16
CO	67	15	14
CO-1	75	44	31
CO-2	8	0.6	2.1
BE ^a	92	31	27
CE ^a	8	1.0	3.5
AM	100	22	10
AM-1	92	21	11
AM-2	100	22	8.0
AM-3	8	1.0	3.4
AM-4 (MA-1, MDMA-3) ^b	42	7.4	10
AM-5	42	0.5	0.7
AM-6	83	11	7.1
AM-7	100	16	9.0
BE^a	92	30	17
BE-1	83	47	31
BE-2	92	19	27
BE-3	25	3.8	6.8
MDMA	100	40	24
MDMA-1 (MDA-6, COD-3) ^b	17	2.4	5.6
MDMA-2	50	5.3	6.0
MDMA-3 (AM-4, MA-1) ^b	42	13	16
MDMA-4 (MDEA-6) ^b	25	12	24
MDMA-5 (MA-4, MDEA-4) ^b	92	28	23

^aDOA was treated both as parent DOA and as metabolite for other DOA to calculate mole fractions

^bTPs that are present in more than one group

3.4.4 Mole fraction of metabolites

The estimated % mole fractions (Mf) and frequency of detection of all TPs are shown in Table 3.6. Target analytes benzoylecognine and cocaethylene are well-known metabolites of cocaine and were included as metabolites for the Mf calculation for cocaine. However, each of them has their own TPs and therefore was also treated as

parent DOA. Same was true with EDDP, a known metabolite of methadone. Based on the mf, all DOAs were classified into three categories: low (0-20%), medium (20-70%) and high-abundance (70-100%). Results in Table 3.6 are sorted according to the parent DOAs Mf category. The Low-abundance category included 12 parent DOAs (out of 15 total) meaning that majority of the parent DOAs are converted to TPs before they enter WWTPs and therefore TPs should always be monitored instead of parent DOAs in this category. Many drugs (i.e. AM and MDA) produce a large number of metabolites so it will be difficult to assess their occurrence and consumption patterns unless extensive monitoring is conducted. AM, BE and MDMA are the DOAs that fall under medium abundance category with respective mole fractions of 22%, 30% and 61%, the category in which it is important to monitor both the parent and its TPs. No DOAs were found to contain Mfs >70% and therefore none was included in high abundance category. When parent drug is found with relatively high Mf (>70%) it is safe to assume that metabolites can be ignored. One such example would be the artificial sweetener, sucralose, which is very persistent in the environment and hence used as tracer to track anthropogenic wastewater intrusions in to the environmental waters [40]. Results presented here clearly highlight the importance of monitoring metabolites rather than the parent molecules for drugs of abuse. The mole fraction distribution of the 10 most abundant TPs from this study were plotted as box plots and shown in Fig. 3.8. The frequency of detection is shown in parenthesis above the box plot. The boundaries of box plot cover 25th-75th percentile, the centerline indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles.

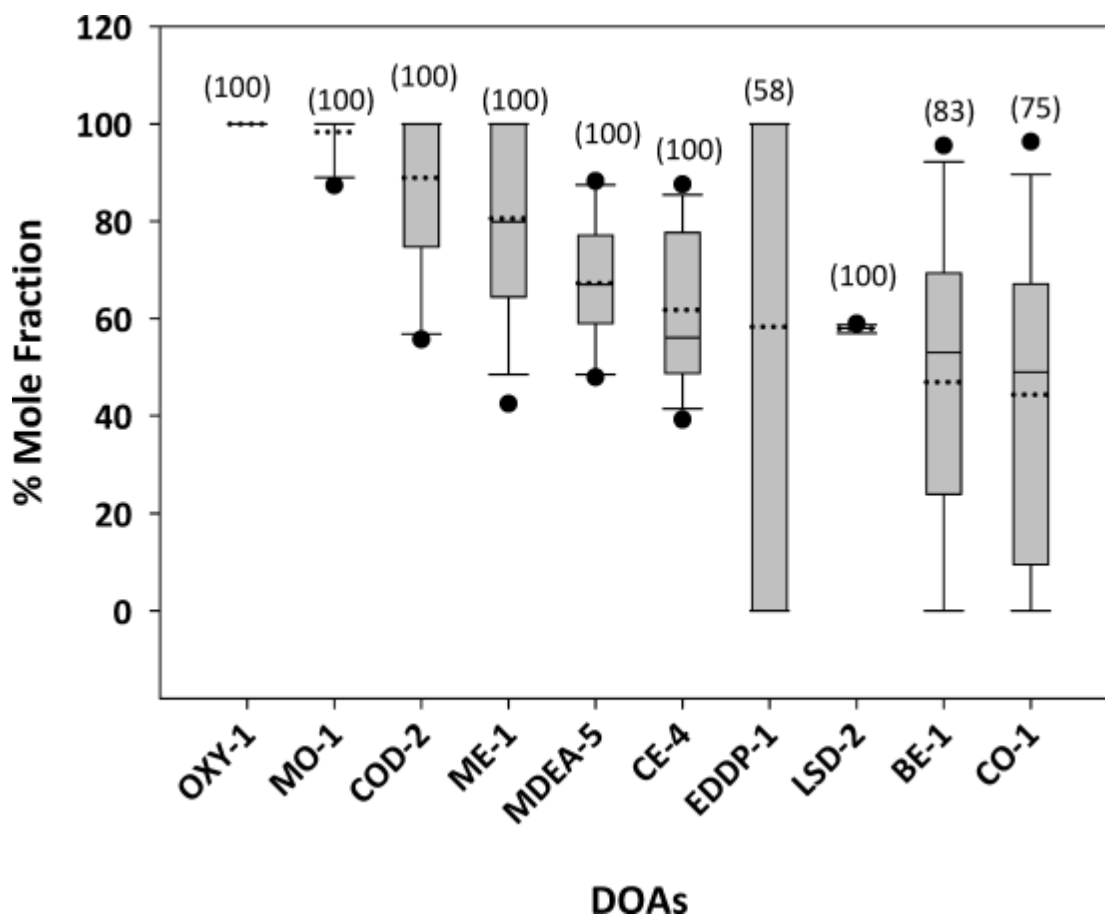


Fig. 3.8. Percent mole fractions of the most abundant TPs in sewage water (frequency of detection shown in parenthesis). The boundaries of box plot cover 25th-75th percentile, the center line indicates median of the sample population, error bars (whiskers) above and below the box refer to 90th and 10th percentiles. Dotted line indicates the mean of the sample population.

3.5 Conclusion

A total of 54 phase I and phase II metabolites of drugs of abuse were successfully detected and identified in raw sewage from a small population setting using high resolution mass spectrometry. Targeted MS² and data-dependent acquisition modes were compared and used in combination with metabolic profiling software to achieve identification of transformation products with a mass accuracy of 4.2 ppm or better. Concentrations of all TPs were estimated based on the parent DOAs response factor and

used to calculate the percent mole fractions (Mf). A group of 9 metabolites, all detected at high frequency, were observed to be more abundant (higher % mole fraction) than the parent compounds, and thus should be used as the main analytes for routine monitoring. Based on this simplistic example, until new technologies and materials for the treatment of emerging contaminants in domestic wastewater streams developed are tested a comprehensive monitoring of degradation products needs to be conducted in order to fully assess their fate, transport, and toxicological impact to the natural environment.

3.6 Acknowledgements

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CHAPTER 4

Characterization of wastewater effluent, surface, and drinking waters by high-resolution mass spectrometry: Understanding the composition of recalcitrant chemicals

4.1 Abstract

Contaminants of emergent concern are continuously reaching waterways through wastewater effluents, storm water and agricultural runoff, and many other sources produced by human activity. The current study investigates the chemical diversity in a typical river basin transect affected by domestic treated water discharges that also serves as source for downstream drinking water production. The trajectory along the model river includes an upstream site (not influenced), the effluent release site (point source), an effluent mixing zone (heavily influenced), a downstream location, the point for drinking water production intake, and finalized treated drinking water. Compound diversity, defined as the number of chemical formulas identifiable from the high-resolution mass spectrometry analysis (HRMS) proved to be greatest in the effluent (97 unique compound classes) and the treated drinking water (95 compound classes) locations. However, the summed response for all the detected formulas was much higher in the effluent than in any other sample in the system. Combination of m/z (accurate mass) vs. retention time scatter plots and Venn diagrams were used to recognize recalcitrant compounds that remained in all the water samples from the effluent release, and also to identify analytes introduced during the drinking water treatment process. A total of 64 compounds originated at the effluent location persisted throughout the entire process. A combination of MS/MS spectral analysis and *in-silico* fragmentation prediction were used to identify and tentatively confirm 4 compounds. From the 3,227 chemical features (individual accurate masses that met selection criteria) present in the drinking water source; 1,152 were retained after the drinking water treatment while 1857 features were introduced to the final drinking water product. This fact clearly shows the potential importance of

treatment byproducts introduced by either water polishing (polymers) or formed during the disinfection processes. The use of high resolution mass spectrometry instruments capable of performing both MS and MS/MS experiments at resolutions above 100K such as the Q-Exactive Orbitrap played a crucial role in this study as it provided both the sensitivity and mass accuracy needed for screening target and non-target components for the complete characterization of the different water matrices.

4.2 Introduction

In order to fulfill human “needs” in our rapidly changing society, a great variety of products ranging from pharmaceuticals and personal care products (PPCPs), drugs (illicit and prescription), artificial sweeteners, nanomaterials, perfluorinated compounds (PFCs), sunscreens, and pesticides to name a few, are being manufactured, consumed, used, and disposed of, on a daily basis in households and industrial settings (Chiaia-Hernandez et al. 2013; Hernández et al. 2014; Richardson and Ternes 2014). A large portion of these compounds (in their unchanged form or as transformation products) reaches wastewater treatment facilities where in many cases they can persist throughout the treatment process. (Huerta-Fontela et al. 2008; Bartelt-hunt et al. 2009; Gros et al. 2010; Barceló et al. 2012). The result of this persistence is a chronic source of contamination to sensitive aquatic ecosystems, which can potentially be affected (Ben-Jonathan and Steinmetz 1998; Rabiet et al. 2006; Kasprzyk-hordern et al. 2008; Barceló et al. 2012; Lacey et al. 2012; Parolini et al. 2013). To further aggravate the increasing pollution, stormwater and agricultural runoff introduces additional inputs of organic pollutants, as these waters

receive inadequate treatment or no treatment before entering waterways (Xiao et al. 2012; Jernberg et al. 2013; Moschet et al. 2013, 2014).

Surface water is in many cases the main source of water to be used for drinking water production. Upon entering treatment facilities, physical treatment (e.g., flocculation, sedimentation, filtration) followed by chemical disinfection (e.g., chlorination, ozone, and/or ultraviolet radiation) is performed to remove suspended matter and pathogens (Barceló et al. 2012; Postigo and Richardson 2014). However, these types of treatments may not be sufficient enough to remove the more persistent compounds. For instance, Segura et al. (2011) reported low levels (parts per trillion, ng/L) of atrazine and carbamazepine in drinking water from cities in Canada. Similarly, the presence of sucralose has previously been reported by our group, in which concentrations for the artificial sweetener reached as high as 465 ng/L in drinking water (Batchu et al. 2013). A recently published review on the transformation pathways of pharmaceuticals during disinfection processes, clearly showed the formation of disinfection byproducts (DBPs) upon treatment with chlorine, chloramine, ozone, chlorine dioxide, UV, or UV/H₂O₂ (Postigo and Richardson 2014).

To better understand chemical diversity of different water matrices, it is essential to take advantage of high-resolution instruments such as the Orbitrap, which offer great sensitivity and optimal resolution capable of resolving thousands of species in a single spectrum with the high mass accuracy needed for molecular assignment (Gonsior et al. 2011; Chiaia-Hernandez et al. 2014). Such features make high-resolution instruments ideal for the characterization of unknowns identification and confirmation of low and high abundant contaminants and their potential transformation products (Moschet et al.

2013; Tseng et al. 2013; Schymanski et al. 2014). Target analysis of known compounds is usually done using a suite of reference standards plus their labeled analogs for quantitation purposes. Multiple methods using this approach have been reported in the literature, for a wide variety of compounds (Postigo et al. 2010; Baker and Kasprzyk-hordern 2011; Panditi et al. 2013; Wang and Gardinali 2013; Castiglioni et al. 2014).

Non-target techniques encompass the analysis of known and unknown compounds using a combination of full scan and MS/MS experiments. One such technique is the so called suspect screening, for which databases (instead of reference standards) are used to tentatively identify and confirm the presence of known analytes on the basis of mass accuracy, retention time, isotopic pattern determination, and structure confirmation using MS/MS experiments (Krauss et al. 2010; González-Mariño et al. 2012; Moschet et al. 2013; Chiaia-Hernandez et al. 2014). In addition, non-target techniques have been implemented for the identification of potential transformation products from known target analytes using metabolite identification tools in conjunction with *in-silico* structural elucidation models followed by confirmation using accurate mass MS/MS data (Moschet et al. 2013; Bletsou et al. 2015; Heuett et al. 2015).

One last, yet more challenging approach is the use of non-target screening techniques for the characterization and tentative identification of unknown compounds in complex matrices such as wastewater. In non-target screening techniques, no prior information is known about the compounds to be detected and thus, it is of utmost importance to acquire the data using high-resolution instruments. Accurate mass data censored to a confidence level (5 ppm) is used to generate a peak list, for which molecular composition is determined using formula generator tools, to then determine possible structures from

databases (Krauss et al. 2010; Bletsou et al. 2015). Studies have been implemented using the above protocols in the evaluation of molecular composition changes from degradation or treatment processes (Gonsior et al. 2011; Tseng et al. 2013; Schymanski et al. 2014); and in the tentative identification of analytes from environmental samples (Müller et al. 2011; Hug et al. 2014; Leerdam et al. 2014).

Understanding the composition of different types of samples of the same matrix that are interconnected to one another could help to potentially pin point sources of pollution, measure treatment efficiency in wastewater and drinking water facilities, and identify the presence of persistent microconstituents. Therefore the goal of this study is twofold: a) to characterize effluent, surface, and drinking water samples linked by a common trajectory using non-target high-resolution mass spectrometry, and b) to classify and compare the chemical diversity in each matrix, to further identify and understand the composition of newly formed or recalcitrant chemicals.

4.3 Experimental

4.3.1 Reagents and chemicals

High performance liquid chromatography (HPLC) grade water, methanol, acetonitrile, formic acid, ammonium formate, and optimal grade methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and used as received. Clear polyethylene terephthalate (PET) bottles for sample collection were purchased from SKS Science (Watervliet, NY, USA). Glass fiber filter were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.3.2 Sample collection

Grab surface water samples (500 mL) were collected on multiple locations along a river system on October 7th and 8th, 2014. Sampling locations included upstream (~800 meters above the wastewater effluent), the outfall itself, an effluent mixing zone (~200 meters downstream from the outfall), and a downstream site (~9 kilometers from the outfall). In addition, samples were also collected at the drinking water treatment plant intake (~13.5 kilometers downstream from the outfall) and at the end of the treated drinking water process (see diagram in Fig. 4.1). Samples were vacuum filtered using a 0.5 um PreSep Prefilter glass fiber filter and stored in the dark at -20 °C until analysis time.

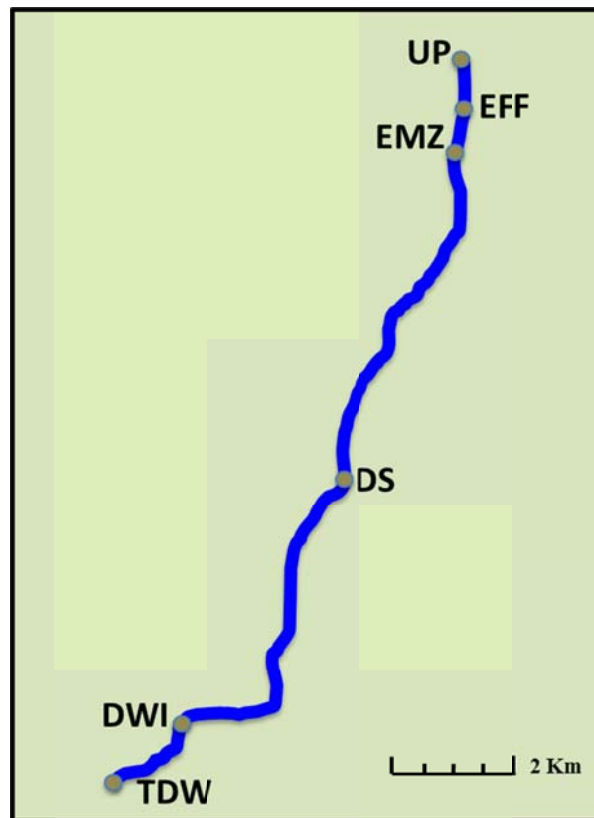


Fig. 4.1. Diagram of sampling locations, upstream (UP), wastewater effluent (EFF), effluent mixing zone (EMZ), downstream (DS), drinking water intake (DWI), and treated drinking water (TDW).

4.3.3 Sample preparation

As a result of the unknown nature of the analytes present in the water samples, exhaustive extraction was performed by liquid-liquid extraction against methylene chloride. Prior to extraction, filtered samples were basified to pH 10 using ammonium hydroxide and placed in individual 1L separatory funnels. Analytes were extracted using a total of 250 mL of methylene chloride. After collecting the organic phase, the remaining aqueous phase was acidified using formic acid to reach pH 4. The acidified sample was then extracted using 250 mL of methylene chloride. The organic phases from the basic and acid extractions were combined to obtain a total sample extract. Organic extracts were dried over anhydrous sodium sulfate and were concentrated using a water bath; dried under a stream of purified nitrogen gas; and reconstituted to 1 mL using methanol before analysis.

4.3.4 UPLC-Orbitrap analysis

Extracts were analyzed on a UPLC high-resolution Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with HESI II electrospray source operated in the positive ion mode. The mass spectrometer was operated in full scan mode (100 – 1000 m/z) at a 140,000 resolution, and MS/MS mode at 35,000 resolution (HCD collision energy 35). Chromatographic separation of analytes was performed using a Hypersil GoldTM aQ analytical column (50 x 2.1 mm, 1.9 μ m) protected by a Hypersil GoldTM aQ guard column (10 x 2.1 mm, 1.9 μ m). The above mentioned method was adapted from an existing online SPE method (Wang and Gardinali 2013), and modified to a direct injection method. Mobile phase composition

consisted of water modified with 0.1% formic acid, and a binary mixture of acetonitrile:methanol (50:50). Details on the modified gradient program and flow rate are shown in table 4.1.

Table 4.1. Analytical pump gradient program for direct injection. Mobile phases used: LC-MS grade water (A), methanol (B), acetonitrile (C), and 0.1% formic acid in water (D).

Time	A	B	C	D	Flow (μ l/min)
0	0	1	1	98	500
0.2	0	1	1	98	500
0.5	0	1	1	98	500
5	0	47	50	3	500
9	0	47	50	3	500
11	0	47	50	3	500
11.5	0	1	1	98	500
13	0	1	1	98	500

4.3.5 Data analysis

Post-acquisition data mining of samples was performed using Compound Discoverer 1.0 software for peak list generation (Thermo Scientific, San Jose, CA, USA). Subsequently, a blank subtraction was done for each of the samples to remove background ions and chemical noise introduced by the lab procedures. Peak lists including exact mass (m/z), their intensity and the retention time for each of peaks found in each one of the blank-subtracted samples were exported and uploaded into an in-house database. Molecular formulae were assigned for all m/z 's that had intensities above 10,000 using the formula generation package from Spidermass. The formula generation software was adapted to apply the seven golden rules (SGR) for heuristic filtering of molecular formulas obtained by high resolution mass spectrometry (Kind and Fiehn 2007). Elements considered in the molecular assignment (and their minimum and

maximum counts) included: C (3-60), H (2-120), N (0-20), O (0-25), P (0-6), S (0-8), F (0-10), Cl (0-10), and Br (0-7). Data was further censored with ring double bond equivalence values (RDBE) not greater than 40, and elemental composition error less than 0.5 mDa from the accurate mass. Element ratios, element probability check and isotopic pattern check were evaluated as part of the SGR approach. Visual representations (e.g. graphs and diagrams) were used to recognize similarities/differences among the samples, as well as to identify compositional patterns.

4.4 Results and discussion

Non-target analysis and post-acquisition strategies were implemented in this study to determine chemical diversity, recognize persistent chemicals, and identify contaminants of emergent concern in surface water samples from a river system that serves both as a point of discharge for treated domestic wastewater upstream and as a source for drinking water production downstream. Liquid-liquid extraction (LLE) was used in this study to screen for as many organic components as possible while avoiding the potential segregation (washout or irreversible retention) of analytes that can result from using a specific packing material as in the case of solid phase extractions. A liquid-liquid extraction was performed at different pH levels (pH 4, 7, 10) using a set of reclaimed water samples ($n = 3$) as a proof of concept test to understand the influence of pH in the number of components extracted. This test demonstrated that under basic conditions (pH 10) a larger number of components with a higher diversity (as depicted by the range of Kendrick mass defect (KMD) values) are extracted in comparison to neutral or acidic pH conditions (data not shown). Therefore, during the extraction procedure for the surface

water samples, pH of the sample was sequentially modified with ammonium hydroxide (pH 10), followed by formic acid (pH 4) in order to be able to extract a the largest array of acidic and basic compounds. A laboratory blank (LC/MS water) was extracted in the same manner and used as background subtraction during the post-acquisition data mining.

4.4.1 Molecular formula generation

Molecular formulae containing C, H, N, O, P, S, F, Cl, and Br elements were generated for all samples using the SGR approach. Neutral masses were obtained from protonated ions by subtracting the proton mass (1.007825 u) from the accurate mass and used to generate formulae. It is important to note that, by assuming protonation of all detected ions, all other possible adducts generated during acquisition are excluded. Initially, several hundreds of thousands of formulae were generated; however, by following the application of the SGR the number of formulas was drastically reduced.

4.4.2 Chemical diversity

Chemical composition was evaluated and compared between the samples to determine chemical diversity among the different sites. A compound class contains an elemental composition with the same heteroatom content (e.g., CHNO), and the variety of compound classes in a sample makes up its diversity. Water samples generated molecular signatures containing a substantial number of compound classes. Table 4.2 outlines the number of compounds detected, the number of molecular assignments and compound classes, as well as the total intensities for each of the sampling locations. Effluent location presented the highest number of detections and compound classes

demonstrating a significant input of a large variety of chemicals in comparison to all the other sites. On the other hand, TDW site showed the least number of formulae, with the lowest total intensity, which is in agreement with the type of location (treated drinking water). However, chemical diversity for this location proved to be very similar (in numbers) to that of the EFF site, indicating a wide variety of chemical species found in the treated drinking water.

Table 4.2. Number of compounds detected, number molecular assignments and compound classes, as well as the total intensities for each of the sampling locations.

Location	Number of masses detected	Number of formulas generated	Number of compound classes	Total intensity
UP	3858	2415	37	5.81e+8
EFF	6690	4817	97	4.99e+9
EMZ	4032	2268	63	3.78e+8
DS	4431	2178	76	3.20e+8
DWI	4381	2438	75	4.01e+8
TDW	3009	1562	95	2.86e+8

4.4.3 Recalcitrant fraction of chemicals

Effluent inputs were evaluated to identify chemicals that survive the river system trajectory and made it into the treated drinking water. The first step consisted of eliminating the molecular entities found in the upstream sample (UP) from all the other samples. In order to isolate these recalcitrant chemicals, the remaining commonalities among the EFF, EMZ, DS, DWI, and TDW locations were assessed by means of a 5-way Venn diagram (Fig. 4.2), and through mass vs. retention time scatter plots (Fig. 4.3). After excluding all chemical inputs from the upstream, a total of 5,978 components were detected in the effluent location (Fig. 4.3a), and only 530 of these components were

observed in the effluent mixing zone (Fig. 4.3b). Nine kilometers down from the effluent at the downstream location, 247 components were found to be common between the EFF, EMZ, and DS sites (Fig. 4.3c), and less than 3% (169 components) of the chemicals detected in the effluent made it to the drinking water intake (Fig. 4.3d). Finally, a total of 64 masses found in the treated drinking water (Fig. 4.3e), were observed to be ubiquitous in all locations (except the upstream) demonstrating their persistence throughout the trajectory.

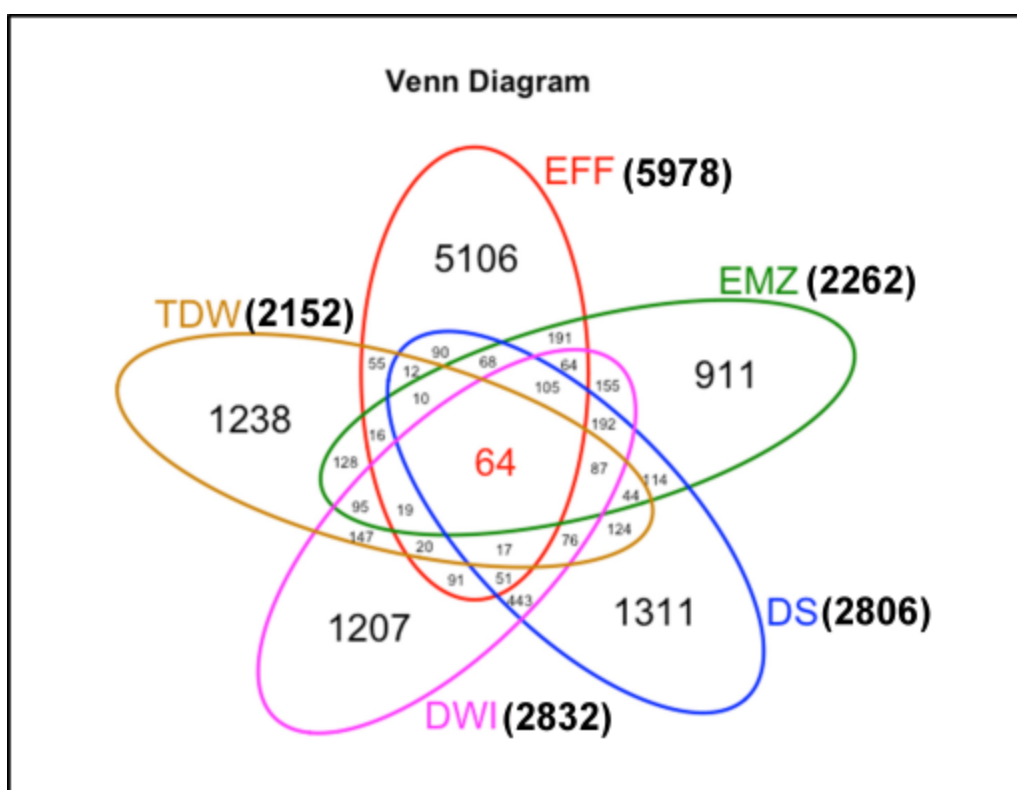


Fig. 4.2. 5-way Venn diagram the effluent (EFF), effluent mixing zone (EMZ), downstream (DS), drinking water intake (DWI), and treated drinking water (TDW) locations.

The Venn diagram in Fig. 4.2 shows the intersection of features among the EFF, EMZ, DS, DWI, and TDW locations indicating the 64 common chemical entities. In addition, m/z vs. retention time scatterplots presented in Fig. 4.3 show the distribution of

masses across a chromatographic run for each of the sampling locations. Early eluting high m/z values, as well as late eluting chemical species that are originally detected in the effluent location disappear gradually in each of the subsequent sites (See Fig. 4.3) and are drastically reduced in the finished drinking water.



Fig. 4.3. m/z vs. retention time scatter plots of analytes originated at the EFF location and remained constant throughout the trajectory.

Target MS/MS spectra for the 64 recalcitrant chemicals species were generated by re-running the samples for the specific 64 accurate masses and the fragmentation patterns obtained were evaluated through *in-silico* fragmentation software packages and spectral libraries (Mass Frontier, MetFrag, MassBank) to recognize and confirm their potential structure. Among the 64 candidates the identity of only four compounds was tentatively confirmed to previously reported chemicals to a mass accuracy threshold of less than 5 ppm (See Fig. 4.4). Compounds were positively identified as: 4-(4-ethylphenyl)butoxymethanol ($C_{13}H_{20}O_2$, m/z 209.1536), 3-(3-cyclohexyl-4-methoxyphenyl)-1-propanol ($C_{16}H_{24}O_2$, m/z 249.1849), nonoxynol-8 ($C_{31}H_{56}O_9$, m/z 573.3997), and nonoxynol-9 ($C_{33}H_{60}O_{10}$, M/Z 617.4255). The two latter compounds are well known as components in nonionic surfactants of the nonoxynol family, and are used in cosmetics, detergents, and contraceptives (Musah et al., 2012). Despite the robustness of the HR-MS/MS approach and the *in-silico* ratification for their unambiguous confirmation to occur authentic standards will have to be used.

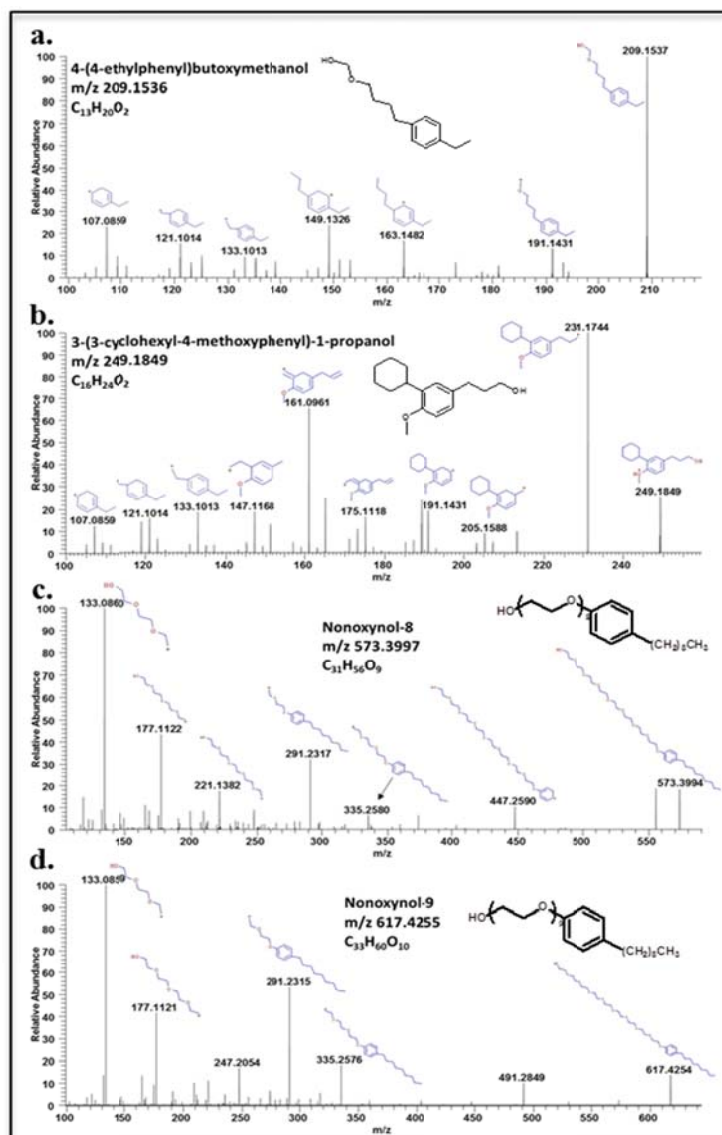


Figure 4.4. MS/MS spectral elucidation of four tentatively confirmed compounds.

4.4.4 Kendrick mass plots

Characterizing and comparing complex matrices is a difficult task because of the large amounts of data generated by high-resolution instruments. One way to simplify mass spectra is through mass defect filtering by plotting the nominal mass as a function of Kendrick mass defect. Mass defect is observed in all compounds as a result of differences in the binding energy of elements, and it is defined as the difference between

a compound's observed mass and its nominal mass. In Kendrick plots, the measured mass is multiplied by $14.0000/14.01565$ (nominal mass/exact mass of CH_2) to simplify the display to rectilinear peak patterns (Sleno 2012). Kendrick mass defect plots were used in this study to visualize shifts in mass spectra for identification of possible distribution patterns among sample locations (Fig. 4.5). Blue areas, showing a positive Kendrick mass defect were ubiquitous in all samples (Fig. 4.5). This positive section of the plots had a chemical composition largely of CHNOS and CHNO compound classes, which is the typical composition of natural organic matter (NOM) (Mesfioui et al. 2012; Tseng et al. 2013). In addition, distribution of the 64 identified recalcitrant compounds (red diamonds in Fig. 4.5) show a positive Kendrick mass defect, falling within the main blue region of the graph. Despite the complexity of the different types of matrices, it is possible to differentiate the chemical diversity inputs coming from the effluent (Fig. 4.5b). Lower purple and green bands, which show to be unique to this location, indicate the presence of compounds abundant in S, P, Cl and Br heteroatoms, as these elements display uniquely negative mass defects (Sleno 2012). Most common compound classes found in this region were CHNOSCl and CHNOCl. Absence of these bands from all the other locations following the effluent suggests possible dilution and/or degradation of this group of compounds. Lower orange band predominantly present in the upstream location (Fig. 4.5a) was mainly comprised of CHNOPS, CHNOF and CHNOSCl compound families. This ubiquitous fraction of compounds (orange) appears to originate from the upstream location or to be part of the natural environment.

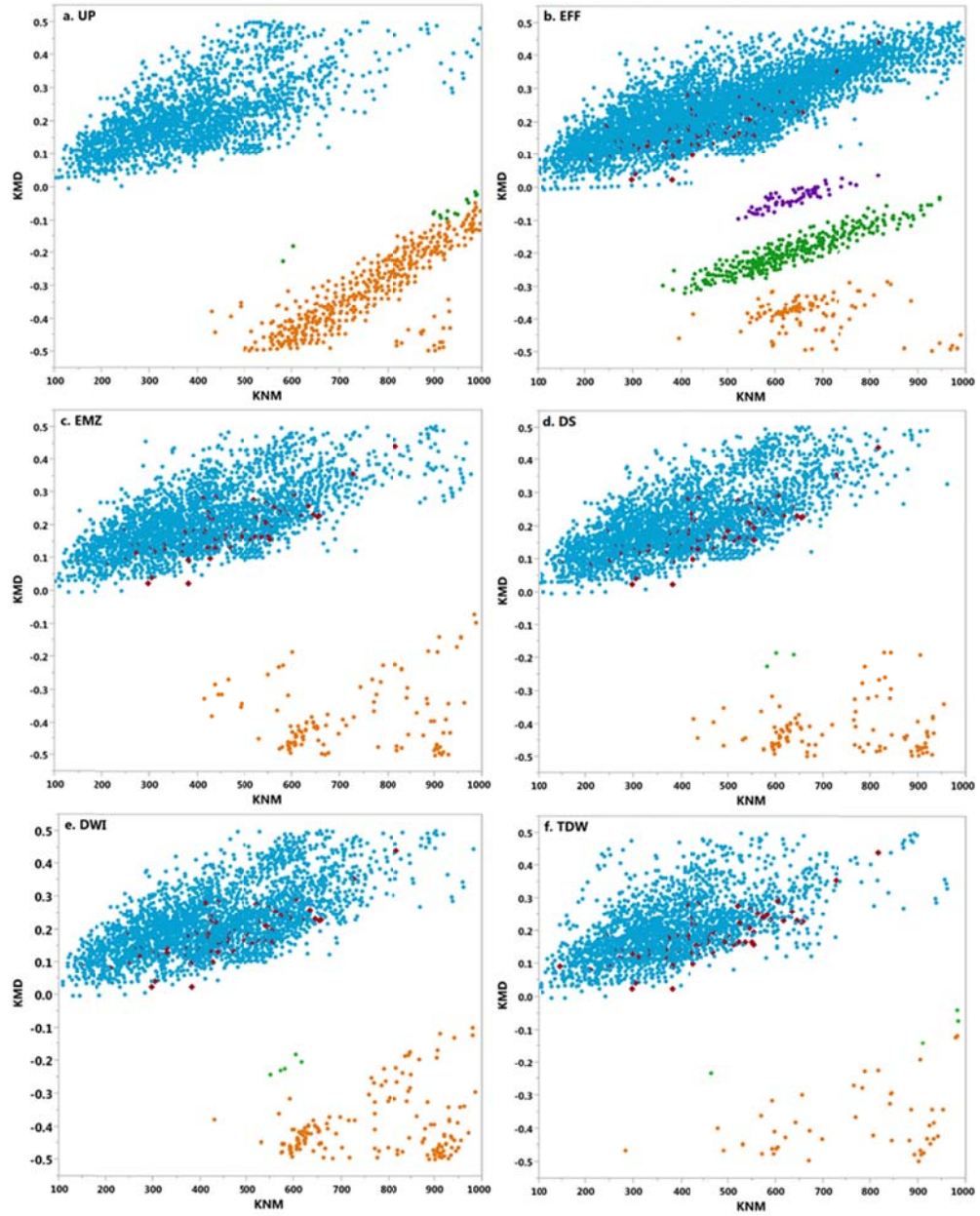


Fig. 4.5. Kendrick plots for each of the locations. a) UP, b) EFF, c) EMZ, d) DS, e) DWI, and f) TDW.

4.4.5 Evaluation of drinking water treatment

Qualitative comparison of DWI and TDW samples using a 2-way Venn diagram shown in Fig. 4.6 provided a general overview of relevant features from the intersections of these two sources. As shown in table 4.2, drinking water intake contained a wide

number of chemicals that are exposed to different types of processes upon entering the treatment facility. Chlorine is typically used as a disinfectant in drinking water and multiple controlled laboratory and real finish drinking water studies have reported the formation of pharmaceutical DBPs upon interaction with chlorine (Deborde and von Gunten 2008; Postigo and Richardson 2014); in addition, other disinfection methods such as the use of Chloramine, Ozone or UV radiation could also introduce additional unintended byproducts to the finished drinking water. Therefore, it is not only important to recognize chemical features that may persist through treatment but also those that appear during treatment. A total of 4,381 features were initially detected in the drinking water intake, and 26% of these masses were additionally identified in the treated water. This percentage represents the fraction of chemicals that withstand treatment. Moreover, treated water demonstrated the presence of several chemical species that were unique to the TDW location (Fig. 4.6).

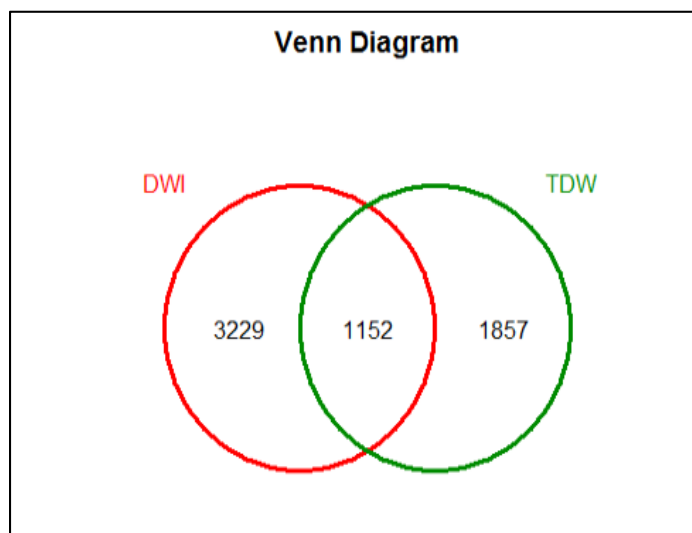


Fig. 4.6. 2-way Venn diagram of DWI and TDW samples.

Sample intensities were used to determine the percent difference between the two sources. Despite limitations introduced by the ionization efficiencies, possible losses during the extraction process and differences associated with the water retention time in the treatment plant, Fig. 4.7 clearly shows a fraction of chemicals that were unique to the drinking water intake (red) and treated drinking water (green) locations. The blue symbols in the central region of the graph represent the chemical entities that were found in both end members (purple diamonds represent the 64 recalcitrant chemicals). This area can be further divided into three: chemicals that showed higher intensity values in the DWI than the TDW location (above the zero line), components in which an increase in signal was observed in the TDW location (below the zero line), and features near the zero line, for which their abundance remained similar before and after treatment.

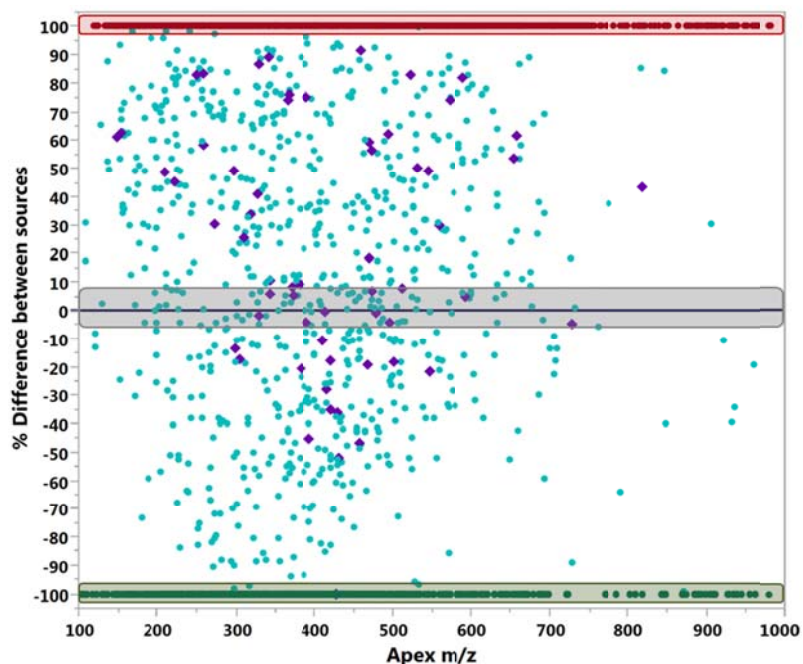


Fig. 4.7. Percent difference between the DWI and TDW locations using the intensities. Red indicates components found only in DWI. Green indicates components found only in TDW. Purple diamonds indicate the 64 recalcitrant chemicals. Blue dots represent components found in both locations. Dots near the zero line represent chemicals that have similar intensities before and after treatment.

4.5 Conclusion

Non-target analysis of treated wastewater effluent, surface water, and treated drinking water using a high-resolution QExactive mass spectrometer revealed the presence of a large array of chemical formulae. Wastewater effluent and treated drinking water proved to be the most chemically diverse samples with 97 and 95 different compound classes respectively. However, total intensities for treated drinking water were more than an order of magnitude lower than those from the effluent. In addition, upon exclusion of any molecular formulae present at the upstream location, it was determined that a total of 64 components introduced from the effluent location persistent through all the samples including the final treated drinking water. Target MS/MS in combination with *in-silico* fragmentation software packages were used to tentatively confirm the identity of four of these 64 recalcitrant chemicals.

Comparison between the drinking water intake and the treated drinking water demonstrated that 74% of the chemicals entering the drinking treatment plant were no longer present in the treated water following treatment. A portion of persistent chemical entities ($n = 1,152$) were observed in both end-members showing differences in intensity before and after treatment. These findings suggest that treatment technologies to date are not fully capable to remove chemical residues which may be present in wastewater and/or drinking water sources.

The combination of high-resolution instruments, fragmentation prediction software packages, and post-acquisition data mining provided the means necessary for characterization, identification, and tentative confirmation of chemicals found in surface waters matrices. This approach can be applied to other scenarios to help recognize

differences and similarities among multiple sites, and to identify potential sources of contamination.

4.6 Acknowledgements

We would like to thank Drs. Susan Glassmeyer and Mark Mills from the US Environmental Protection Agency (USEPA) for providing all of the samples for this work as part of the WW2DW project. NH acknowledges the support from FIU's University Graduate School through the award of a Dissertation Year Fellowship (DYF). We would also like to thank Thermo Scientific for providing partial analytical support. Special thanks to Miguel Parra for providing statistical analysis and software support.

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CHAPTER 5

Conclusions

The overall objectives of this study were to develop target and non-target analytical methodologies on the basis of high-resolution mass spectrometry for the analysis and assessment of drugs of abuse (DOA) from raw sewage waters in a college campus, the identification of phase I and phase II DOA transformation products, and the identification and characterization of unknown recalcitrant compounds in different types of interconnected water matrices.

During the first part of this research, the successful development of a fully automated online-SPE-LC-HRMS method accomplished, and implemented to the comprehensive analysis of 18 drugs of abuse including some of their well-known metabolites. Two different locations (dorms and main campus) within a college campus yield detections for 14 DOAs. The two most abundant and prevalent drugs found among the two locations were amphetamine and THC's main metabolite (THC-COOH). Maximum concentrations observed throughout the one-year study were 5,956 ng/L and 2,413 ng/L for amphetamine and THC-COOH respectively.

Drug consumption estimates were determined for amphetamine, THC, heroin, and cocaine in the dorms and main campus. Low Levels of consumption (<3 doses/day/1000 people on average) were found for heroin and cocaine in both locations. Amphetamine intake range between 10 and 20 doses/day/1000 people (with higher levels being in the dorms), and remain constant throughout the beginning, middle and end of the semester in the dorms. THC consumption estimates were significantly higher in the dorms (233 ± 199 doses/day/ 1000 people) than those in the main campus (46 ± 26 doses/day/1000 people). In addition, an increase in consumption for THC was noticeable and at the end of the semester in the dorms location.

In the second portion of this study, two different acquisition modes (target MS/MS and data-dependent MS/MS) were compared and used in combination to metabolic identification and structural elucidation software to identify and confirmed a total of 35 phase I and phase II DOA metabolic transformation products. Even though, data-dependent scan is a faster approach towards the potential identification of metabolites and acquires MS/MS data for all precursor ions above a specific intensity, target MS/MS was selected because it offered an acceptable and consistent number of data points across the chromatographic peak needed for the reliable confirmation of TPs.

Raw sewage samples collected in the main campus were used in this portion of the study, and concentrations for all detected TPs (n=54) were estimated based on the parent DOAs response factor and used to calculate the percent mole fractions (Mf). High frequency and abundance (compared to the parent compound) for nine of the TPs identified, solidifies the importance of evaluating the presence of TPs in water matrices to assess treatment efficiency in wastewater and drinking water treatment facilities; and to determine their fate, transport and potential toxicity in aquatic ecosystems.

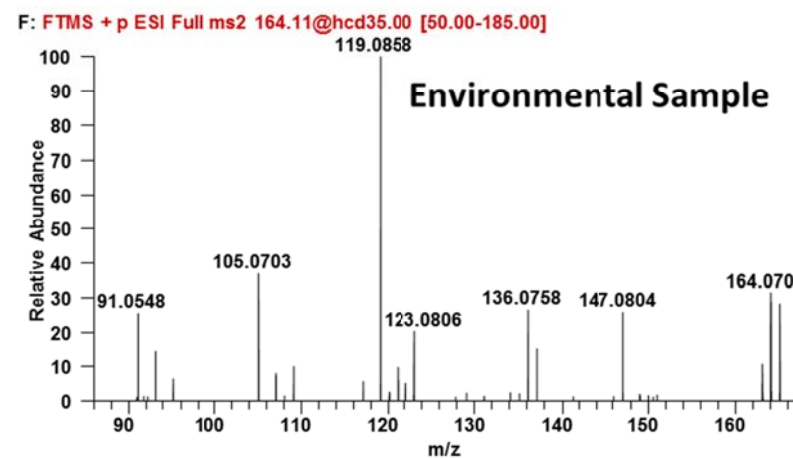
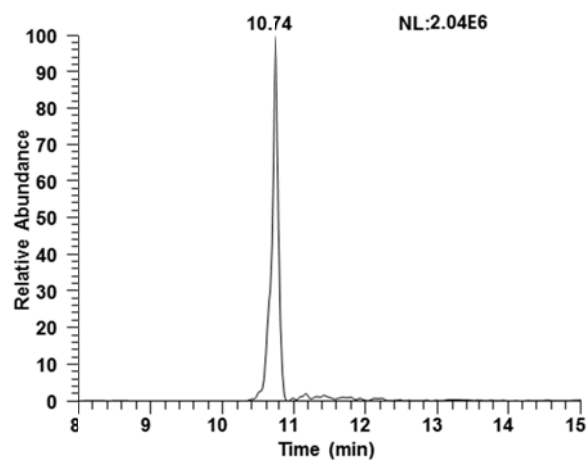
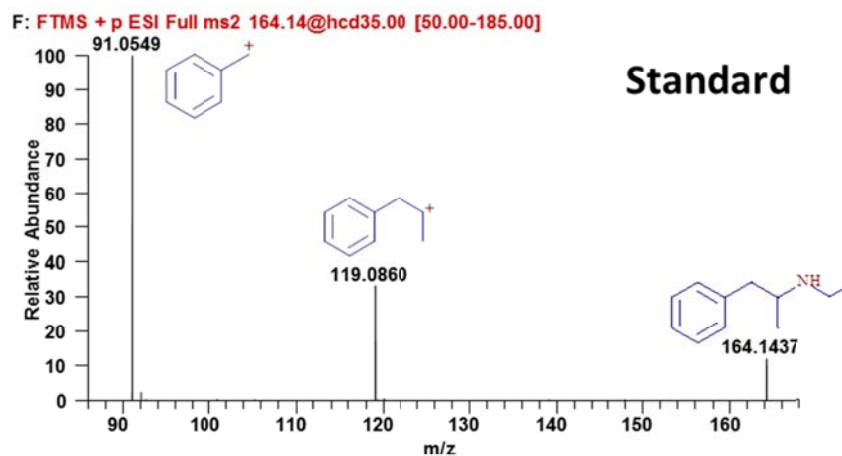
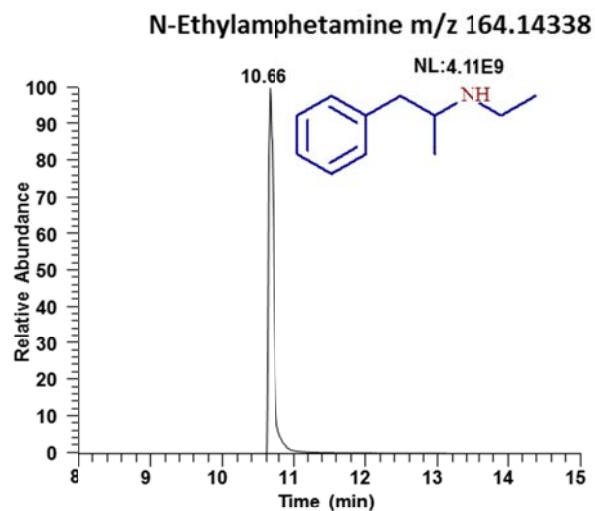
Lastly, non-target analysis was carried out on six samples, from locations covering about nine miles of upstream (UP), effluent wastewater (EFF), effluent mixing zone, downstream (EMZ), drinking water intake (DWI), and treated drinking water (TDW). Full-scan post-acquisition data was mined following a series of heuristic steps for determining molecular compositions to establish their abundance (in number of formulas), and identify compound diversity among the locations.

Graphical interpretations of data (plots and diagrams) were used to identify relationship among locations following the effluent, and between the drinking water

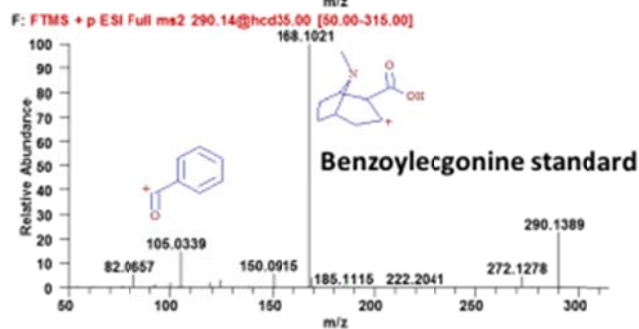
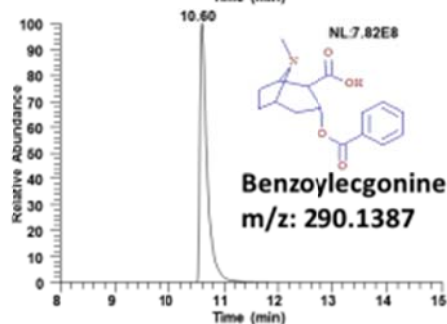
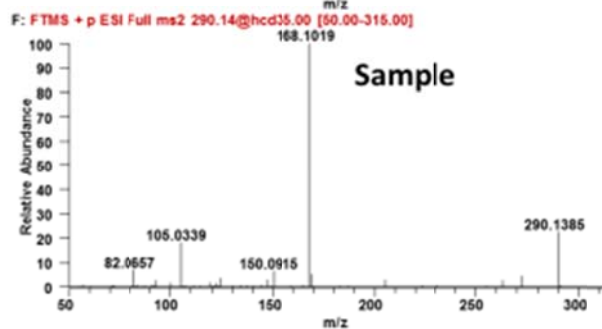
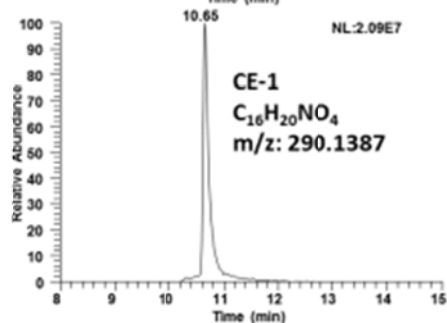
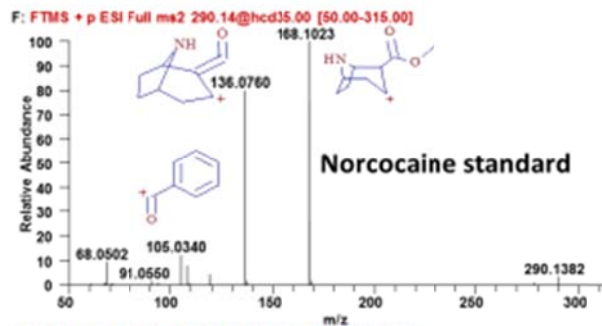
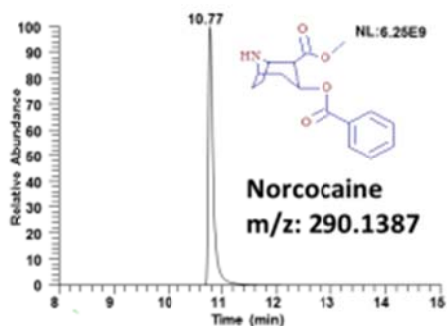
intake and the treated drinking water. Results show that a total of 64 compounds between the EFF-TDW trajectory remain persistent throughout this process. In addition, when comparing DWI and TDW, it was observed that 1,152 compounds survive drinking water treatment, and 1,857 analytes are formed during treatment. This demonstrates that treatment technologies to date are not designed to remove a substantial quantity of compounds.

APPENDICES

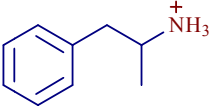
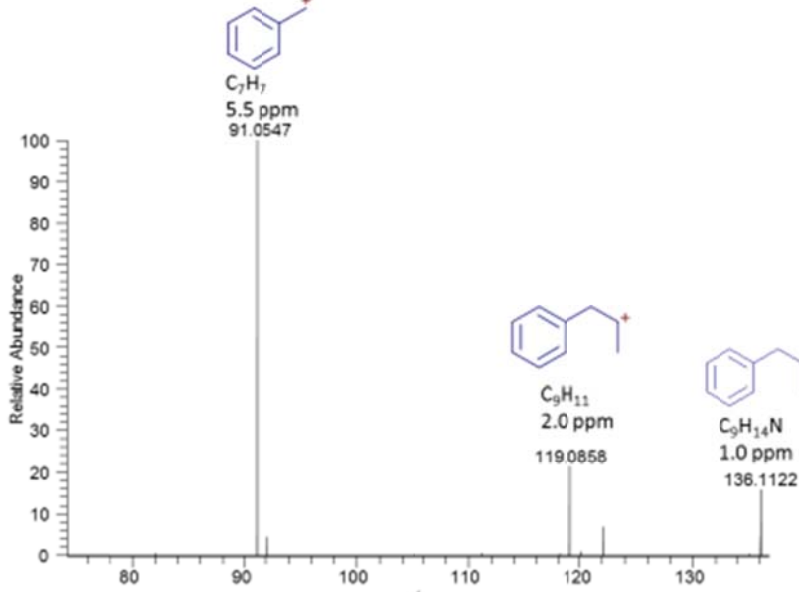
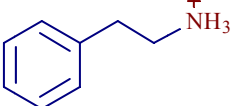
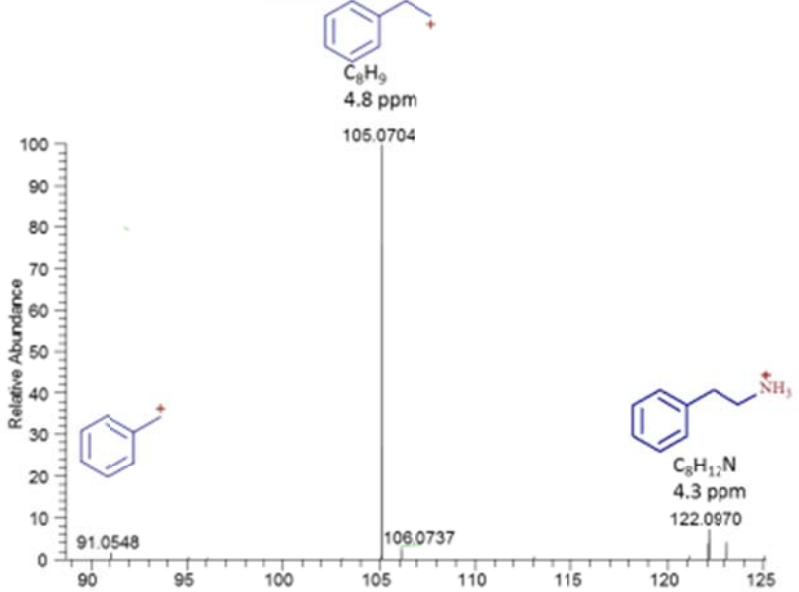
Appendix 1. MS/MS spectral comparison for TP identified as N-ethylamphetamine from TraceFinder database, and formed from methamphetamine (MA-4), MDEA (MDEA-4), and MDMA (MDMA-5) against an N-methylamphetamine reference standard.

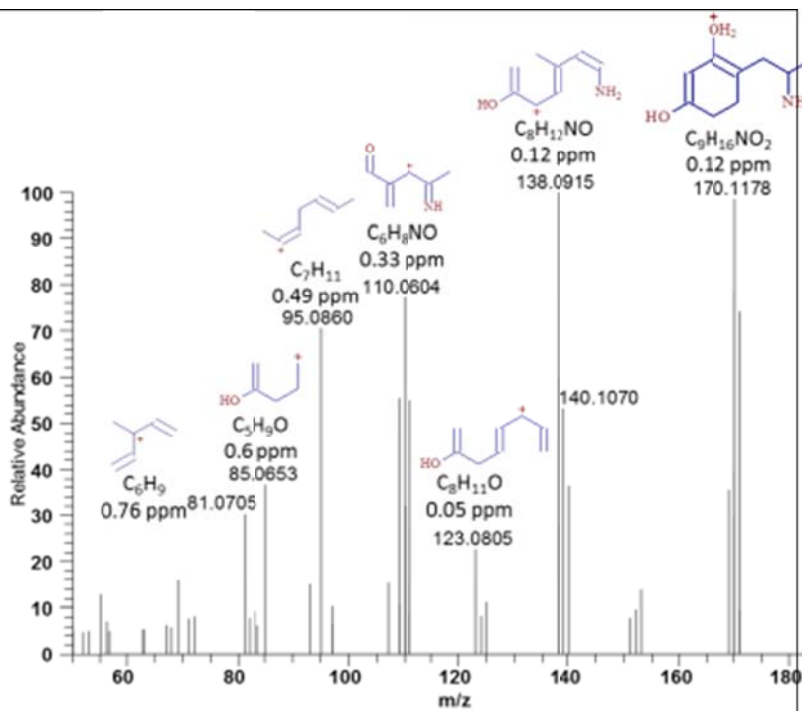
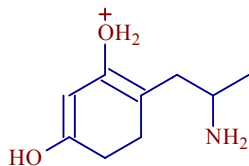
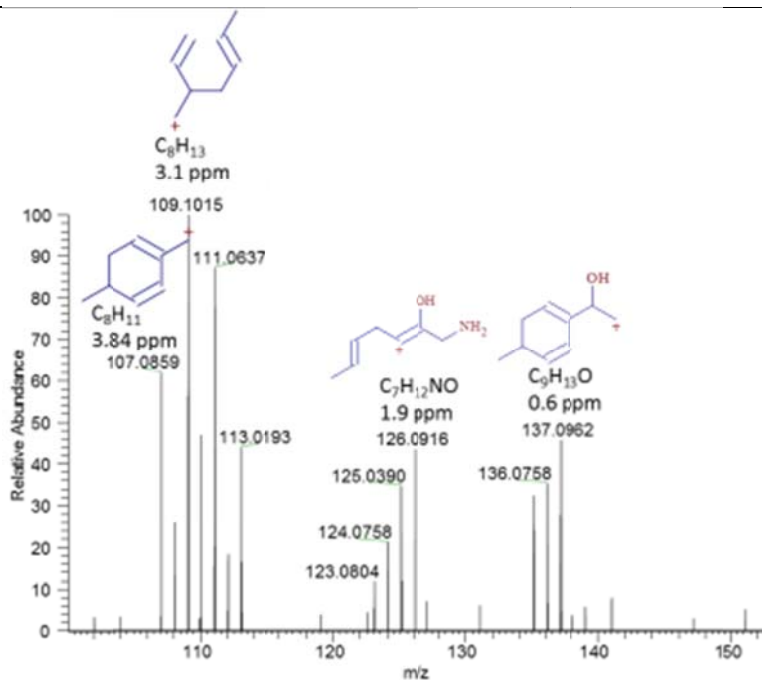
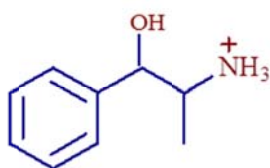


Appendix 2. MS/MS spectral comparison for TP identified as CE-1 formed from cocaethylene, had the same m/z 290.1387 as norcocaine and as the target metabolite benzoylecgonine. MS/MS fragmentation of sample was compared to both reference standards.

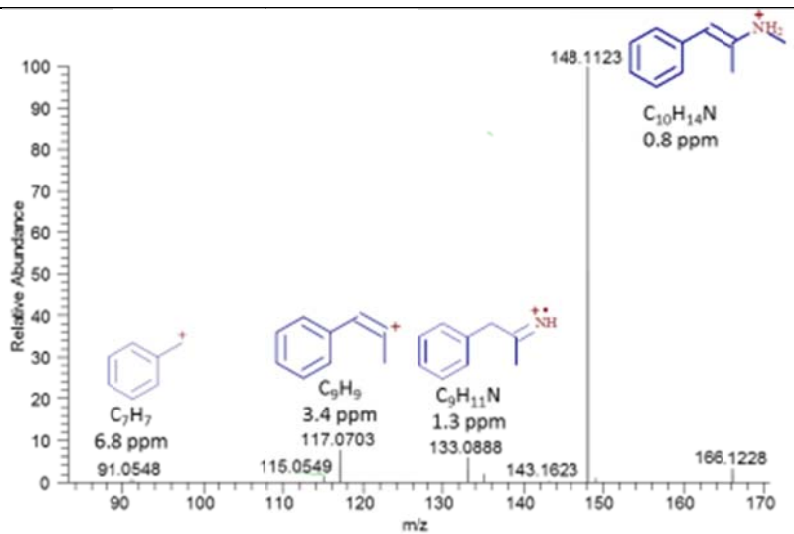
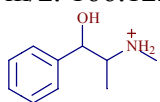


Appendix 1. MS/MS spectral elucidation for all target analytes (parents and their metabolites) and spectral elucidation for some of the identified TPs, showing tentative structure, chemical formulas, and mass error (ppm) is demonstrated. MS/MS spectra for all 54 TPs is presented.

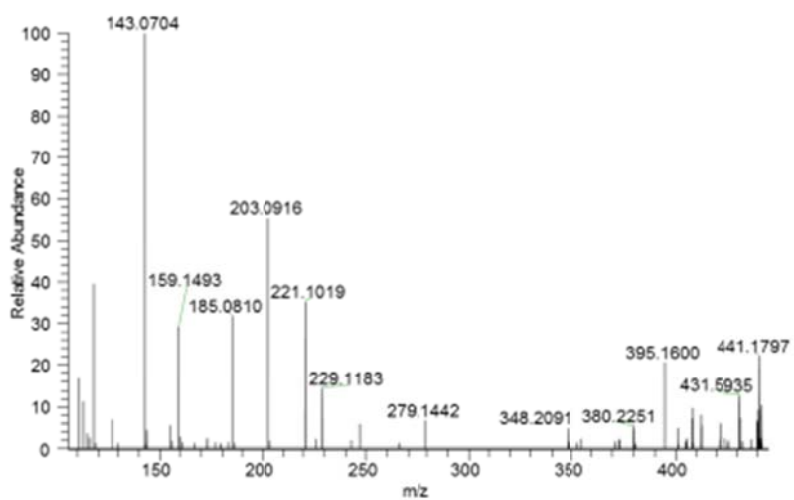
Analyte/TPs	MS/MS Spectra
<p>Amphetamine (AM) $C_9H_{14}N$ m/z: 136.1121</p> 	 <p> C_7H_7 5.5 ppm 91.0547 </p> <p> C_9H_{11} 2.0 ppm 119.0858 </p> <p> $C_9H_{14}N$ 1.0 ppm 136.1122 </p>
<p>AM-1* $C_8H_{12}N$ m/z: 122.0964</p> 	 <p> C_8H_9 4.8 ppm 105.0704 </p> <p> $C_8H_{12}N$ 4.3 ppm 122.0970 </p> <p> 91.0548 </p>

AM-2* $C_9H_{16}NO_2$ m/z : 170.1176**AM-3*** $C_9H_{16}NO$ m/z : 154.1226

AM-4, MA-1, MDMA-3
C₁₀H₁₆NO
m/z: 166.1226



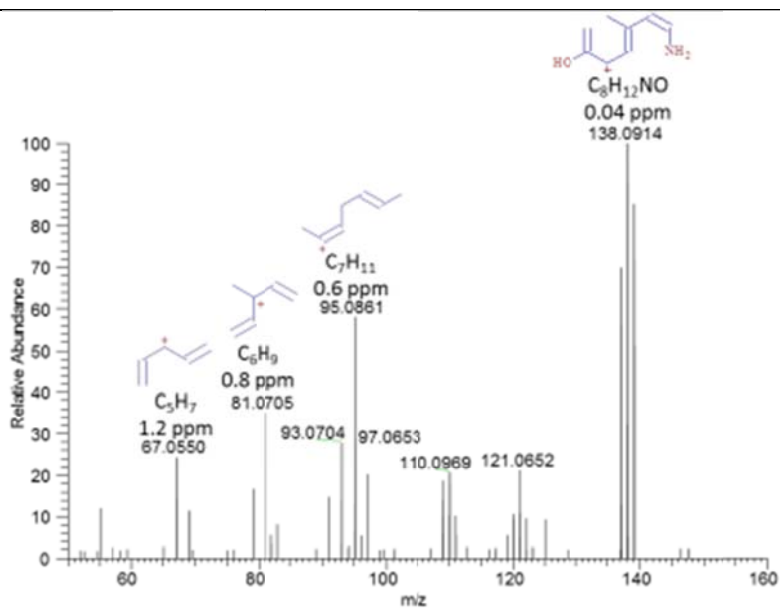
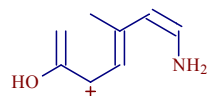
AM-5*
C₁₉H₂₉N₄O₆S
m/z: 441.1802



AM-6*

$C_8H_{12}NO$

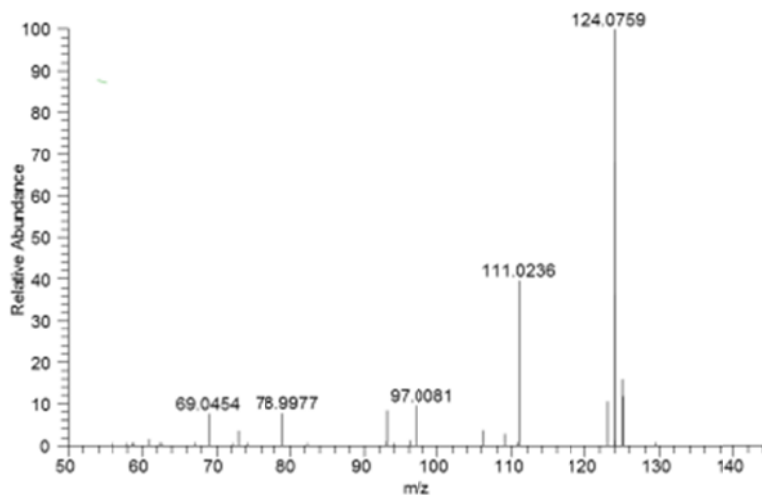
m/z : 138.0913

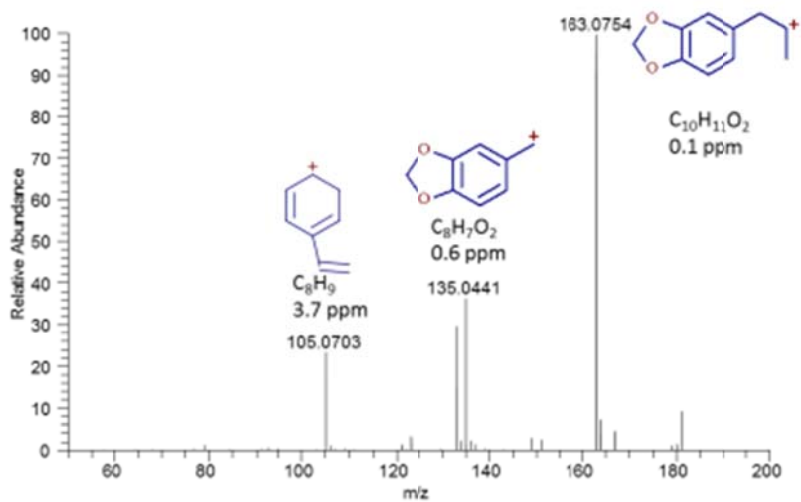
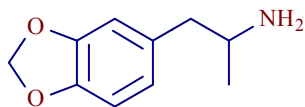
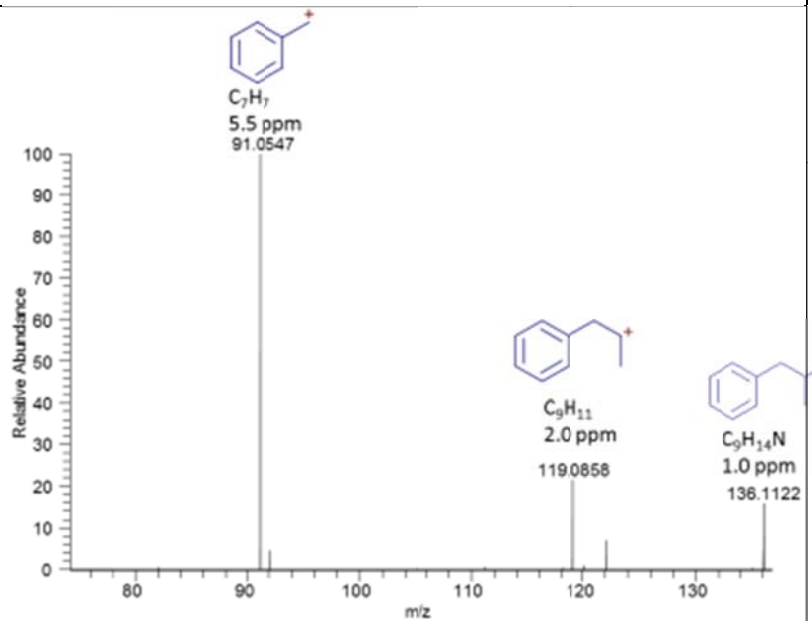
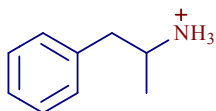


AM-7*

$C_7H_{10}NO$

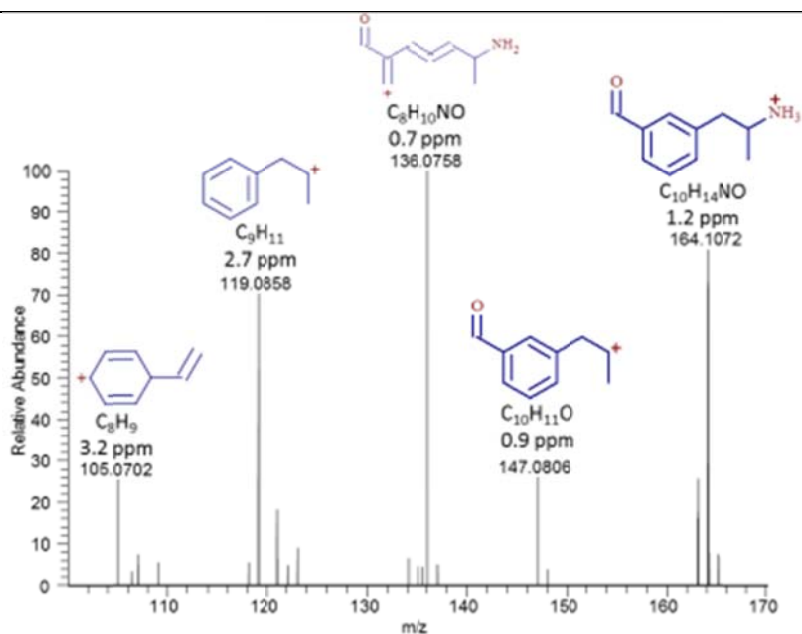
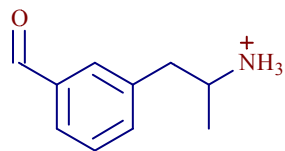
m/z : 124.0757



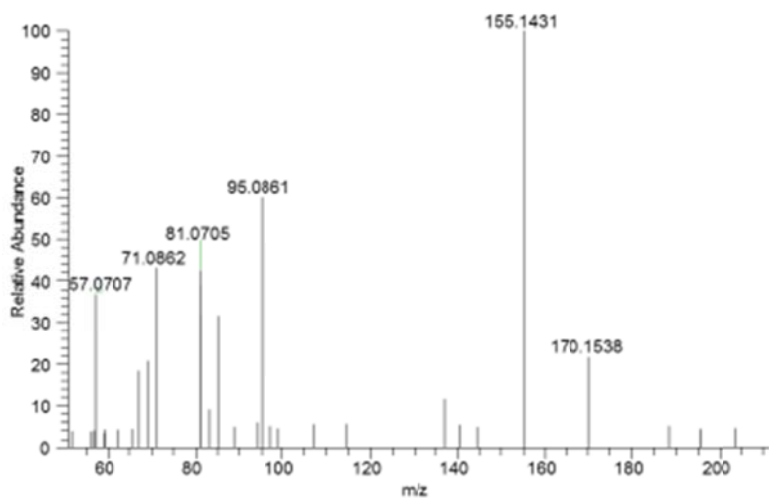
MDA $C_{10}H_{14}NO_2$ m/z : 180.1015**MDA-1, AM, MA-2** $C_9H_{14}N$ m/z : 136.1121

MDA-2*C₁₀H₁₄NO

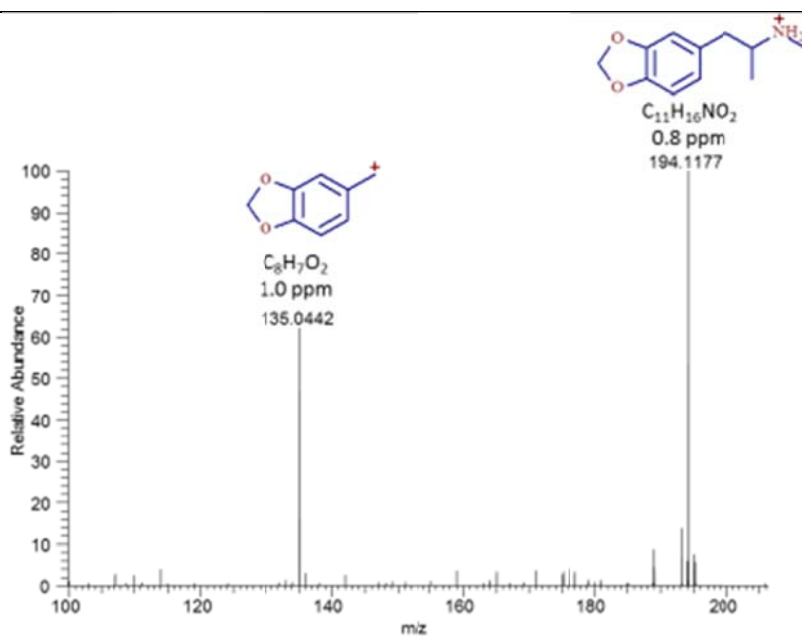
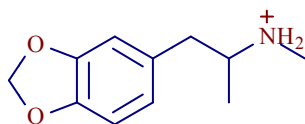
m/z: 164.1070

**MDA-3***C₁₀H₁₄NO₄

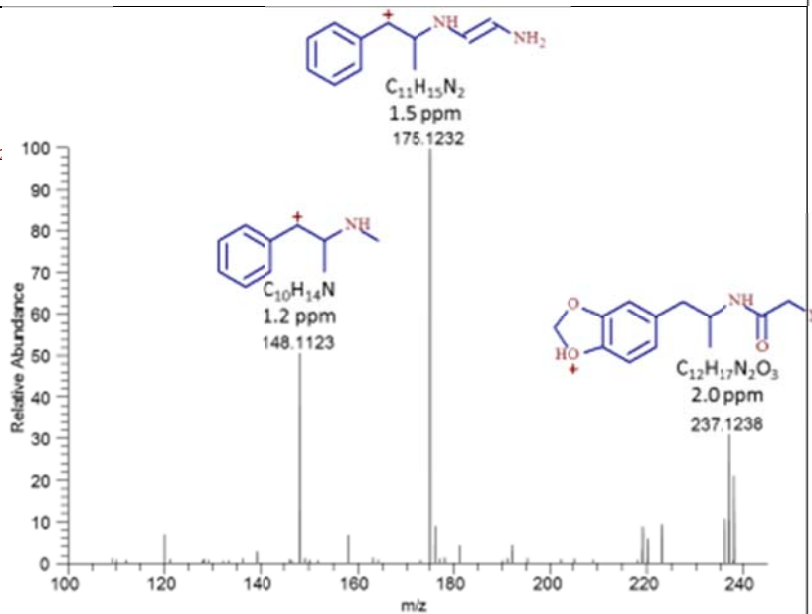
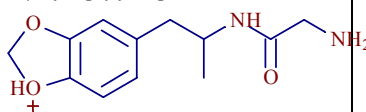
m/z: 212.0917



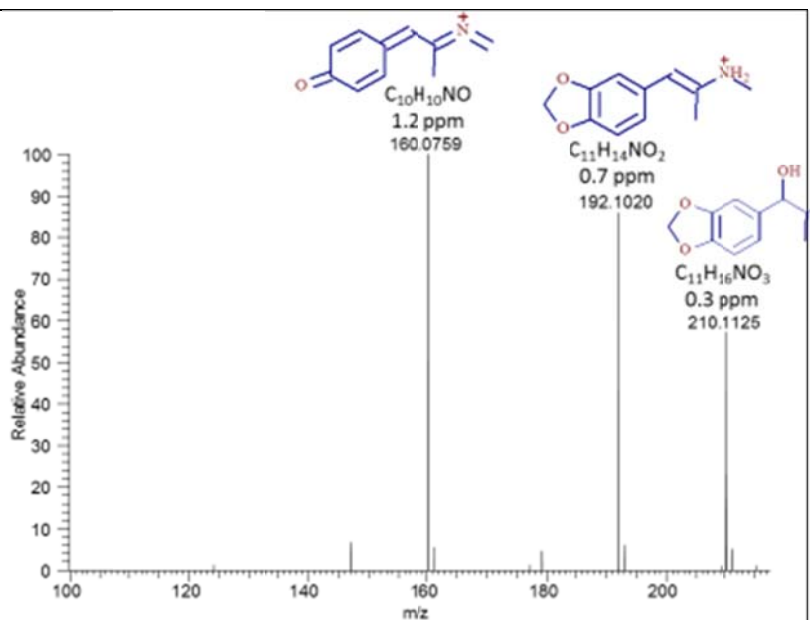
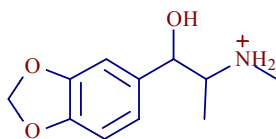
MDA-4, **MDMA**,
MDEA-2
 $C_{11}H_{16}NO_2$
 m/z : 194.1176



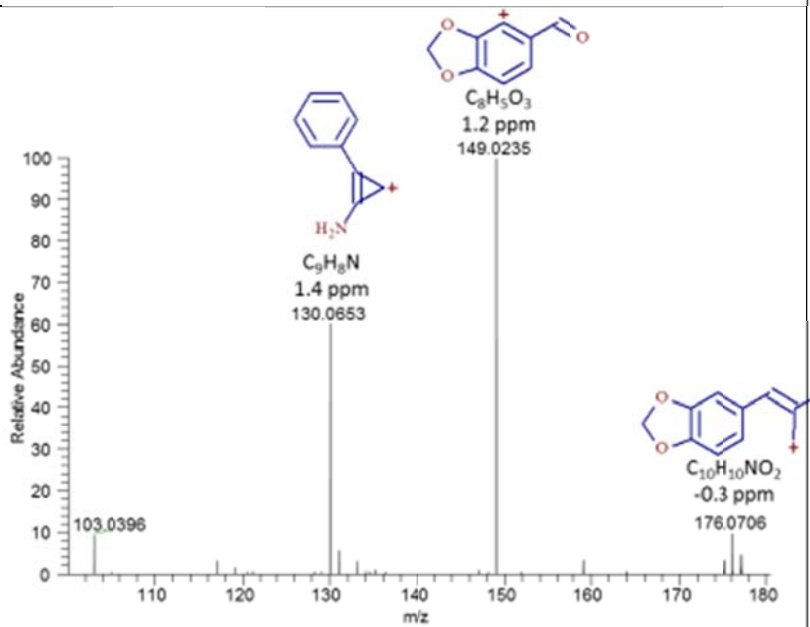
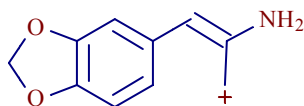
MDA-5*
 $C_{12}H_{17}NO_3$
 m/z : 237.1234



MDA-6*, MDMA-1,
COD-3
 $C_{11}H_{16}NO_3$
 m/z : 210.1125

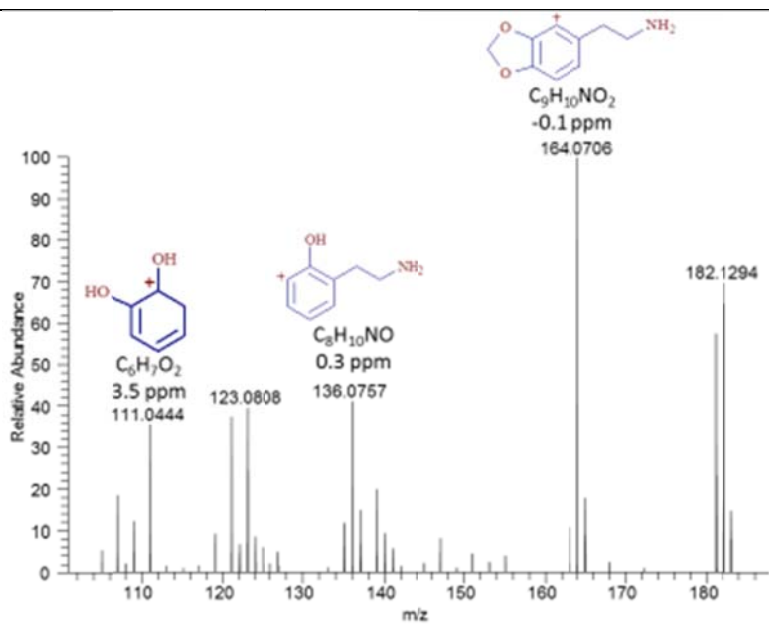
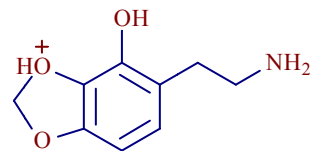


MDA-7*
 $C_{10}H_{10}NO_2$
 m/z : 176.0706

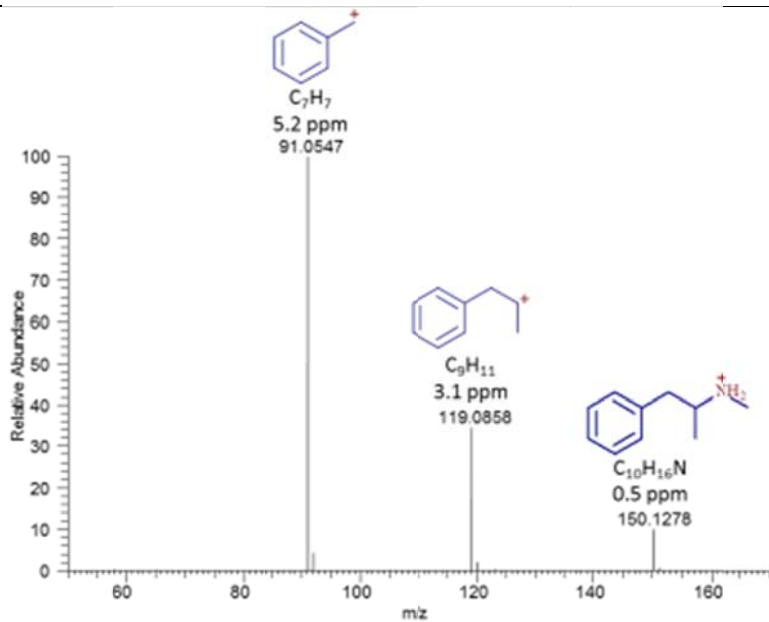
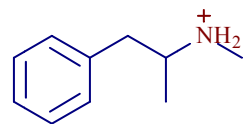


MDA-8*C₉H₁₂NO₃

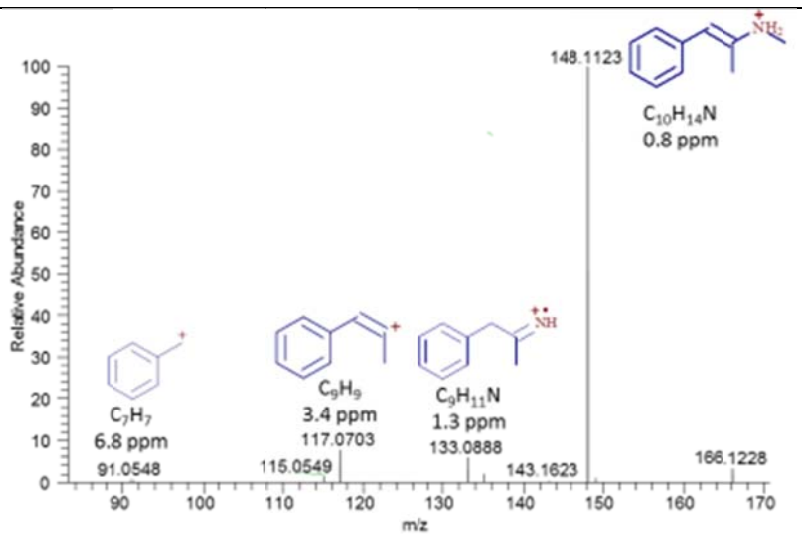
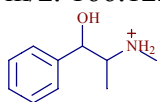
m/z: 182.0812

**Methamphetamine (MA)**C₁₀H₁₆N

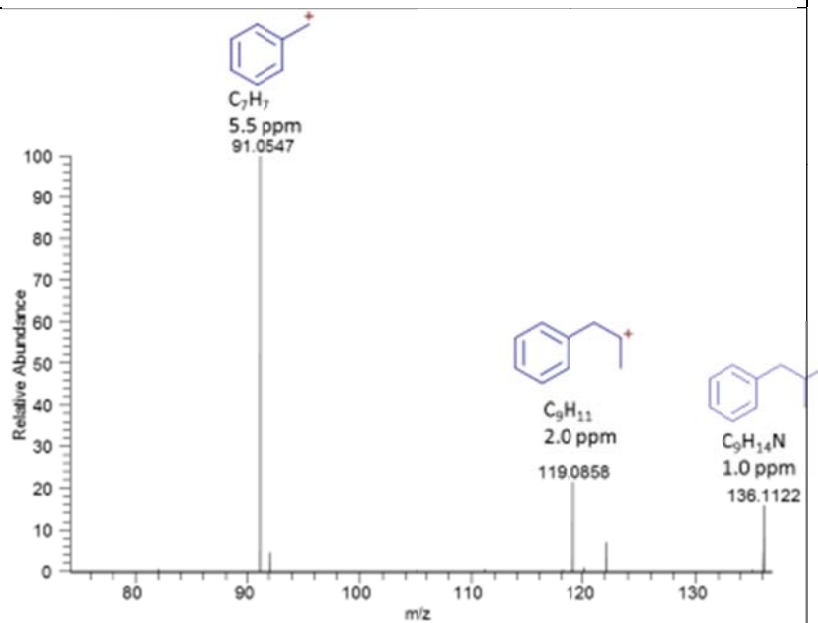
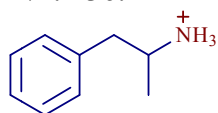
m/z 150.1277



MA-1, AM-4, MDMA-3
C₁₀H₁₆NO
m/z: 166.1226

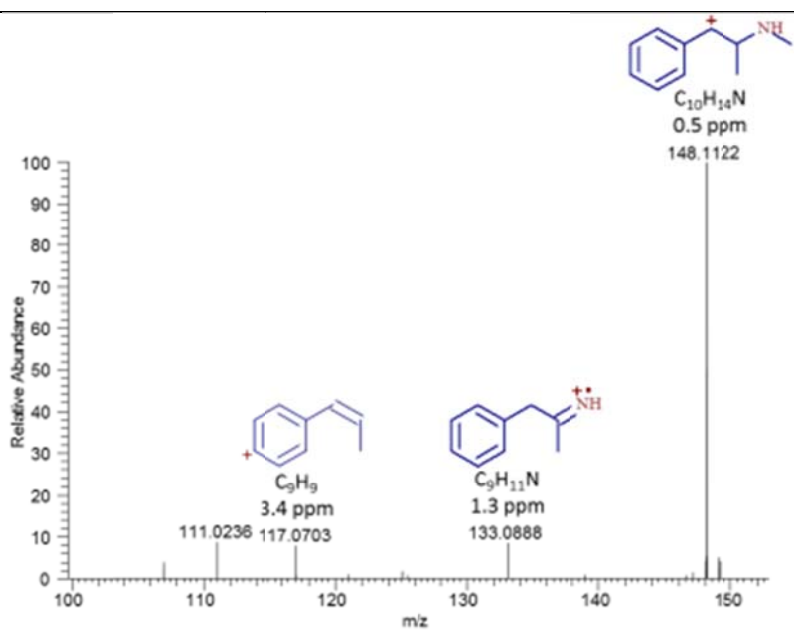
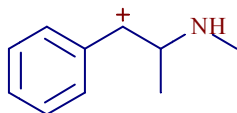


MA-2, AM, MDA-1
C₉H₁₄N
m/z: 136.1121

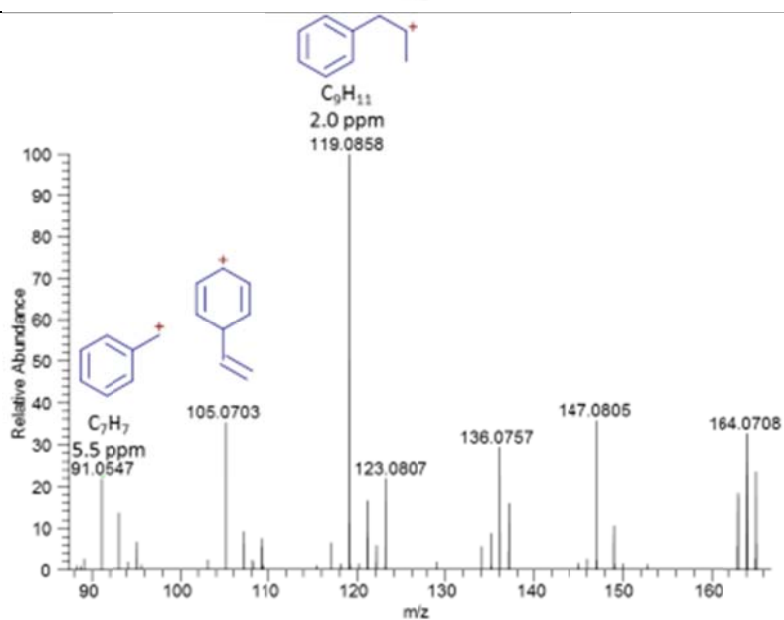
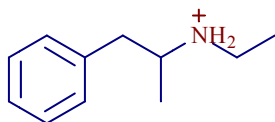


MA-3*C₁₀H₁₄N

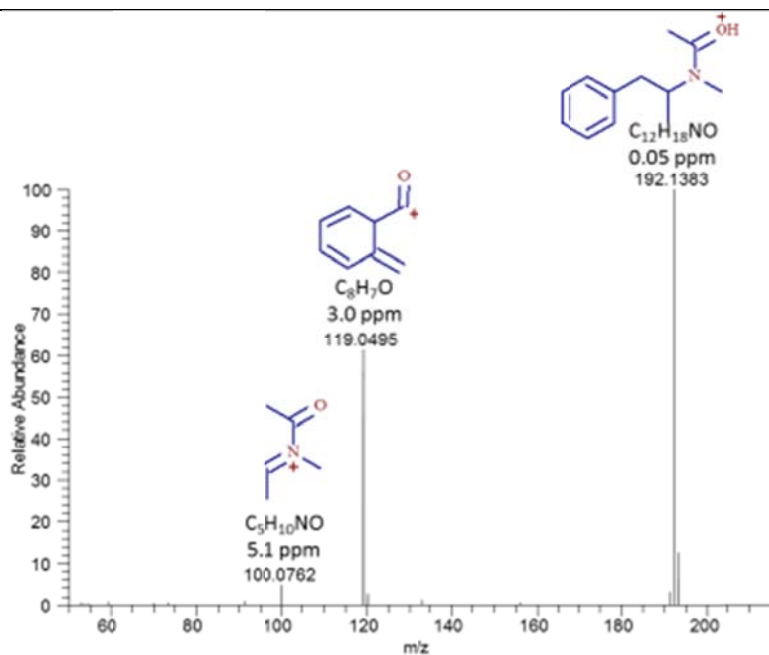
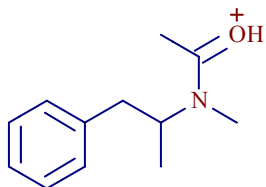
m/z 148.1121

**MA-4, MDEA-4,****MDMA-5**C₁₁H₁₈N

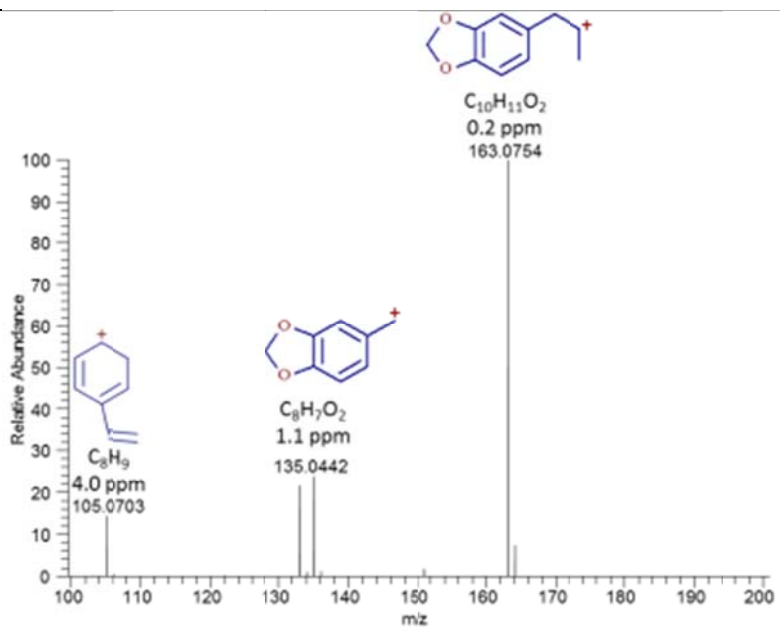
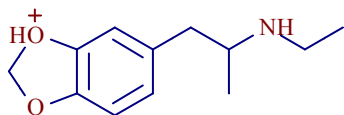
m/z: 164.1434

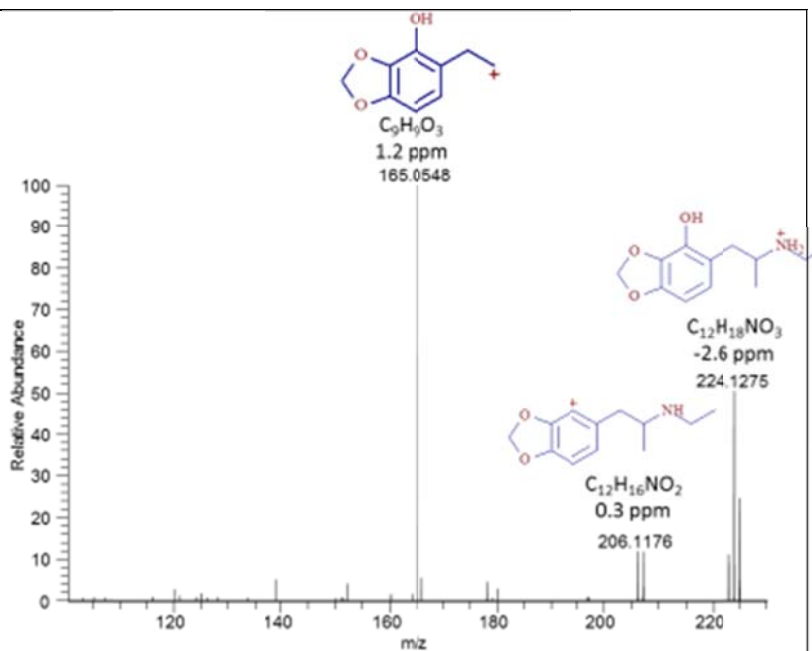
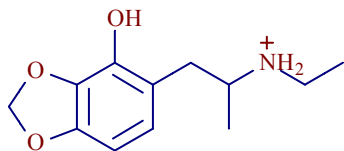
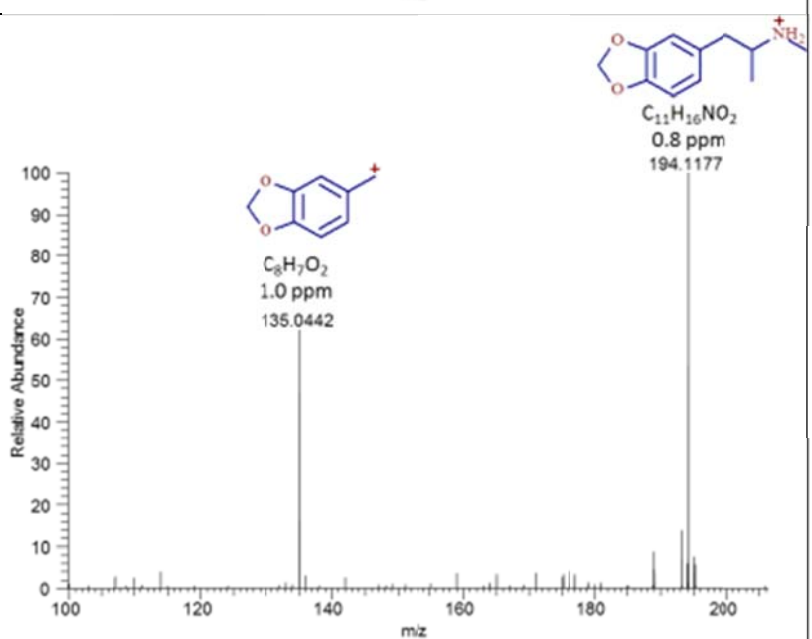
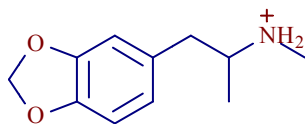


MA-5*, MDEA-5
 $C_{12}H_{18}NO$
 m/z 192.1383

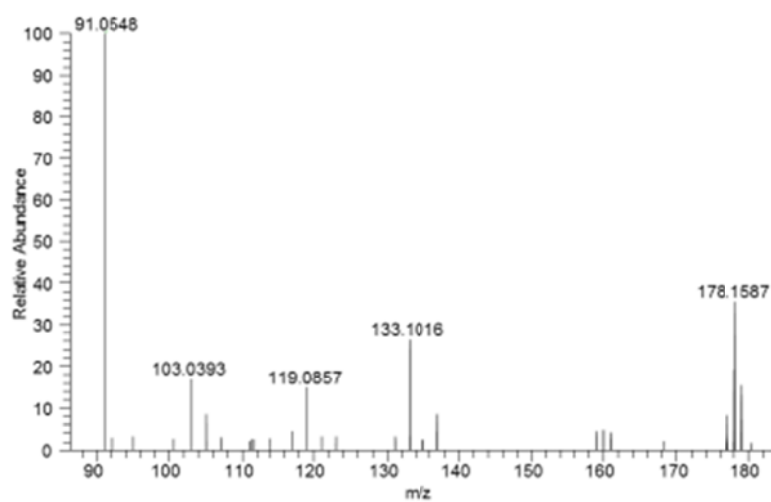


MDEA
 $C_{12}H_{18}NO_2$
 m/z 208.1332

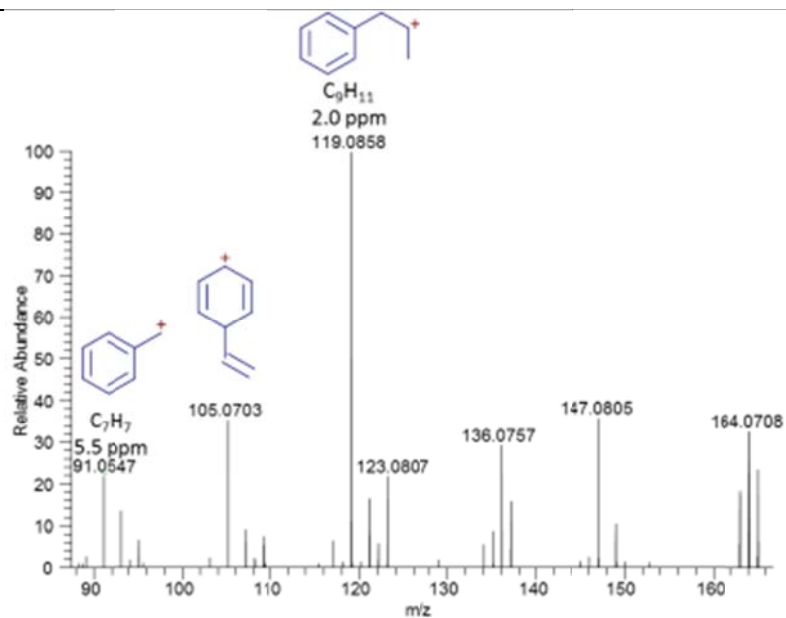
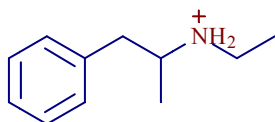


MDEA-1* $C_{12}H_{18}NO_3$ m/z 224.1281**MDEA-2, MDA-4,****MDMA** $C_{11}H_{16}NO_2$ m/z : 194.1176

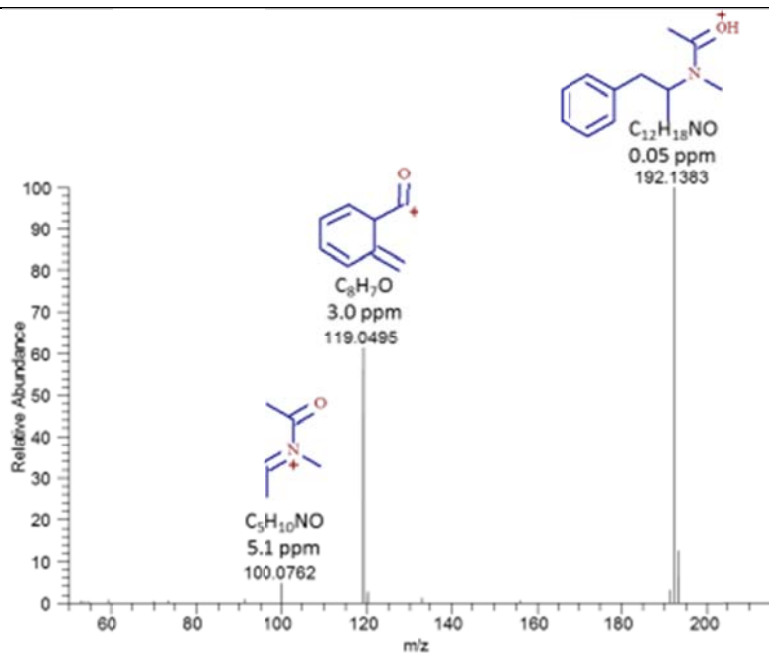
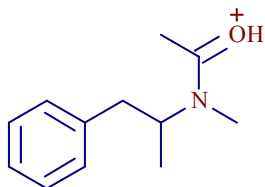
MDEA-3*
 $C_{12}H_{20}N$
 m/z : 178.1590



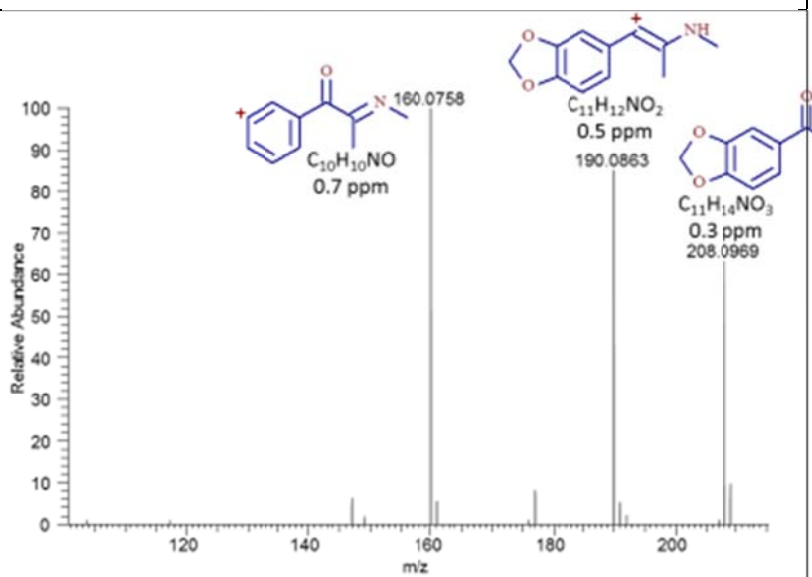
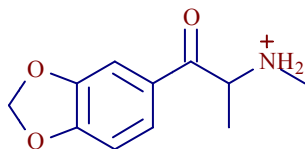
MDEA-4, MDMA-5,
 MA-4
 $C_{11}H_{18}N$
 m/z : 164.14338



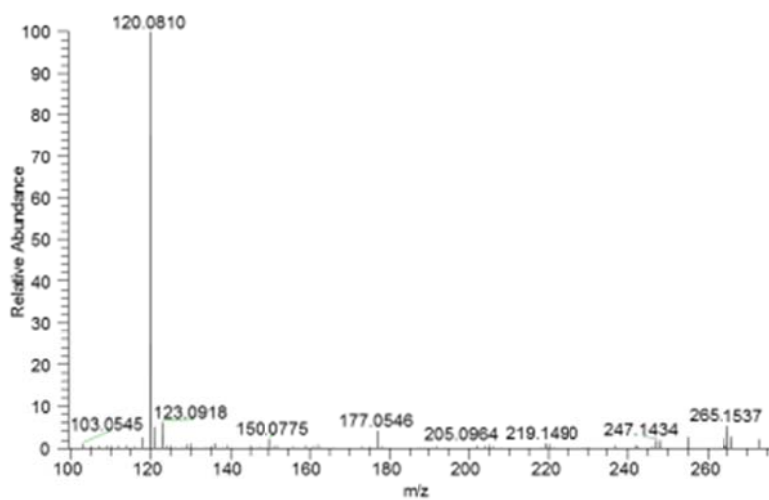
MDEA-5*, MA-5
 $C_{12}H_{18}NO$
 m/z 192.1383



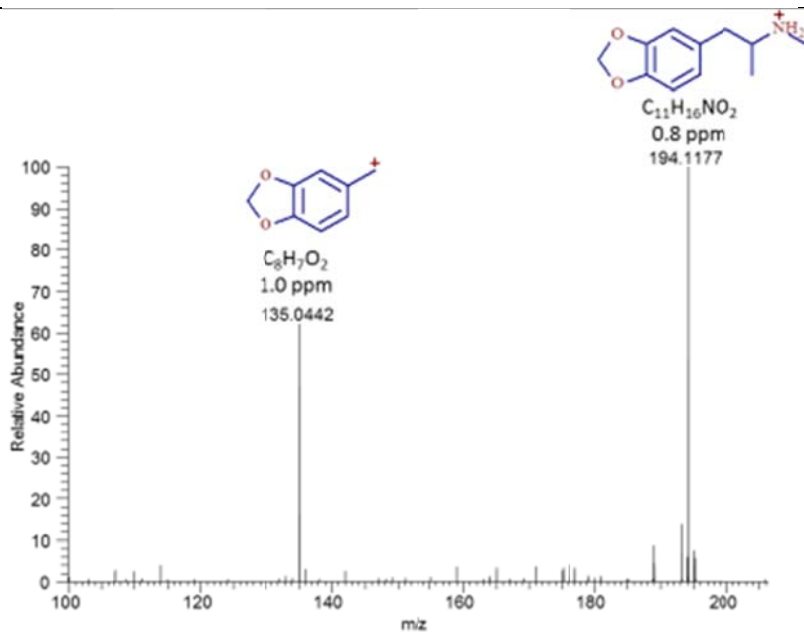
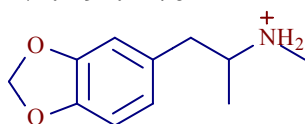
MDEA-6, MDMA-4
 $C_{11}H_{14}NO_3$
 m/z 208.0968



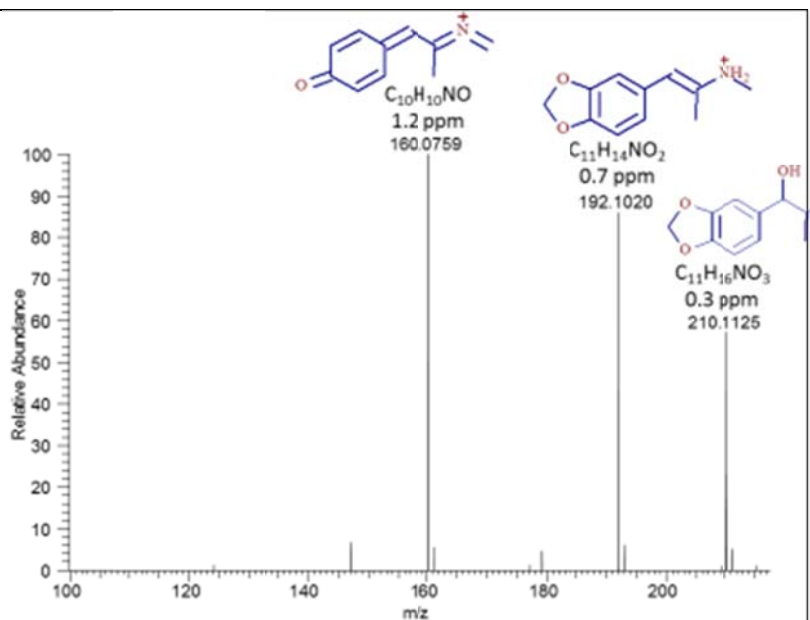
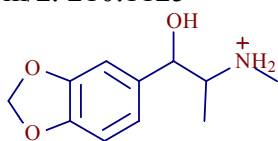
MDEA-7*
 $C_{14}H_{21}N_2O_3$
 m/z 265.1547



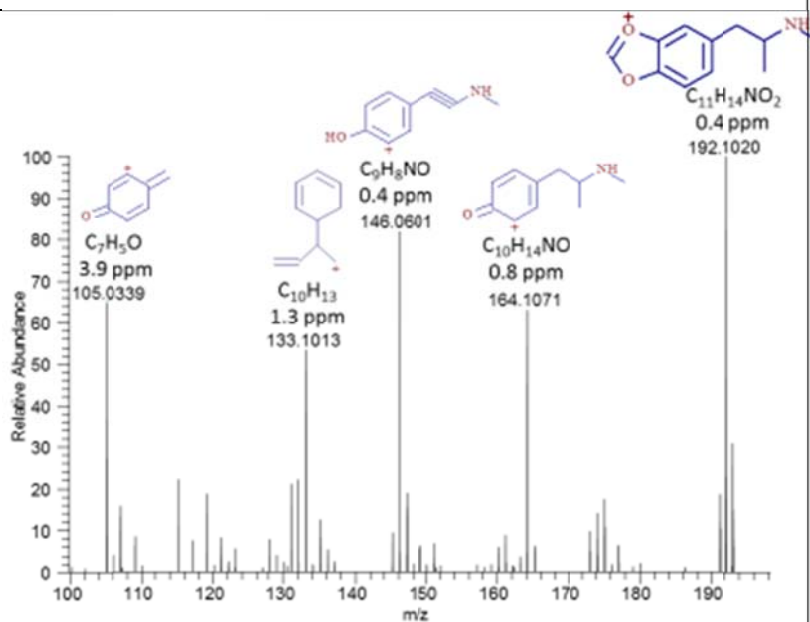
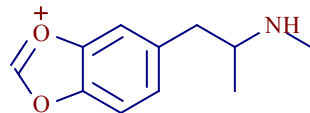
MDMA
 $C_{11}H_{16}NO_2$
 m/z : 194.1176



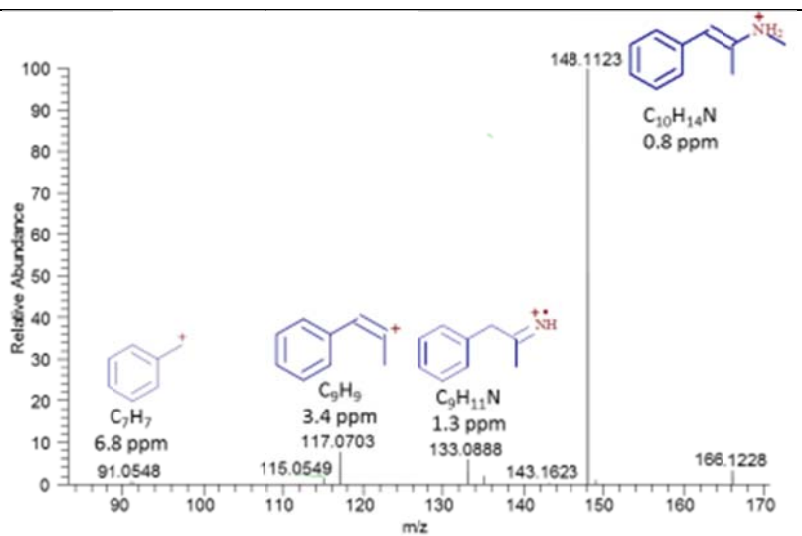
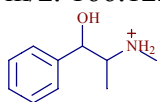
MDMA-1*, MDA-6,
COD-3
 $C_{11}H_{16}NO_3$
 m/z : 210.1125



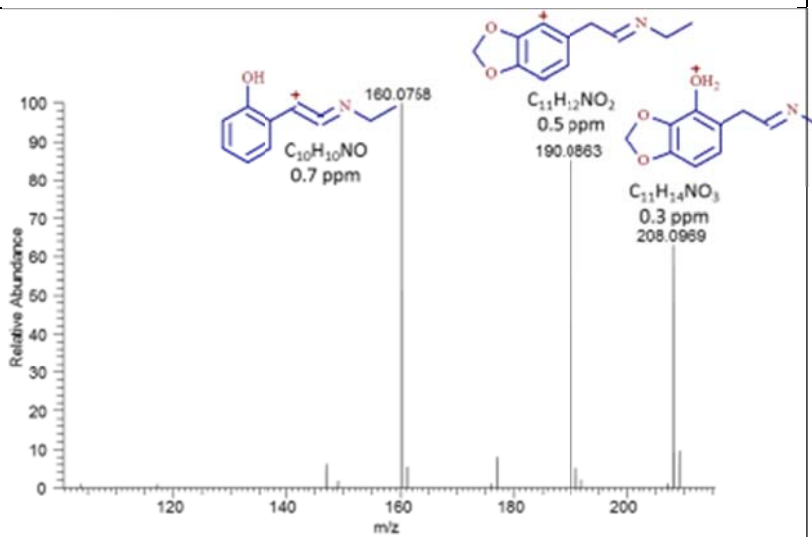
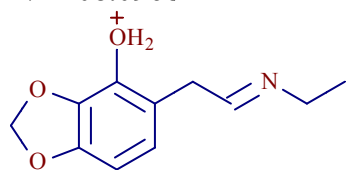
MDMA-2*
 $C_{11}H_{14}NO_2$
 m/z : 192.1019



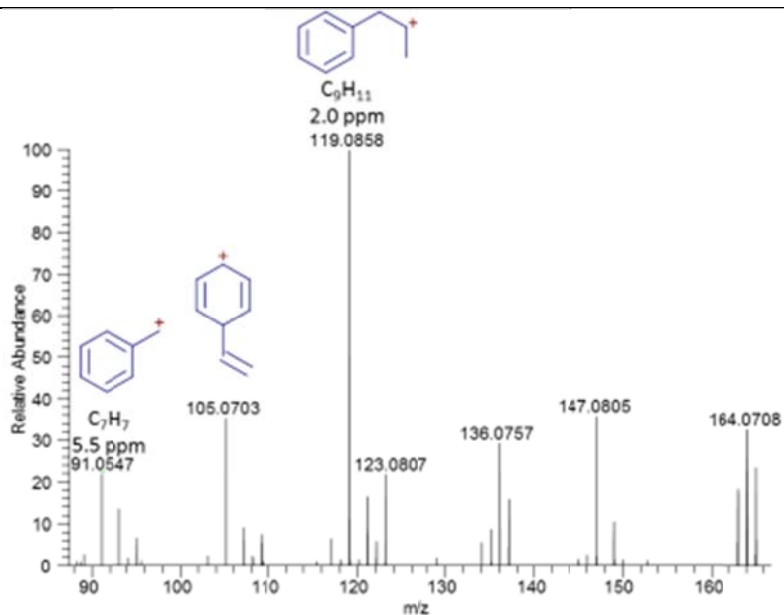
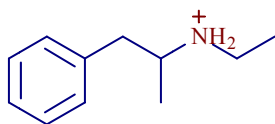
MDMA-3, MA-1, AM-4
C₁₀H₁₆NO
m/z: 166.1226



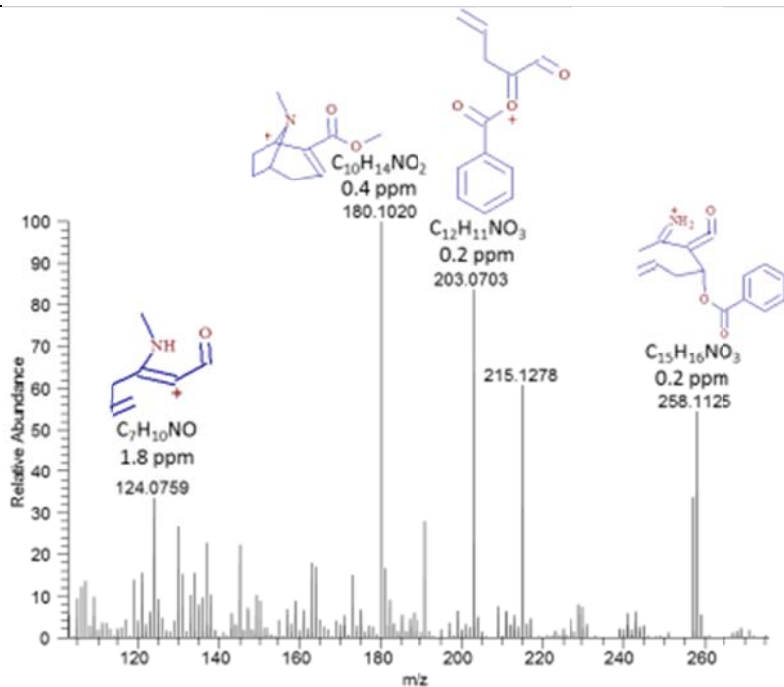
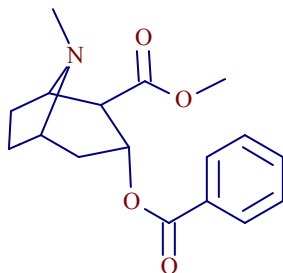
MDMA-4, MDEA-6
C₁₁H₁₄NO₃
m/z 208.0968

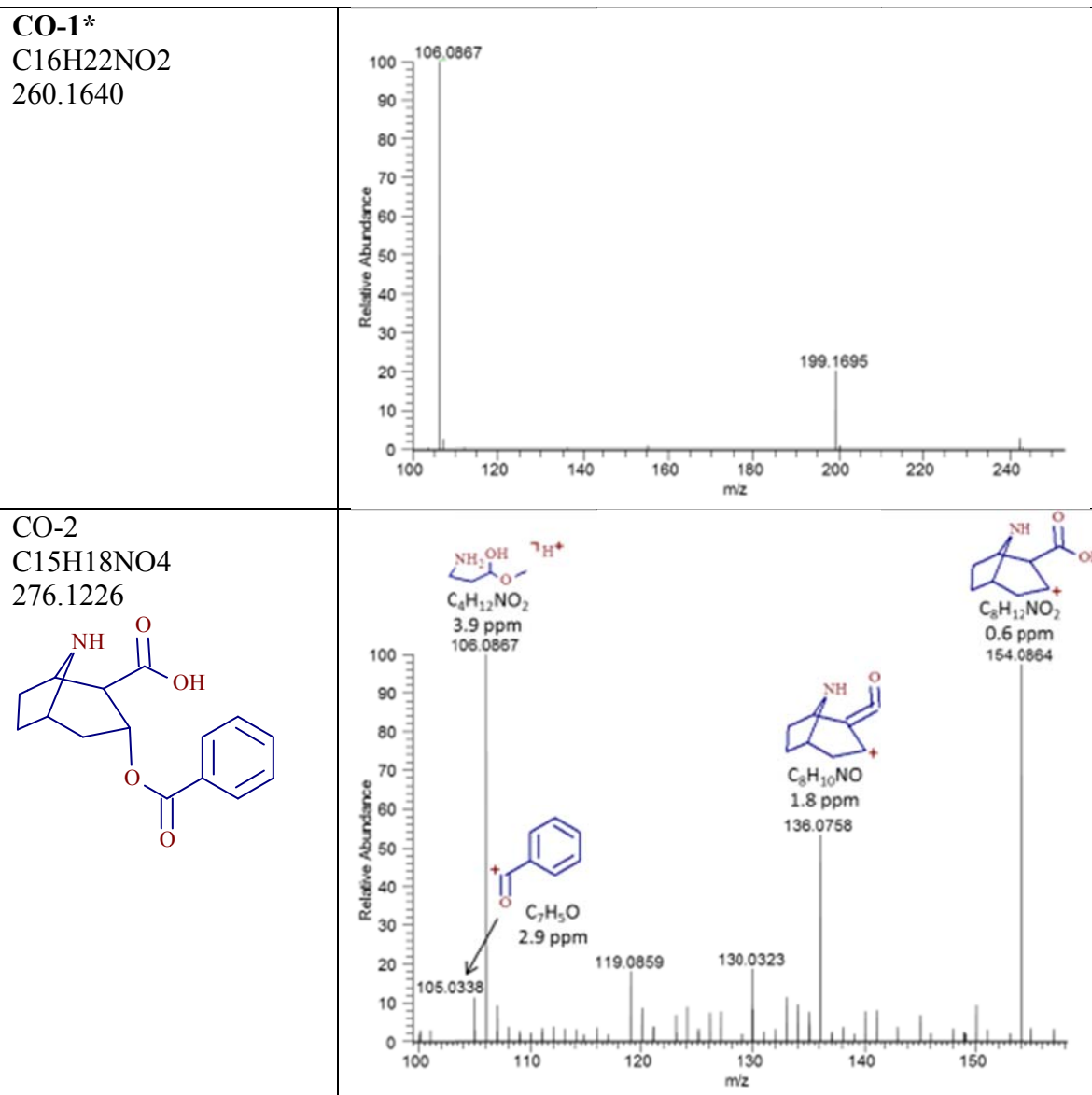


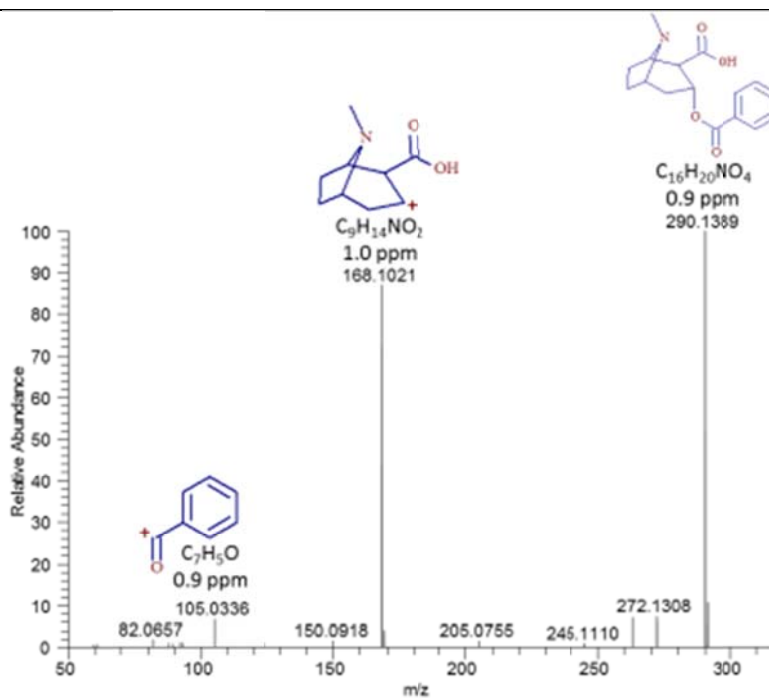
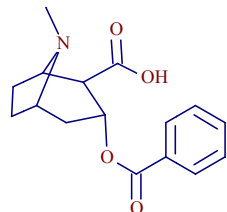
MDMA-5, MDEA-4,
MA-4
C₁₁H₁₈N
m/z: 164.14338



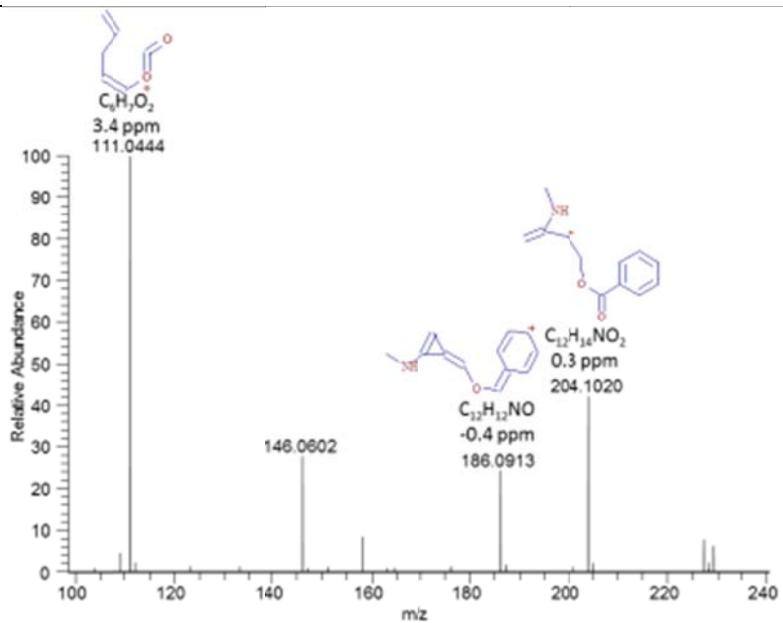
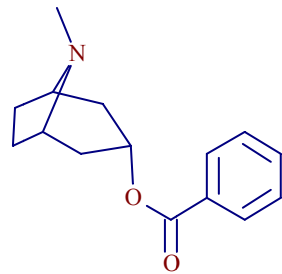
Cocaine (CO)
C₁₇H₂₂NO₄
304.1544





Benzoylecgonine (BE) $C_{16}H_{20}NO_4$ m/z : 290.1387**BE-1*** $C_{15}H_{20}NO_2$

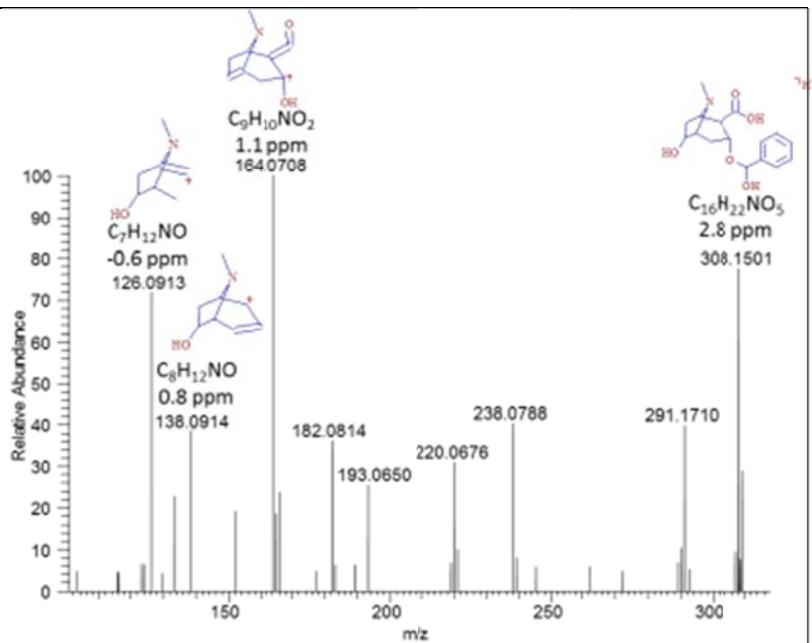
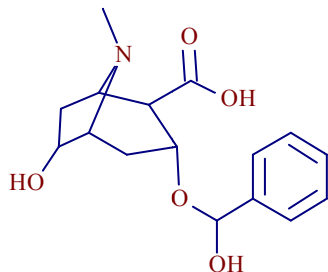
246.1483



BE-2*

$C_{16}H_{22}NO_5$

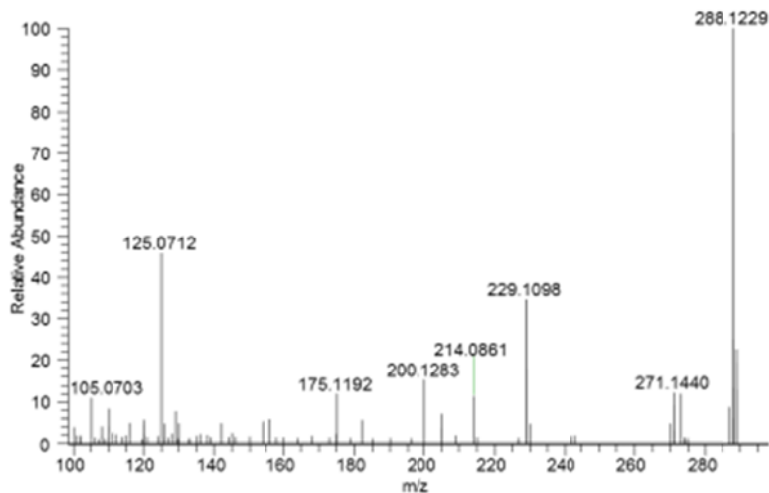
m/z: 308.1492



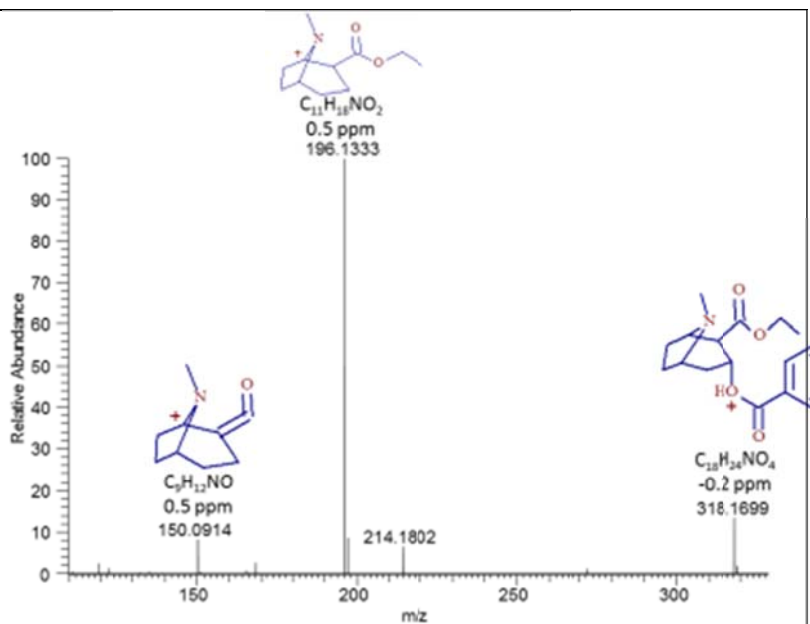
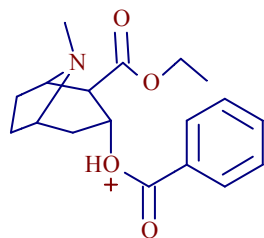
BE-3*

$C_{16}H_{18}NO_4$

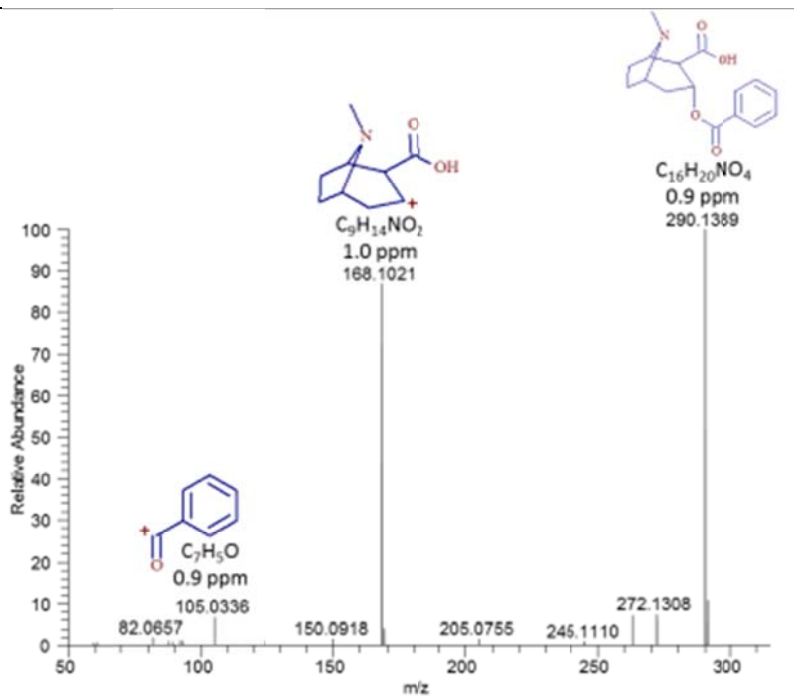
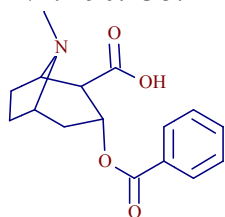
m/z: 288.1230



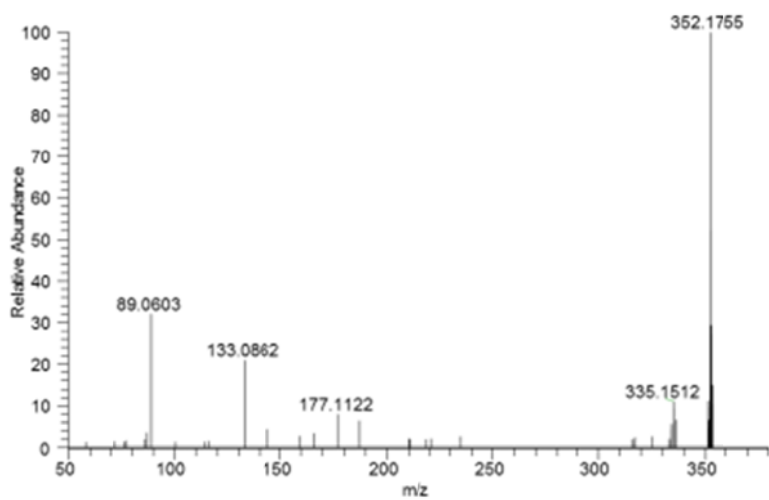
Cocaethylene (CE)
 $C_{18}H_{24}NO_4$
 m/z : 318.1700



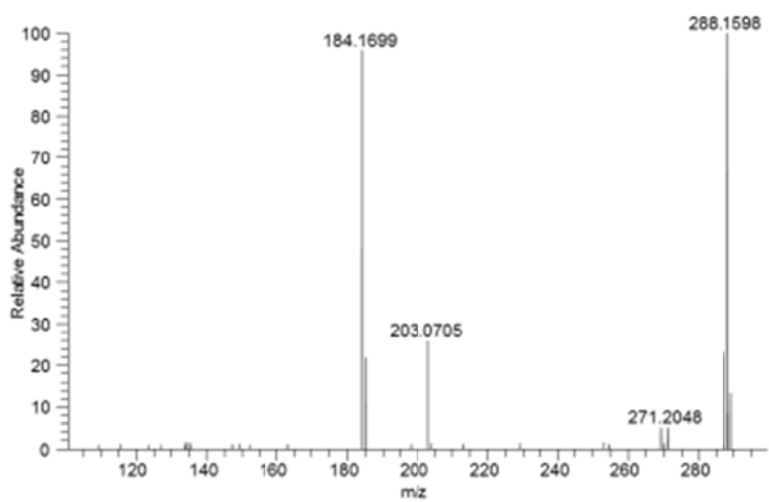
CE-1, BE
 $C_{16}H_{20}NO_4$
 m/z : 290.1387

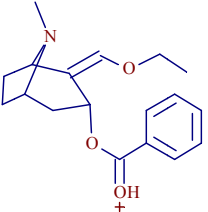
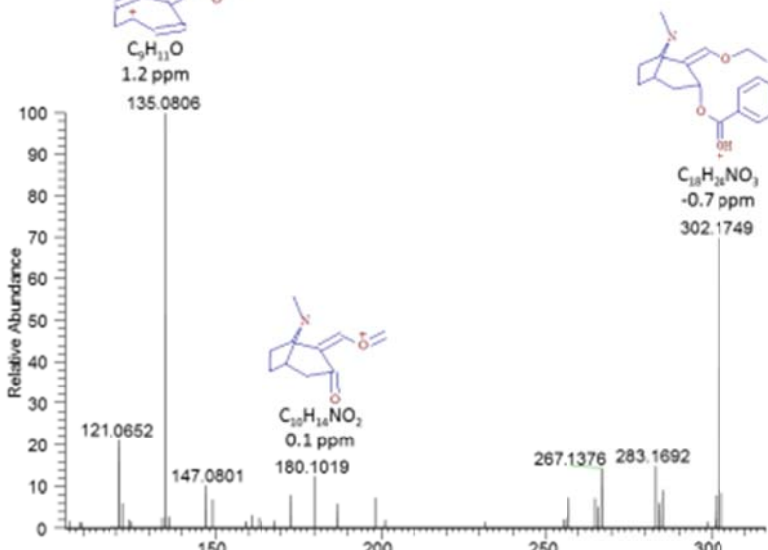
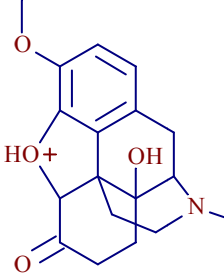
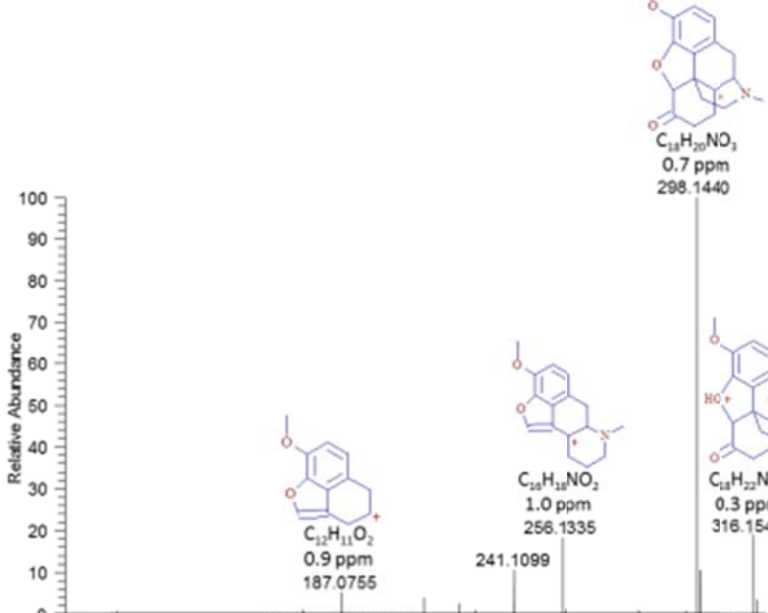


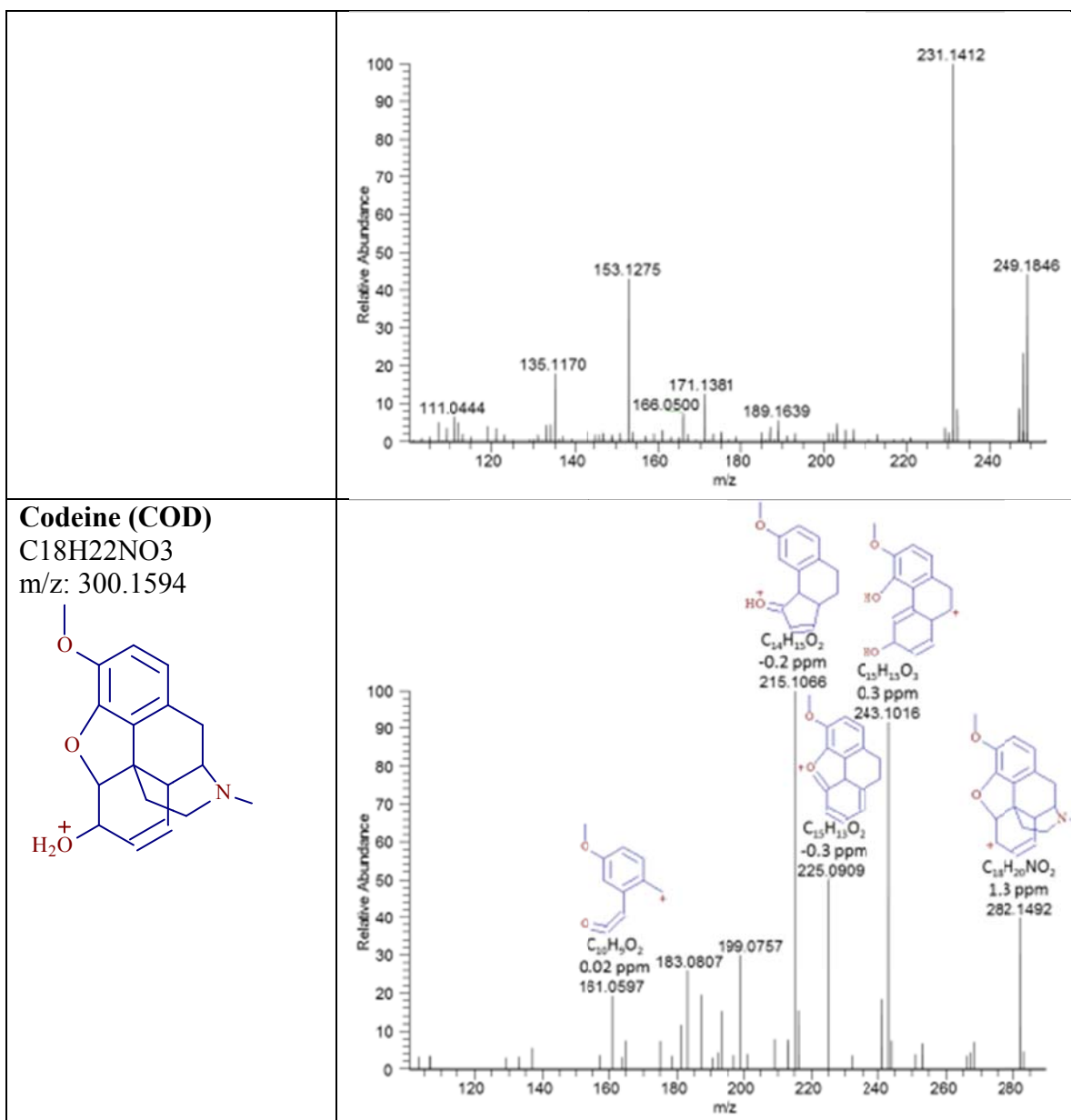
CE-2*
C18H26NO6
m/z: 352.1755

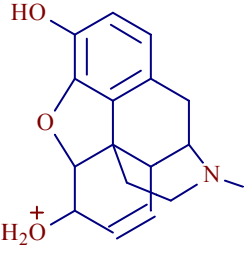
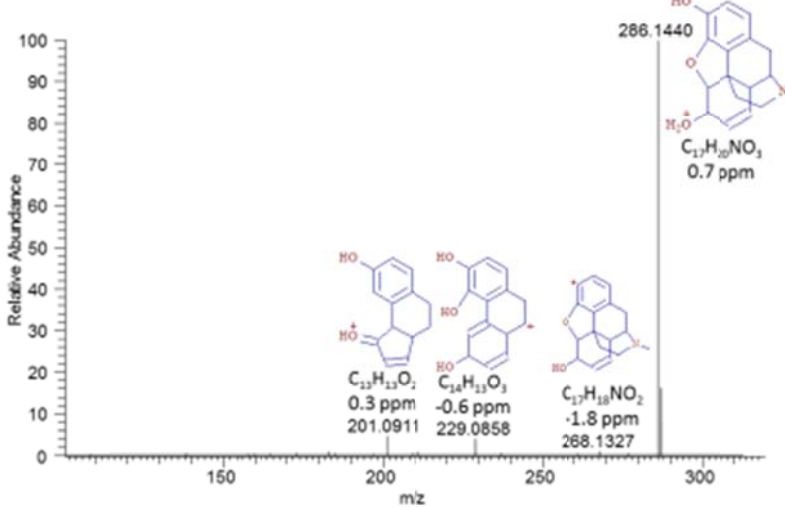
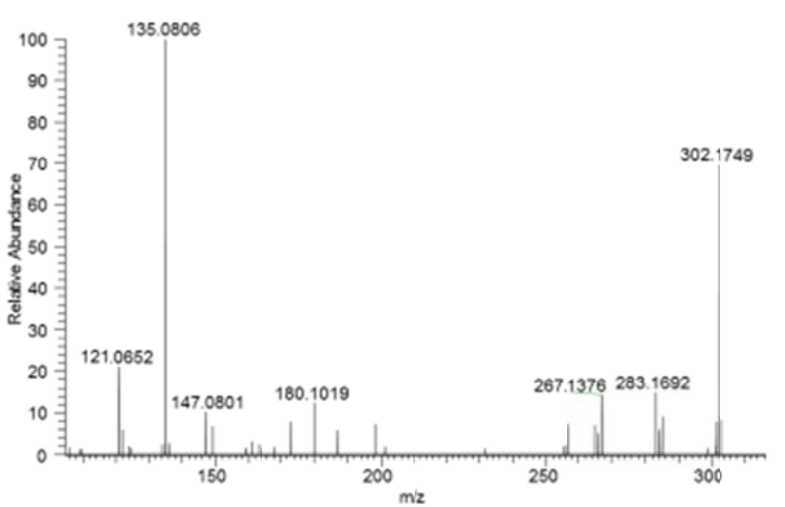
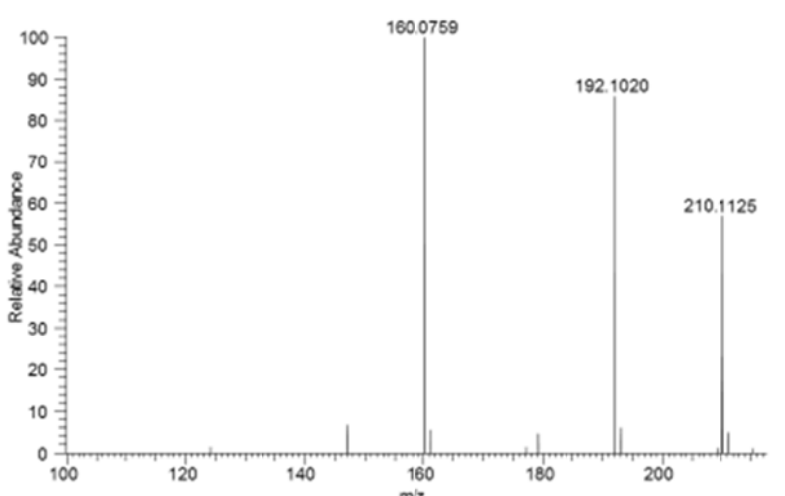


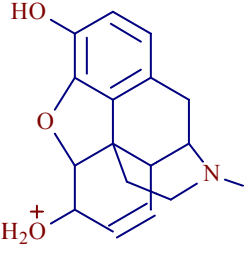
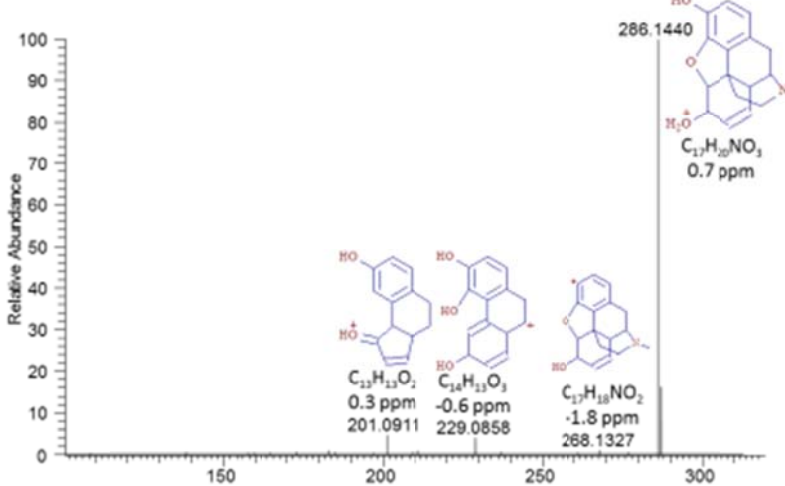
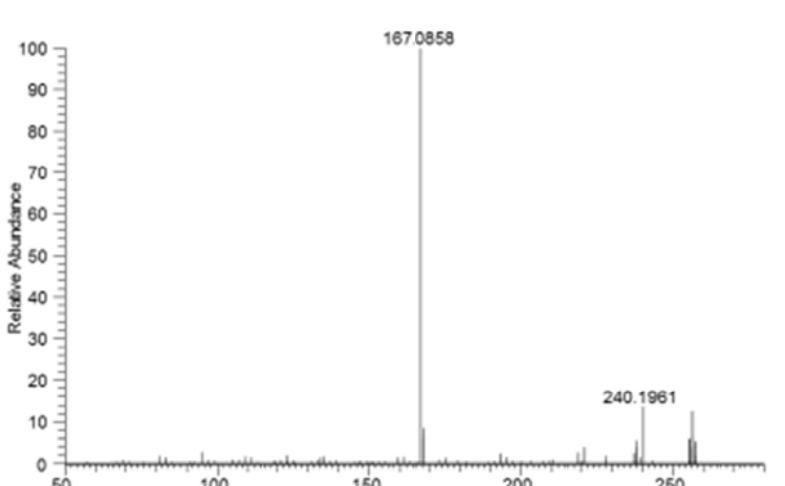
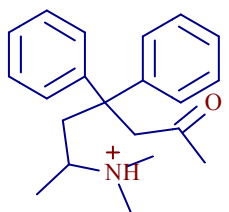
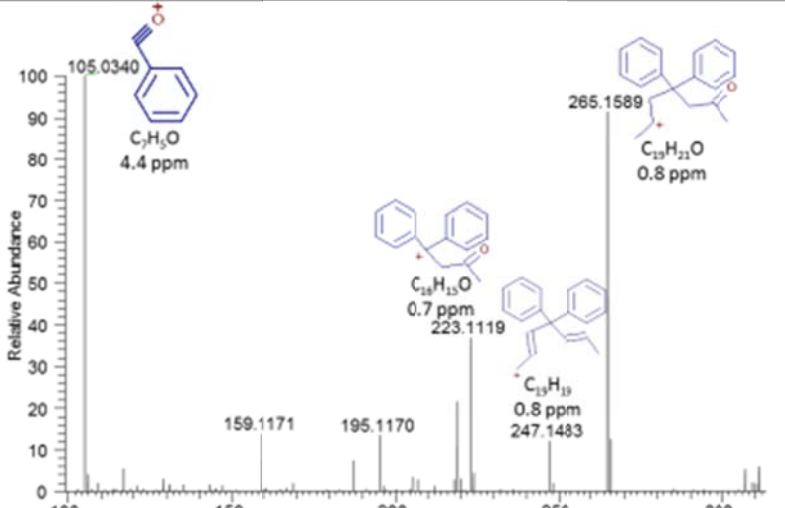
CE-3
C17H22NO3
m/z: 288.1594



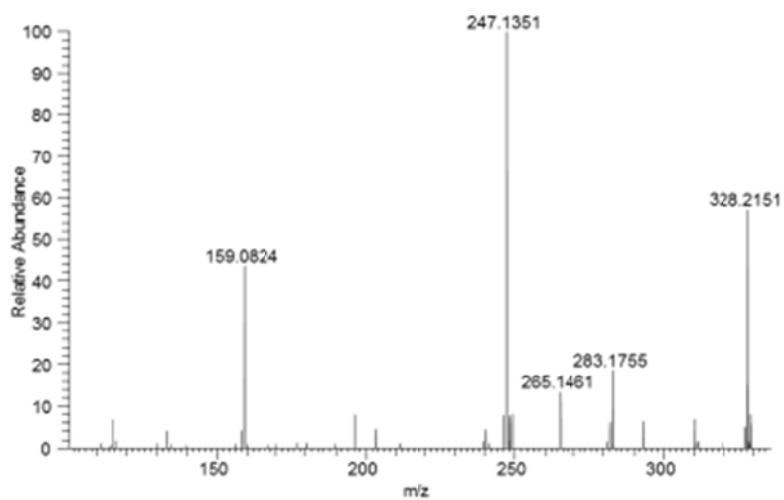
<p>CE-4*, COD-2 $C_{18}H_{24}NO_3$ $m/z: 302.1751$</p> 	 <p>Chemical structure of CE-4*, COD-2: $C_9H_{13}O$, 1.2 ppm, 135.0806</p> <p>Chemical structure of CE-4*, COD-2: $C_{18}H_{24}NO_3$, -0.7 ppm, 302.1749</p> <p>Chemical structure of CE-4*, COD-2: $C_{10}H_{14}NO_2$, 0.1 ppm, 180.1019</p> <p>Other peaks: 121.0652, 147.0801, 267.1376, 283.1692</p>
<p>Oxycodone (OXY) $C_{18}H_{22}NO_4$ $m/z: 316.1543$</p> 	 <p>Chemical structure of Oxycodone (OXY): $C_{18}H_{20}NO_3$, 0.7 ppm, 298.1440</p> <p>Chemical structure of Oxycodone (OXY): $C_{12}H_{11}O_2$, 0.9 ppm, 187.0755</p> <p>Chemical structure of Oxycodone (OXY): $C_{18}H_{22}NO_4$, 0.3 ppm, 316.1544</p> <p>Chemical structure of Oxycodone (OXY): $C_{18}H_{22}NO_4$, 1.0 ppm, 256.1335</p> <p>Other peaks: 241.1099</p>
<p>OXY-1* $C_{15}H_{22}NO_2$ $m/z: 248.1670$</p>	



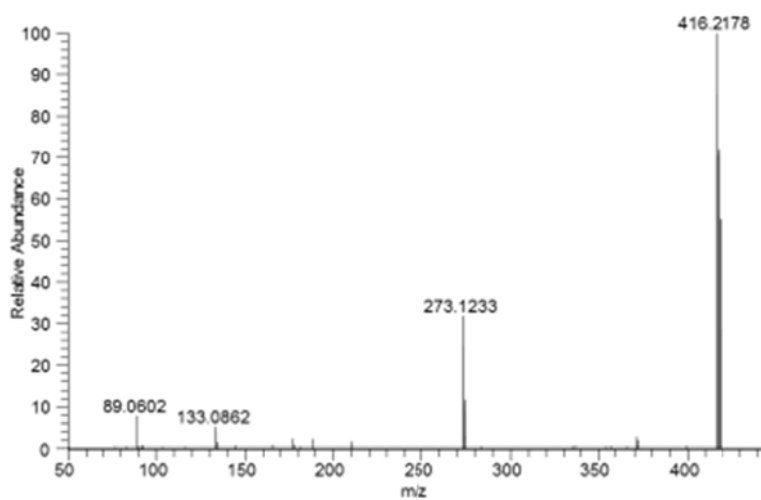
<p>COD-1, MO $C_{17}H_{20}NO_3$ m/z: 286.1438</p> 	
<p>COD-2*, CE-4 $C_{18}H_{24}NO_3$ m/z: 302.1751</p>	
<p>COD-3*, MDMA-1, MDA-6 $C_{11}H_{16}NO_3$ m/z: 210.1125</p>	

<p>Morphine (MO) $C_{17}H_{20}NO_3$ m/z: 286.1438</p> 	
<p>MO-1* $C_{17}H_{22}NO$ m/z: 256.1696</p>	
<p>Methadone (ME) $C_{21}H_{28}NO$ m/z: 310.2165</p> 	

ME-1*
C21H30NO2
m/z: 328.2271

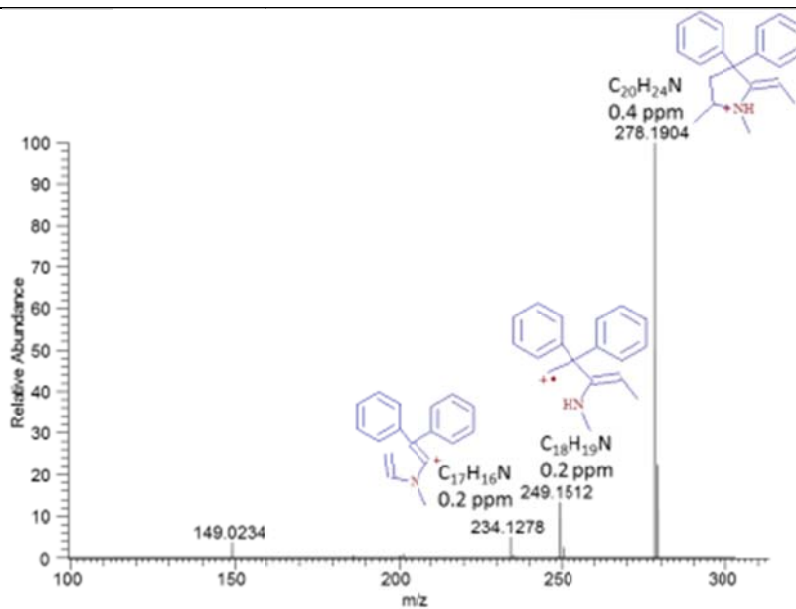
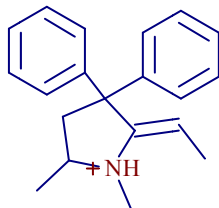


ME-2*
C23H33N2O3S
m/z: 417.2206

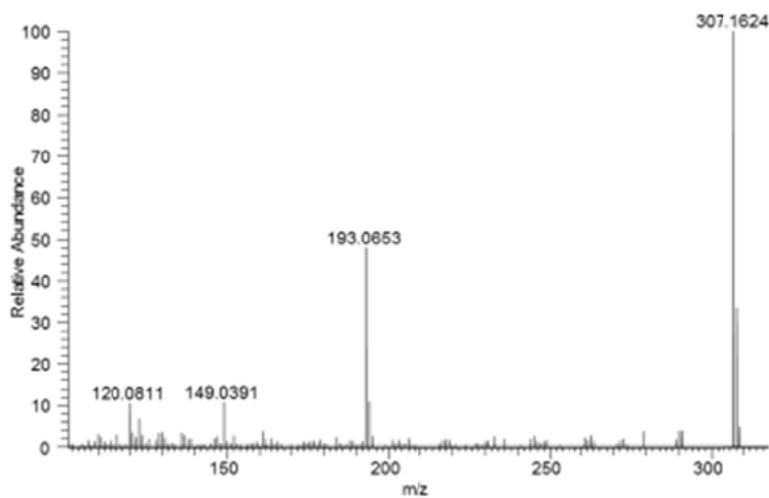


EDDPC₂₀H₂₄N

m/z: 278.1903

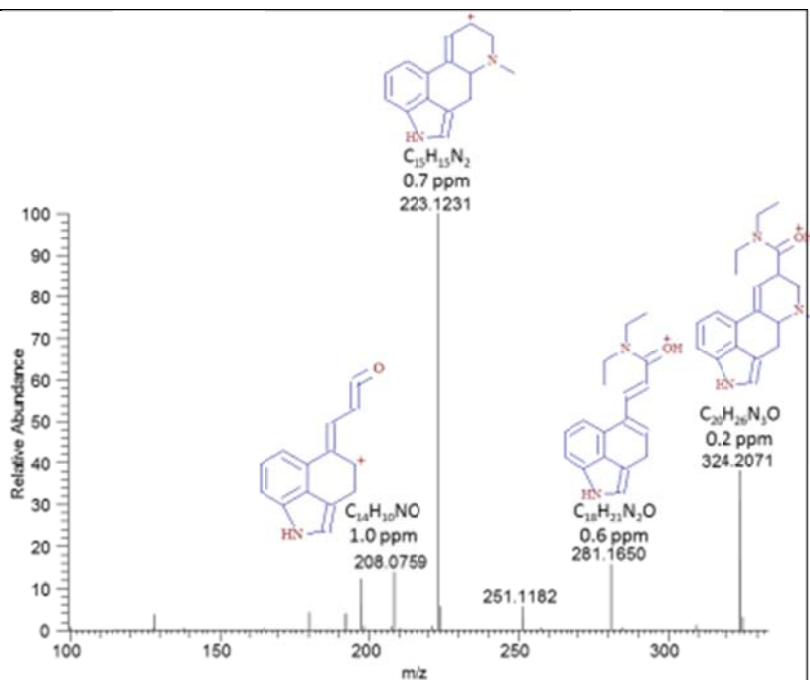
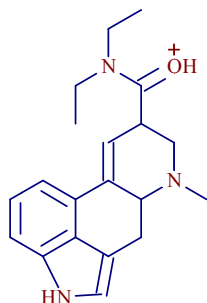
**EDDP-1***C₂₀H₂₂NO₂

m/z: 308.1645

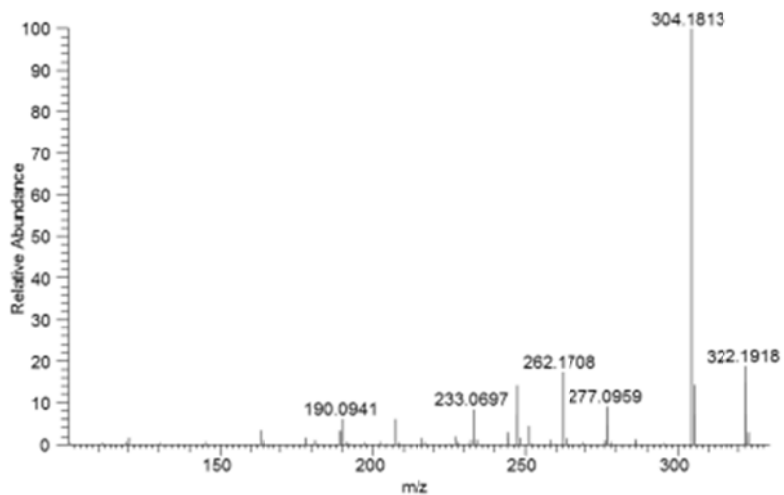


LSDC₂₀H₂₆N₃O

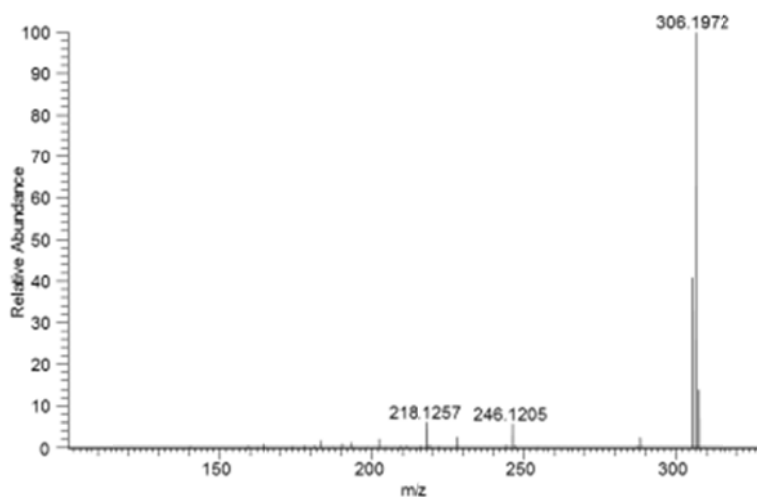
m/z: 324.2070

**LSD-1***C₂₀H₂₄N₃O

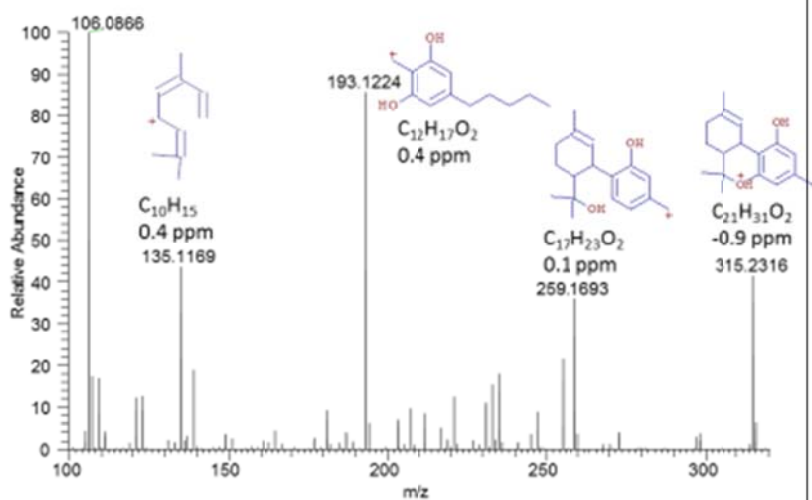
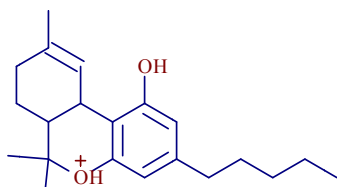
m/z: 322.1914



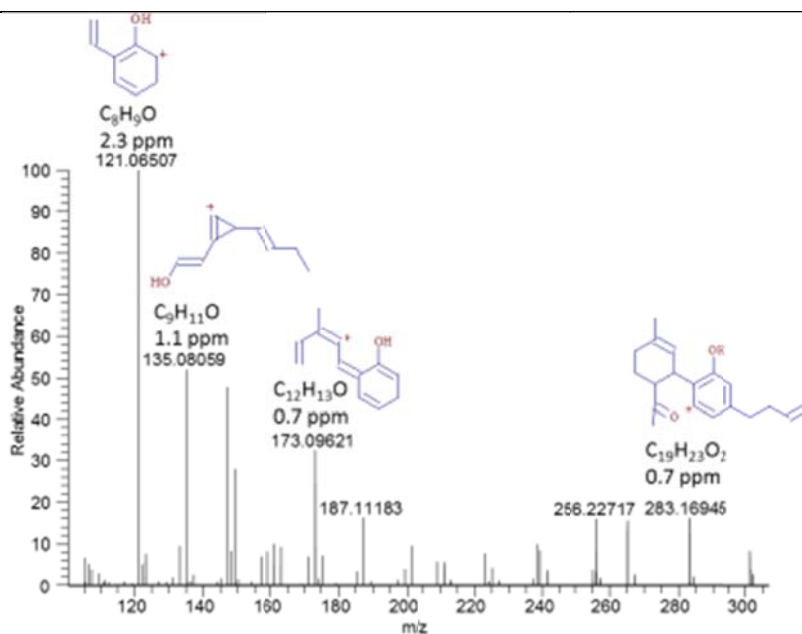
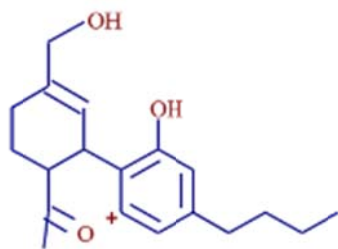
LSD-2*
 $C_{20}H_{24}N_3$
 m/z : 306.1965



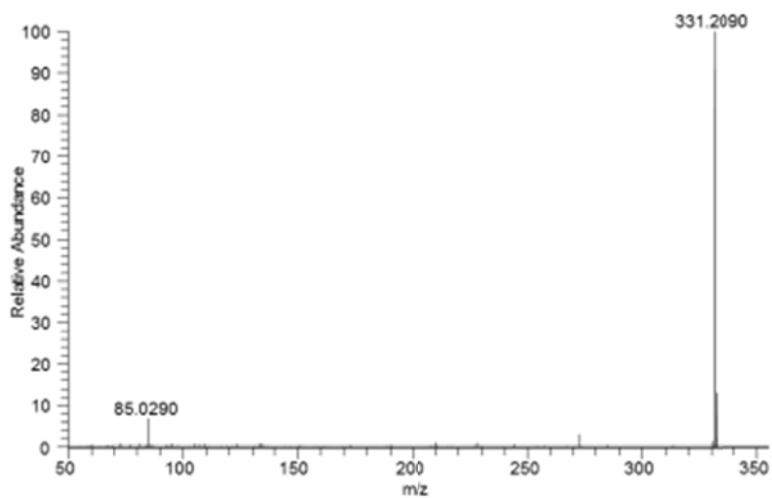
THC
 $C_{21}H_{31}O_2$
 m/z : 315.2319

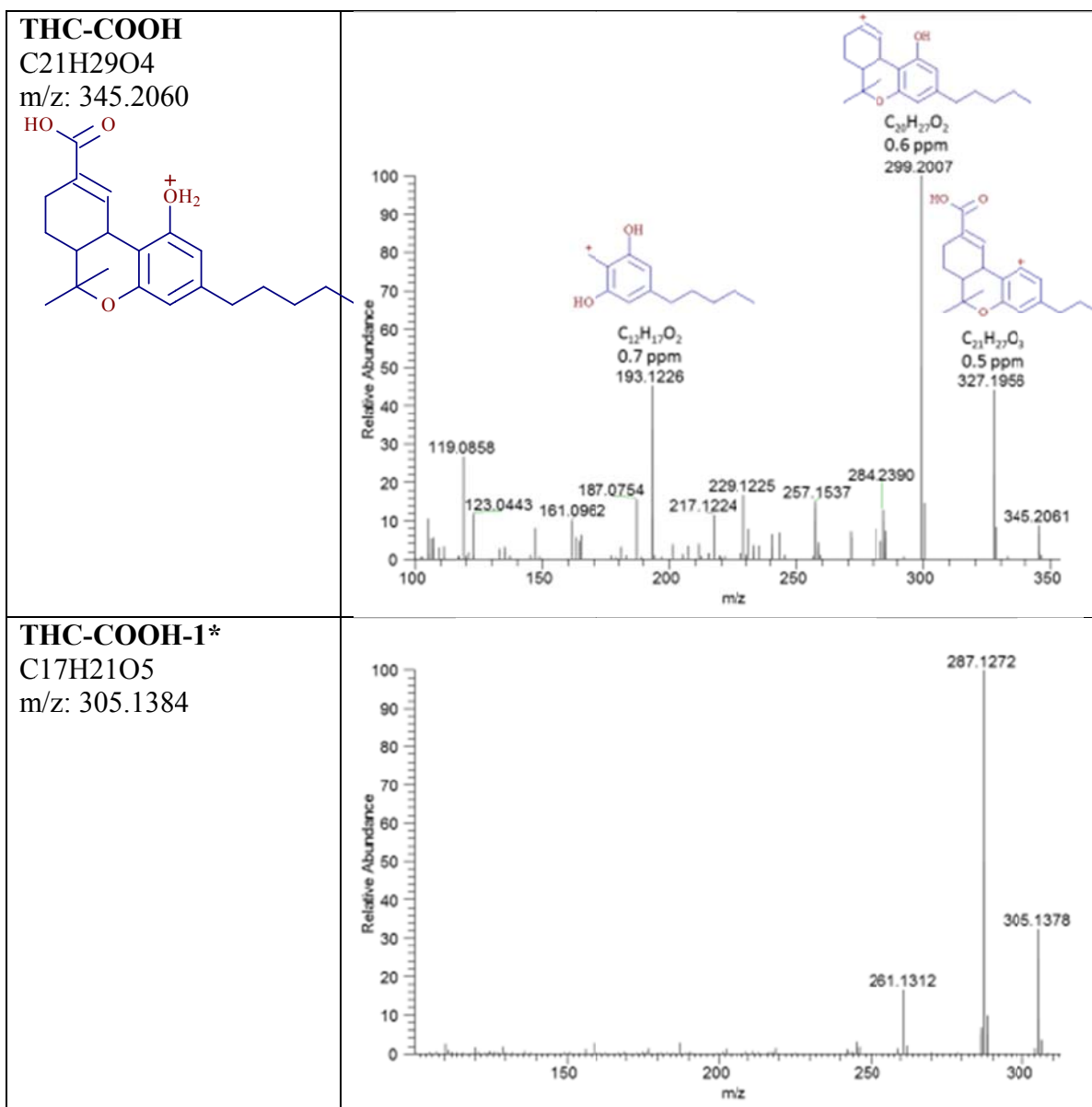


THC-1*
 $C_{19}H_{25}O_3$
 m/z : 301.1798



THC-2*
 $C_{21}H_{31}O_3$
 m/z : 331.2268





* New tentatively identified TPs in raw influent sewage water.

VITA

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PUBLICATIONS AND PRESENTATIONS

Nubia V. Heuett, Cesar E. Ramirez, Adolfo Fernandez, and Piero R. Gardinali. (2015). Analysis of Drugs of Abuse by Online SPE-LC High Resolution Mass Spectrometry: Communal Assessment of Consumption. *Science of The Total Environment* 511: 319–330.

Nubia V. Heuett, Sudha Rani Batchu, and Piero R. Gardinali. (2015). Understanding the Magnitude of Emergent Contaminant Releases through Target Screening and Metabolite Identification Using High Resolution Mass Spectrometry: Illicit Drugs in Raw Sewage Influent. *Journal of Hazardous Materials* 282: 41–50.

Jaume Aceña, Sandra Pérez, Piero Gardinali, José Luis Abad, Peter Eichhorn, Nubia Heuett, and Damià Barceló. (2014). Structure Elucidation of Phototransformation Products of Unapproved Analogs of the Erectile Dysfunction Drug Sildenafil in Artificial Freshwater with UPLC-Q Exactive-MS. *Journal of Mass Spectrometry* 49: 1279–1289.

Nubia V. Heuett, and Piero R. Gardinali. Characterization of influent, surface, and drinking waters by high-resolution mass spectrometry: Understanding the composition of recalcitrant chemicals. Poster Presentation. SETAC Europe 35th Annual Meeting. Vancouver, Canada. May 3-7, 2015.

Nubia V. Heuett, Cesar E. Ramirez, Sudha Rani Batchu and Piero R. Gardinali. Target and non-target analysis of drugs of abuse by high resolution mass spectrometry. Platform Presentation. SETAC North America 35th Annual Meeting. Vancouver, Canada. November 9-13, 2014.

Nubia V. Heuett, Cesar E. Ramirez, Sudha Rani Batchu and Piero R. Gardinali. Communal assessment of drugs of abuse and identification of their transformation products by online SPE-LC-HRMS. Platform Presentation. 248th ACS National Meeting & Exposition. San Francisco, CA. August 10-14, 2014. (Award for best student platform presentation).

Nubia V. Heuett, Cesar E. Ramirez, Sudha Rani Batchu and Piero R. Gardinali. Communal assessment of drugs of abuse and identification of their transformation products by high-resolution mass spectrometry. Platform Presentation. Third Annual Forensic Science Symposium. Miami, FL. May 1st, 2014.

Nubia V. Heuett, Cesar E. Ramirez, and Piero R. Gardinali. Analysis of drugs of abuse and their metabolites via online-SPE-HPLC-MS/MS for communal assessment of consumption. SETAC North America 33rd Annual Meeting. Long Beach, CA. November 15, 2012.