AN ABSTRACT OF THE THESIS OF

Aurea C. Chiaia Hernandez for the degree of Master in Science in Chemistry presented on June 17, 2008.

Title: Large Volume (1,800 μL) Injection HPLC/MS/MS for the Quantitative Determination of Illicit Drugs and Human Urinary Biomarkers in Municipal Wastewater

Abstract approved:

_____

Jennifer A. Field

A sensitive, selective, rapid analytical method based on large-volume injection (LVI) liquid chromatography/ tandem mass spectrometry was developed using commercially-available hardware that eliminates the need for either off-line or on-line solid phase extraction. Centrifugation followed by the direct injection of 1,800 μL was used for the quantification of illicit drugs, metabolites, and human urinary biomarkers in municipal wastewaters. The accuracy of the method as indicated by standard addition was calculated for analytes with concentrations ranging from 4 to 3,500,000 ng/L. The average precision of the method, as indicated by relative standard deviation is 7%. Detection limits range from 2.5 ng/L to 250 ng/L. As a

demonstration of the method, the temporal trend in illicit drugs, selected metabolites and human urinary biomarkers was determined for 24-hr flow-normalized composite samples of raw influent collected from a single municipal wastewater treatment plant over the course of three weeks.

Large Volume (1,800 μL) Injection HPLC/MS/MS for the Quantitative Determination of Illicit Drugs and Human Urinary Biomarkers in Municipal Wastewater

by

Aurea C. Chiaia Hernandez

A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 17, 20008
Commencement June 2009

Master of Science thesis of Aurea C. Chiaia Hernandez presented on June 17, 2008.

APPROVED:

Major Professor, representing Chemistry

Chair of the Department of Chemistry

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Aurea C. Chiaia Hernandez, Author

ACKNOWLEDGEMENTS

I would like to thank many people who help me through my thesis. First I would like to give special thanks to my advisor Dr. Jennifer Field for her guidance, support, enthusiasm and friendship. I would like to thank my family, specially my mother who always emphasized the importance of education, and my sisters Itzel and Rocio for encouraging me to keep studying and helping me through tough times. I would also like to thank my partner Andreas for his endless help, motivation and patience all these years. I like to thank Carl, Carin, Rob, Ralph and Amanda for their help in the laboratory and for sharing their knowledge. Many thanks to my academic committee: Dr. Vince Remcho, Dr. Dan Sudakin and Dr. Brady Gibbons, and finally I would like to thank my friends for their support and good times.

TABLE OF CONTENTS

Page

1. Introduction....1

2. Experimental Section....5

2.1. Standards and Reagents....5
2.2. Sample Collection and Preservation....7
2.3. Sample Preparation and Large-Volume Direct Injection-Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)....8

3. Method Validation....16

3.1. Standard Addition....16
3.2. Recovery....16

4. Results and Discussion....18

4.1. Method Optimization for LVI....18
4.2. Accuracy and Precision....25
4.3. Temporal Trend in Psychoactive compounds, Opiates and Human Urinary Biomarkers in Wastewater....30

5. Conclusions....45

6. Acknowledgments....46

7. References....47

Appendices....52

Appendix A....53

Appendix B....66

LIST OF FIGURES

Figure Page

1. Modified injection of an Agilent 1100 HPLC with the locations of the1400 μL stainless steel seat extension loop and seat capillary indicated. 10

2. Separation of stimulants (ephedrine, amphetamine, MDMA and methamphetamine) in raw influent wastewater acquired by LVI (1,800 μL)-LC/MS/MS conditions. 19

3. Chromatograms for .prescription opiates (oxycodone, hydrocodone and methadone) and cocaine and its metabolite benzoylecgonine in raw influent wastewater acquired by LVI (1,800 μL)-LC/MS/MS conditions. 20

4. Chromatograms for human urinary biomarkers (cotinine, caffeine and creatinine) in raw influent wastewater acquired by LVI (1,800 μL)-LC/MS/MS conditions. .. 21

5. LVI injection valve in the 'by-pass' position at the beginning of the injection sequence when the mobile phase by-passes the injector when sample is being withdrawn and placed in the 900 μL sample loop extension and 1,400 μL seat capillary..... 23

6. LVI injection valve in the 'main-pass' position when mobile phase is pumped through the 900μL sample loop extension and 1,400 μL seat capillary tubing in order to transfer the sample to the analytical column. 24

7. Methamphetamine loads ± standard deviation (mg/day/person). 32

8. Amphetamine loads ± standard deviation (mg/day/person). 33

9. Methamphetamine, amphetamine and ephedrine concentrations ± standard deviation for the three week sampling period. 33

10. Ephedrine loads ± standard deviation (mg/day/person)..... 34

11. Loads of MDMA ± standard deviation (mg//day/person) for a three week period. 35

12. Ketamine loads ± standard deviation (mg/day/person) over a three week sampling period. 36

LIST OF FIGURES (Continued)

Figure Page

13. Hydrocodone loads ± standard deviation (mg/day/person) for the three week sampling period. 37

14. Oxycodone loads ± standard deviation (mg/day/person) 37

15. Flow ± standard deviation of wastewater over the sampling period 38

16. Methadone loads ± standard deviation (mg/day/person). 38

17. Cocaine loads ± standard deviation (mg/day/person). 39

18. Benzoylecgonine loads ± standard deviation (mg/day/person). 40

19. Ratios of benzoylecgonine to cocaine ± standard deviation for the three week sampling period. Line at ratio 7.0 represents the average of ratio for the three week period 41

20. Mass of Cotinine (g) ± standard deviation 42

21. Mass of Caffeine (kg) ± standard deviation 43

22. Mass of creatinine (kg) ± standard deviation 44

23. Creatinine (mg) vs Flow (L) ± standard deviation 44

24. Ratios of benzoylecgonine to creatinine ± standard deviation for the three week sampling period 45

LIST OF TABLES

Table Page

1. Legal and illegal drugs, metabolites, and human urinary biomarkers analyzed and the optimized mass spectrometer acquisition parameters including precursor and product ions, cone voltage, collision energy, and internal standards used for quantification. 13

2. Internal standards and their precursor and product ions, cone voltage, and collision energies used for their acquisition..... 14

3. Accuracy determined by standard addition indicated concentrations determined by standard addition were statistically equivalent to concentrations determined from solvent-based calibration curves at the 95%. 26

4. The slopes of individual regression for the % recovery vs. mass for each analyte in standard addition experiments. Values of $t_{calculated}$ indicated by Equation 2 indicate the slope is not statistically different from zero at the 95% CI. The averaged recoveries from the standard addition data are reported ± standard deviation..... 28

5. Instrumental detection (IDL) and limits of quantification (LOQ) and whole method precision within a day (intra-day) and between days (day-to-day..... 30

6. List of illicit and legal drugs, metabolites, and human urinary biomarker names and classification..... 31

7. Dose excreted in percentage of BE or cocaine when injected, smoked or snortes. 41

1. Introduction

In recent years, researchers have showed that illicit drugs occur at quantifiable concentrations in the raw influent and effluent of wastewater treatment plants (WWTP), rivers *(1-6)*, surface water *(4, 7)*, and in lakes *(8)*. Interpretation of analytical data is then used to estimate levels of community drug use and consumption in locations including Ireland, Italy, Switzerland and England *(9, 10)*. The measurement of these illicit drugs and their metabolites in WWTPs allows for the analysis of trends in drug use over time and between locations since WWTPs have known catchment areas with specific geographic boundaries and population estimates *(5, 9)*. In addition, influent and effluent data are used to quantify the removal of illicit drugs and their metabolites during wastewater *(3)*.

Wastewater components such as cotinine, the metabolite of nicotine, and caffeine are recognized as molecular markers of wastewater *(11-15)*; however, reports to date focus only on the frequency of detection and corresponding concentrations. Alternatively, we hypothesize that creatinine, which is the end product of phosphocreatinine degradation *(16)*, can be quantified in wastewater due to its universal excretion by humans and, thus, potentially serve as an additional, perhaps more universal, human urinary biomarker than either cotinine or caffeine. Creatinine concentrations excreted by humans range from 0.5 to 3 g/L *(17)* and data from a recent National Health and Nutrition Examination Survey (NHANES) indicated an average creatinine excretion of 1.3 g/L ± 0.8 for a 7,500 participant study (http://www.cdc.gov/nchs/about/major/nhanes/nhanes2007-2008/nhanes07_08.htm). Creatinine is used during work-place drug testing to determine that a urine sample is

authentic and to correct/normalize for dilution and variability in excretion rates when examining measured drug concentrations in a urine sample *(17-19)*. Although creatinine normalization for drug testing has some disadvantages such as the affect in concentrations according to age, gender, and muscle mass *(18, 19)* and stability, hypothesizing that creatinine can be used as a human urinary biomarker in raw municipal wastewater. The interest in human urinary biomarkers stems from the hypothesis that they can potentially be used as a more dynamic indicator of population over a 24 hr period for a given WWTP than the population obtained from census data. To our knowledge, the occurrence of creatinine in wastewater has not yet been studied.

Due to the low concentration of analytes in wastewater, such as pharmaceuticals and illicit drugs, current analytical methods include sample concentration steps such as solid phase extraction (SPE). Off-line SPE concentrate analytes from sample volumes ranging from 50 mL *(1)* to 1000 mL *(2, 3)* onto a range of reverse-phase sorbents. However, after eluting the sorbent cartridges, only a small fraction of the final SPE extract volume is actually injected for analysis with typical injection volumes of 10-20 μL. Fully automated on-line SPE was recently introduced and requires a sample processor Prostek-2 configured for high sample volumes (5 mL) *(5)*. Despite the perceived advantages of SPE including sample concentration and clean up, there are several reports of low and variable recovery of analytes, loss of enrichment factor resulting from the injection of an aliquot *(20)*, contamination of SPE materials in the case of fluorochemicals *(21)*, the time

consuming nature of SPE, and the cost associated with the manufactured SPE materials.

Large volume injection (LVI) is a technique that dates back to the early 1980s *(22, 23)* and involves the direct injection of samples (30 – 2000 μL) larger than the conventionally-injected volumes of 10 - 20 μL. During the injection of a large volume of solvent or low elutropic strength, which is effectively the initial mobile phase, leads to the concentration of the injected analytes onto the head of the analytical column while the solvent (e.g., water or non-aqueous solvents such as soil and vegetable extracts), salts, and other matrix components that do not partition into stationary phase flow un-retained through the column and are run to waste rather than to the detector *(24)*. After the injection phase, which is analogous to the sample concentration phase of SPE, the elutropic strength of the mobile phase is increased to promote elution and separation of the concentrated analytes.

LVI offer several advantages such as an increase in sensitivity and accuracy, since there is minimal sample handling as the pre-concentration and analytical separation steps are linked and operated automatically by the instrument autosampler. In addition, the total sample volume required is smaller than for off-line SPE since the entire volume can be injected and analyzed *(20)*. .The use of LVI results in high rates of sample throughput since the analyses are performed with the minimal sample preparation.

The benefits of LVI are recognized by a variety of applications primarily in the agricultural section such as the determination of phenylthiohydantoin-derivatized amino acids *(25)*, biogenic amines *(26)*, trace analysis of pesticides, herbicides and

fungicides in vegetables and soils *(27-31)*. Additionally, LVI has been used in the determination of micropollutants in surface waters such as pesticides *(24, 32-34)*, herbicides *(35-37)*, fungicides *(34)*, and fluorinated alkyl substances *(21, 38)*. Previous work in our laboratory has demonstrated the use of large volume injection (e.g., 900 μL) for the analysis of polar organic contaminants such as fluorochemicals *(21, 38)* and for the quantification of fullerenes using normal-phase LC *(39)*.

Despite the potential benefits and early applications, LVI has received relatively little attention. This apparent lack of interest in the peer-reviewed literature may stem from the concern over matrix effects or the potential for carryover, and because LVI conditions deviate significantly from conventional chromatographic practice. Historically, reports that describe LVI methodology utilize only external calibration that do not explicitly address matrix effects, and do not provide enough detail on the necessary hardware and software modifications and their operation.

The objective of this research was to develop and rigorously validate a large-volume (up to 1,800 μL) injection LC-MS/MS method as an expeditious approach for the measurement of illicit drugs and their metabolites as well as human urinary biomarkers in raw municipal wastewater. LVI were optimized and evaluated by examining matrix effects, accuracy, and precision through standard addition experiments. The detection and quantification limits of the instrument and method detection limits were then determined using the optimized conditions. In addition, the stability of samples under storage conditions was evaluated. Finally, the analytical method was applied to 24hr, flow –normalized composite samples of raw influent collected from a single WWTP in order to determine the temporal trends in the loads

(mg/person/day) of illicit drug, metabolite, and human urinary biomarker for the community for a period of three consecutive weeks.

2. Experimental Section

2.1. Standards and Reagents

The standards and reagents (analytical grade >99%) of interest that were purchased from Cerilliant Corporation (Round Rock, TX) at concentrations of 1 mg/ml in methanol or acetonitrile included the following: (±)-3,4-methylenedioxethylamphetamine (MDEA), (±)-N-methyl-1,3-benzodioxole-5-butanamine (MBDB), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-3,4-methylenedioxyamphetamine (MDA), (±)-amphetamine, (±)-methamphetamine, (1S, 2R)(+)-ephedrine hydrochloride, cocaine, benzoylecgonine, LSD, 2-oxo-3-hydroxy-LSD, (-)-cotinine, (-) nicotine, oxycodone, hydrocodone, (±)-methadone, caffeine, ketamine hydrochloride, norketamine hydrochloride, phencyclidine (PCP) and flunitrazepam. Creatinine was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Deuterated standards (analytical grade >99%) of (±)3,4-methylenedioxymethamphetamine-D_5 (±)MDMA-D_5), (±)3,4-methylenedioxyamphetamine-D_5 (±)MDA-D5), (±)amphetamine-D_6, (±)-methamphetamine-D_5, cocaine-D_3, benzoylecgonine-D_3, -(±)cotinine-D_3, oxycodone-D_3, hydrocodone-D_6, -(±)methadone-D_9, PCP-D_5, (1S, 2R)(+)ephedrine-D_3

hydrochloride, LSD-D_3, flunitrazepam-D_7 were purchased from Cerilliant Corp.(Round Rock, TX) at concentrations of 100 μg/ml in methanol or acetonitrile. Caffeine-$^{13}C_3$ (trimethyl-$^{13}C_3$) was purchased by Sigma-Aldrich Corp. (St. Louis, MO) and creatinine-D_3 was purchased from US Biological (Swampscott, MA).

Individual stock solutions of analytes and internal standards were prepared in methanol or acetonitrile to match the solvent in which the standard was shipped at concentrations of 39.6 and 3.6 μg/mL, respectively, and all were stored in the dark at -80°C. Creatinine and internal standard stock solutions were prepare by appropriate dilution at concentration of 250 mg/L and 25 mg/L respectively in 35% methanol and kept at -80°C. Working stock solutions containing mixtures of standards were further prepared in methanol or acetonitrile and stored in the dark at -20 °C.

Working solutions of internal standards, except for creatinine-D_3, caffeine-^{13}, (1S, 2R)(+)ephedrine-D_3, and (±)cotinine-D_3, were prepared by appropriate dilution in methanol or acetonitrile at concentrations of 63.36 μg/L. An additional internal standard mixture included caffeine-$^{13}C_3$, (1S, 2R)(+)ephedrine-D_3 and (±)cotinine-D_3 was prepared in methanol at concentration of 158 μg/L. Small quantities of the standard and internal standard solutions were kept at 4 °C for daily analysis and were replaced every two weeks.

HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid was purchased from EMD Chemicals (Gibbstown, NJ). Mobile phase (0.5% acetic acid) was prepared daily and filtered through hydrophilic polypropylene membrane filters 0.45 μm purchased from Pall Corp (Ann Arbor, MI).

2.2. Sample Collection and Preservation

Twenty 24 hr flow-normalized composites of raw wastewater influent were collected for a period of three weeks between March and April 2008. The WWTP sampled is located in the Pacific Northwest and serves a population of 55,000 and treats around 90% domestic and 10% industrial waste. The composite samples were acquired from an automated flow sampler set to collect a fixed volume of sample proportional to the flow every hr for 24 hrs in a single container that was housed in a 4 °C compartment during collection. The 24 hr flow-normalized composites were transferred to 150 mL high density polyethylene (HDPE) bottles (VWR International, West Chester, PA) and frozen until shipment. The frozen samples were shipped on ice and stored at -20 °C until analysis upon receipt at Oregon State University. The samples were analyzed within two weeks of collection.

Due to high microbial activity in raw municipal wastewater, other have observed cocaine degradation under what conditions 4 ^{o}C *(1)* . Due to the potential instability of the unpreserved raw influent samples, a storage stability analysis was conducted prior to sample collection to determine the hold times stored at -20 ^{o}C. A single large volume (20 L) of raw influent wastewater was collected and aliquot into 140 bottles of 150 mL HDPE. Seventy of the bottles were spiked with 600 µL of 6 M hydrochloric acid (HCl) to decrease the pH of the samples to 2 while another 70 bottles were left un-acidified. All the aliquots were spiked to give a concentration of at least 200 ng/L to ensure that, at the onset of storage, all analytes were present. The un-acidified samples were separated into two different groups of n=35. One group was immediately placed in a -20 °C freezer as a control, while the other group of n=35

was kept at 4 °C for 24 hrs to simulate the time during collection of the 24 hr flow-normalized composite. After 24 hrs at 4 °C, the 35 bottles were placed in a -20 °C freezer. Sets of n=4 samples were analyzed on the first four days after preparation and at the end of the first, second, and third week of storage.

The acidified samples were separated into two groups of n=35 and kept at 4°C for 24 hrs to simulate the time during collection of the 24 hr flow-normalized composites. After 24 hrs at 4 °C, one group of n=35 was left at room temperature for 24 hrs to simulate storage during transit in the mail and then placed into storage at -20 °C. The other group of n=35 was left at room temperature for an additional 48 hrs to simulate second-day mail delivery and then placed into storage at -20 °C. Groups of samples (n=4) were then analyzed on the first four days and at the end of the first, second, and third week of storage. All the acidified and no-acidified samples were immediately refrozen until creatinine storage stability was performed. For future studies, creatinine storage was also studied by collecting a single large sample (4L) of raw influent and distributing it into 140 ,50 mL polypropylene centrifuge tubes. Seventy of the bottles were spiked with 6 M HCl to reduce the pH to 2 and the other 70 bottles were left un-acidified. The design and the analysis were performed in the same manner as described above.

2.3. Sample Preparation and Large-Volume Direct Injection-Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

For the analysis of all analytes except creatinine, frozen samples were brought to room temperature and a 7 mL of aliquot volume was centrifuged in a IEC clinical centrifuge (Thermo IEC, Nutley, NJ) for 30 min at a maximum speed of 7100 rpm

(5125 g). The samples were re-frozen immediately for the analysis of creatinine. After centrifugation, supernatant was transferred into a 6 mL autosampler glass vial and spiked with stable-isotope labeled internal standards including 380 pg (±)MDMA-D_5, (±)MDA-D_5, (±)amphetamine-D_6, (±)-methamphetamine-D_5, cocaine-D_3, benzoylecgonine-D_3, (±)cotinine-D_3, oxycodone-D_3, hydrocodone-D_6, -(±)methadone-D_9, PCP-D_5, LSD-D_3, flunitrazepam-D_7, 1100 pg of caffeine-$^{13}C_3$, (±)-cotinine-D_3, and (1S, 2R)(+)ephedrine-D_3 (Table 1). All samples were analyzed within 24 hrs of preparation. Samples for the analysis of creatinine were brought to room temperature and a 2 mL of aliquot was centrifuged in an Eppendorf centrifuge 5415 C for 30 min at a maximum speed of 14,000 rpm (10,000 g). After centrifugation, 1.2 mL of supernatant was transferred to a 2 mL glass autosampler vial and spiked with 900 ng of the creatinine-D_3 internal standard. After preparation, the samples were and analyzed within 24 hrs.

Large-volume injections and separations were performed on an Agilent 1100 HPLC system (Santa Clara, CA) that was modified by adding a commercially-available 900 µL "Injection Upgrade Kit" (Agilent part no. G1363A) that consisted of a 900µL analytical head, a 900 µL stainless steel sample loop extension, and a 900 µL needle. To reach a capacity of 1,800 µL, a commercially-available 1400 µL stainless steel seat extension loop (Agilent part no. G13G13-87308) was installed between the seat capillary fitting and port 5 (injection valve) of the analytical head (Figure 1).

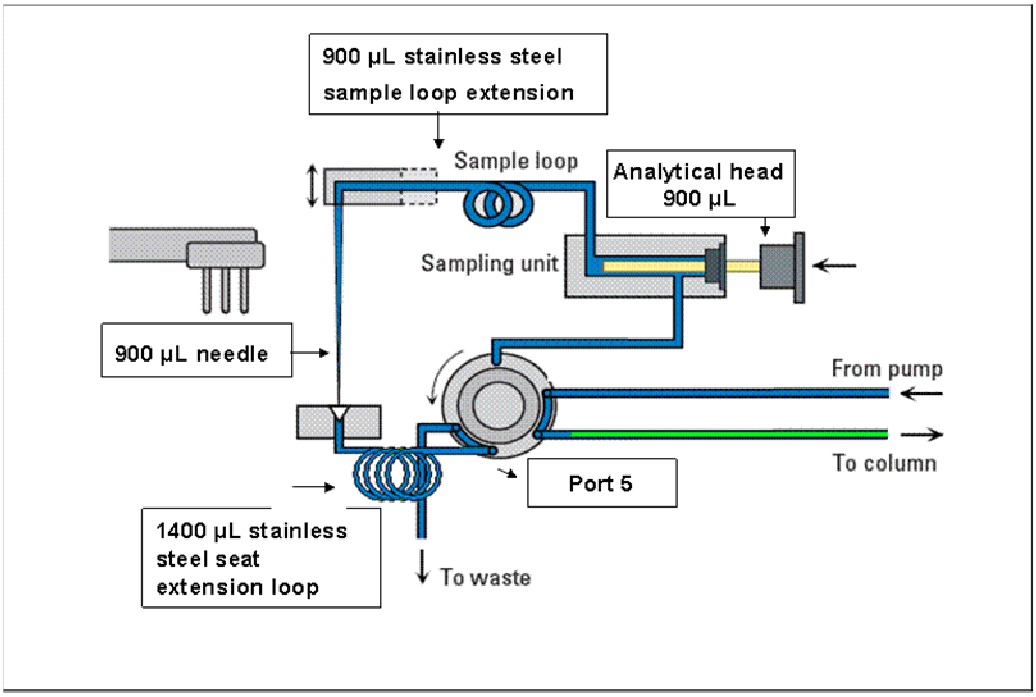


Figure 1. Modified injection of an Agilent 1100 HPLC with the locations of the1400 µL stainless steel seat extension loop and seat capillary indicated.

Given these hardware modifications, 1,800 µL samples were directly injected into a 2.0 X 4.0 mm C18 security guard cartridge (Phenomenex, Torrance, CA) that was connected to a 150 X 4.6 mm 5 µm particle size Atlantis T3 C18 column (Waters Corporation, Milford, MA). The column temperature was 35 °C and the flow rate was held at 500 µL/min. The injection program initiated with a needle wash followed by withdrawal of 900 µL of sample, which is stored in the 1,400 µL seat capillary. This step was repeated to give a total sample volume of 1,800 µL. During injection, the injection valve is kept in the 'main-pass' position for eight min after which the injection is switched to the 'by-pass' position. In addition, for the first nine min, the divert valve located after the analytical column and before the ESI interface is switched to waste to protect the detector from unwanted material.

The mobile phase consists of 0.1% acetic acid in 5% methanol (A) and acetonitrile (B). The gradient consists of holding A (90%) for 8 min, then increasing B to 25% in 6 min followed by a ramp of B to 100% in 10 min and 100% B is maintained for 2 min. The gradient is brought to initial conditions (90% A) and is held for 9 min for the recalibration of the column, giving a total run time of 35 min.

It is important to note that takes approximately 8min to load 1,800 µL of sample into the system. During this time, the gradient is running at initial conditions. Therefore, the 8 min of sample loading where added as a part of the recalibration time of the column. As a result, before an injection is made, the column is re-calibrated for at least 17 min, which corresponds to more than six column volumes of the initial mobile phase.

Creatinine analysis was also performed on the Agilent 1100 HPLC system that was modified with a 900 µL Injection Upgrade Kit. However, the 1,400 µL seat-extension loop was not necessary for the analysis of creatinine since an injection volume of 100 µL was used. The 100 µL sample volume was directly injected into a 2.0 X 4.0 mm C18 security guard cartridge that was attached to a 150 X 4.6 mm 5 µm particle size Luna C18 column (Phenomenex, Torrance, CA). Isocratic conditions with a mobile phase of 10 mM ammonium acetate in 5% methanol were used at a column temperature of 35 °C and a flow rate of 500 µL/min.

Mass spectrometry was performed on a Waters Quattro Micro tandem mass spectrometer (Milford, MA) operated in a positive mode with an electrospray ionization (ESI) interface. The source and desolvation temperature were set to 150 °C and 450 °C, respectively. A total of 43 transitions were acquired to quantify analytes

in multiple reaction monitoring (MRM) mode (Table 1 and Table 2). An interchannel and scan delay of 0.03 s between groups of transitions was used to enhance sensitivity. Peak retention times were used to determine the appropriate time windows for each group of transitions monitored in MRM mode. Analytes were monitored using a single transition with the exception of methamphetamine, amphetamine, benzoylecgonine, and norcocaine which were monitored using two transitions with the second transitions used as a visual check.

Table 1 Legal and illegal drugs, metabolites, and human urinary biomarkers analyzed and the optimized mass spectrometer acquisition parameters including precursor and product ions, cone voltage, collision energy, and internal standards used for quantification.

Name	Precursor Ion (m/z)	Product Ion (m/z)	Cone (V)	CE[a] (E)	Internal Standard	Group[b]
Methamphetamine	150	91.1	20	15	Methamphetamine-D_5	B
	150	119.2	20	10		B
Amphetamine	136	91.1	20	20	Amphetamine-D_6	B
	136	119.3	20	10		B
Ephedrine	165.9	148.4	20	10	Ephedrine-D_3	B
Cocaine	304.1	182.3	40	25	Cocaine-D_3	A
Benzoylecgonine	290.2	168.4	30	20	Benzoylecgonine-D_3	B
		105.1	30	20		B
Norcocaine	290.3	168.4	10	15	Cocaine-D_3	A
		136.3	10	25		A
Norbenzoylecgonine	276.3	154.4	15	15	Benzoylecgonine-D_3	B
Hydrocodone	300.2	199.4	35	30	Hydrocodone-D_6	B
Oxycodone	316.2	298.5	25	20	Oxycodone-D_3	B
Methadone	310.3	265.5	25	15	Methadone-D_9	A
MDA	180	105	20	20	MDA-D_5	A
MDMA	194.1	163.4	20	10	MDMA-D_5	A
MDEA	208.2	163.3	20	10	MDMA-D_5	A
MBDB	208.22	177.3	20	10	MDMA-D_5	A
Ketamine	238.1	125.2	30	25	Ketamine-D_4	A
Norketamine	224.2	125.2	20	20	Ketamine-D_4	A
2-oxo-3-hydroxy-LSD	356.4	222.4	25	25	LSD-D_3	A
LSD	324.2	223.3	25	20	LSD-D_3	A
PCP	244.2	159.4	20	10	PCP-D_5	A
Flunitrazepam	314.2	268.4	25	25	Flunitrazepam-D_7	A
Caffeine	195.2	138.3	30	20	Cotinine-D_3	C
Cotinine	177.1	80	25	20	[^{13}C]Caffeine	C
Creatinine	113.91	43.8	10	15	Creatinine-D_3	D
		86	25	10		D

[a] Collision Energy

[b]Analytes were divided in four different groups to match four different calibration groups running simultaneously during analysis. Group A range from 2.5 ng/l to 250 ng/L. Group B from 10 ng/L to 2000 ng/L, Group C 250 ng/L to 80,000 ng/L and Group D 50,000 ng/L to 10,000,000 ng/l (0.50 to 10 mg/L).

Table 2. Internal standards and their precursor and product ions, cone voltage, and collision energies used for their acquisition.

Compounds	Precursor Ion (m/z) [M+H]	Product Ion (m/z)	Cone V	CE[a] E
Methamphetamine-D_5	155.1	91.7	20	20
Amphetamine-D_6	142	93.1	15	15
Ephedrine-D_3	169.1	151.4	15	15
Cocaine-D_3	307.3	185.5	30	20
Benzoylecgonine-D_3	293.2	171.4	30	20
Hydrocodone-D_6	306.3	202.4	40	30
Oxycodone-D_3	319.3	301.5	25	20
Methadone-D_9	319.4	268.5	25	15
MDA-D_5	185.2	168.5	15	10
MDMA-D_5	199.2	165.4	25	20
Ketamine-D_4	242.1	129.3	30	25
LSD-D_3	327.3	226.5	35	25
PCP-D_5	249.4	164.4	15	15
Flunitrazepam-D_7	321.2	275.4	30	25
Caffeine C_3^{13}	198.1	140.3	35	20
Cotinine-D_3	180.2	80.1	20	25
Creatinine-D_3	116.9	46.9	20	10

[a] Collision Energy

Linear regressions with R^2 values greater than 0.98 were obtained with 1/X-weighting and that were not forced through the origin. Seven-point calibration curves were prepared in deionized water daily. During the development of this study, the analytes investigated for this study exhibited a wide range in concentrations. For this reason, analytes were separated into three different groups (low, medium and high) according to concentrations measured for raw influent samples and appropriate

calibration ranges were determined for each analytes. For example, compounds including cocaine, norcocaine, norbenzoylecgonine, MDMA, MDA, MDEA, MBDB, ketamine, norketamine, LSD, 2-Oxo-3-hydroxy-LSD, PCP, flunitrazepam, and methadone were measured at low concentrations in raw influent so calibration curves were constructed for these analytes over a range from 2.5 ng/L to 250 ng/L. Hydrocodone, oxycodone, amphetamine, methamphetamine, ephedrine, benzoylecgonine, and norbenzoylecgonine occurred at higher concentrations so calibration curves ranging from 10 ng/L to 2000 ng/L were constructed for these analytes. Calibration curves used to quantify caffeine and cotinine ranged from 250 ng/L to 80,000 ng/L and creatinine was analyzed with a calibration curve that ranged from 50 to 10,000 μg/L (50,000 ng/L to 10,000,000 ng/L).

Three quality control standards were used after each batch of five samples to evaluate the performance of the instrument during analysis. Deviations of the quality control standards by more than 30% were rejected and the samples between the rejected quality control sample and the last quality control sample that was not rejected were re-analyzed. In each analysis, 20% of the samples were randomly analyzed in duplicate and the average was reported.

Instrumental blanks were run before and after every batch of samples to monitor carryover, instrument background and sample preparation; no instrument blanks showed carry over or contamination

3. Method Validation

3.1. Standard Addition

Standard addition was performed for all analytes in raw municipal wastewater in order to determine if matrix effects could be compensated for with the use of well-matched stable-isotope internal standards. The initial concentrations of analytes present in a single sample of raw influent were deduced from n=4 replicates using the solvent-based calibration curves. For the standard addition experiments, each analyte was spiked into four additional replicate aliquots of the same raw influent in order to increase the background signal 1.5 to 3 times that of the original signal *(40)*. For example, if the initial mass was 360 pg, the sample was spiked to give a final mass of 540 pg, 720 pg, 900pg and 1080 pg, which is equivalent to 1.5, 2, 2.5 and 3 times the original mass present in the sample. The mass added (x) was plotted against the mass calculated by solvent-based calibration curve (y) in mass units. A line was then fit from which the x-intercept was taken as equivalent to the background mass present in the un-spiked samples. The uncertainty of the background mass determinate by standard addition was calculated at the 95% CI with n-2 degrees of freedom *(40)*. The background mass and the 95% CI determined by standard addition regression was then compared using the student's t test with the mass calculated from the solvent-based calibration curves in the un-spiked samples.

3.2. Recovery

Percent recoveries were calculated from the four individual aliquots spiked with a single mass of analyte that were used as part of the standard addition

experiments. Recoveries were calculated using Equation (1) where m_f is the measured mass in the spiked sample and m_i is the original mass in the sample. Both values were calculated from the solvent-based calibration curves since subsequent analysis indicated that the solvent-based calibration curves and standard addition gave statistically-equivalent concentrations at the 95% CI. To test this alternative method for calculating recoveries, the mass added (pg) was plotted against the calculated % recovery. Linear regression was used to determine the slope and standard deviation for each analyte. Values of Student's *t* at the 95% CI were used to compare the slopes of the individual regressions in order to test if the slopes were statistically different from zero. The observed t value ($t_{calculated}$) was computed using Equation (2) where x is the slope, *s* is the standard deviation of the slope, and n is the number of observations per regression. The value of $t_{calculated}$ was then compared with the critical t value (t_{table}) at the 95% CI.

$$\% \text{ Recovery} = \frac{\left| m_f - m_i \right|}{\text{mass added}} \times 100 \tag{1}$$

$$t_{calculated} = \frac{\left| \bar{x} - 0 \right|}{s} \times \sqrt{n} \tag{2}$$

Intraday precision was evaluated by analyzing four spiked aliquots (n=4) from a single sample of raw influent on a single day. Between-day precision was estimated by subdividing a single sample of raw influent into 16 aliquots and analyzing n = 4 on each of the four consecutive days during a single week. A pooled percent relative standard deviation (RSD) was then computed for each analyte so that the uncertainty

around single measurements could be reported during the demonstration phase of the research.

The instrument detection limits (IDL) were calculated by spiking low level standards in distilled water with concentrations ranging from 0.5 ng/L to 7 ng/L and the IDL was defined as that concentration needed to achieve a signal/noise (S/N) ≥ 3. The method limits of quantification (LOQ) were defined as the lowest point on the calibration curve with a S/N $\geq$10. In the case of human urinary biomarkers (caffeine, cotinine and creatinine) the lowest point on the calibration curve gave S/N that were significantly than 10 since the analytes occur in wastewater at such high concentrations.

4. Results and Discussion

4.1. Method Optimization for LVI

The direct injection of volumes greater than the conventional 10-20 μL volumes onto an analytical column is similar to frontal chromatography in which a large sample volume relative to the void volume of a column is introduced continuously rather than in a small volume as a band. As example, the low elutropic strength of water entering a C_{18} column results in the focusing of analytes at the head of the analytical column. The injection of the large 1,800 μL volume did not adversely affect chromatographic behavior due to the high retention factor (k') of the analytes for the C_{18} phase. Example chromatograms obtained under LVI conditions illustrate the separation of legal and illegal stimulants and rave drugs (Figure 2), prescription opiates, cocaine and its metabolites (Figure 3), and human urinary

biomarkers (Figure 4) in actual samples of raw influent in analytes occur as symmetrical peaks with signal/noise ratios well above background and little or no evidence of band broadening. The observed quality of the chromatography is consistent with other studies using LVI which show great reproducibility of retention times for volumes higher than 1000 µL *(22)* and *(34, 41)*.

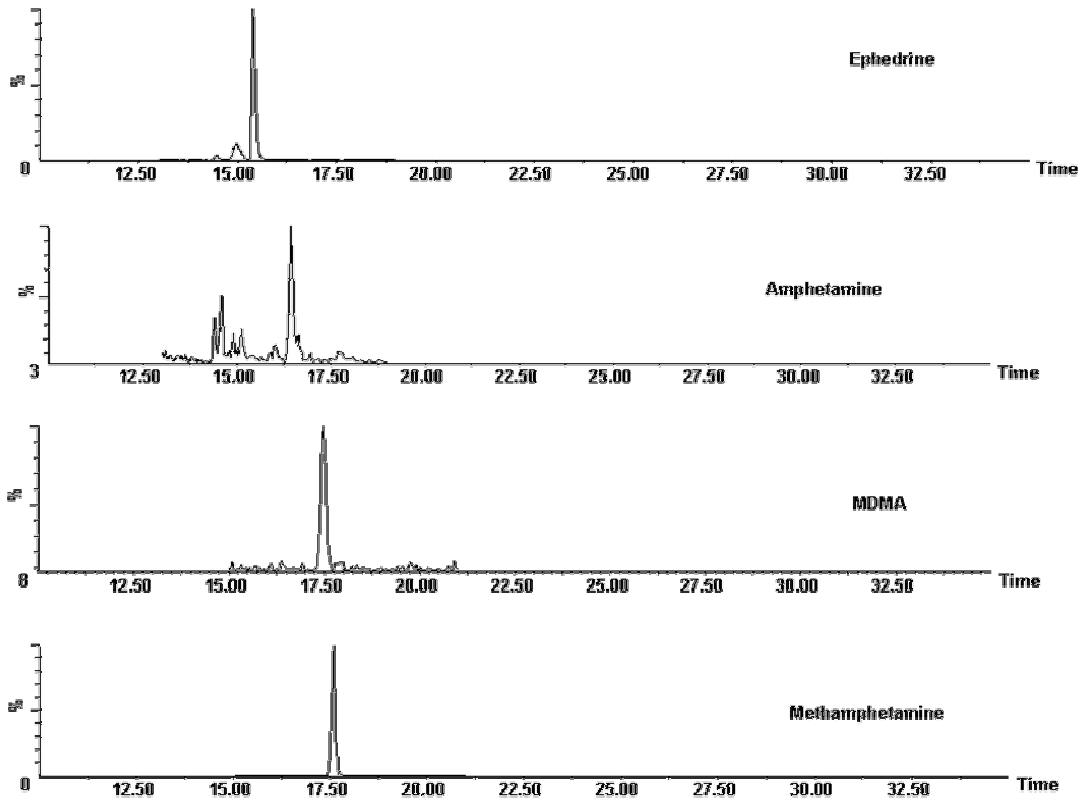


Figure 2. Separation of stimulants (ephedrine, amphetamine, MDMA and methamphetamine) in raw influent wastewater acquired by LVI (1,800 µL)-LC/MS/MS conditions.

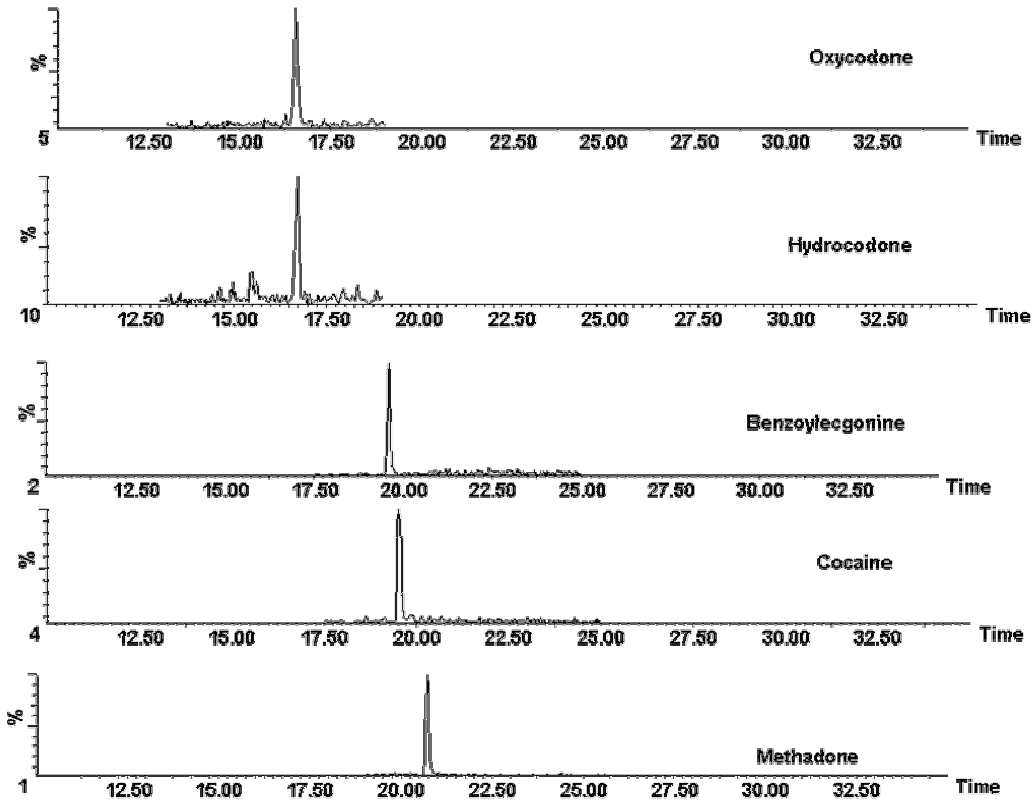


Figure 3. Chromatograms for .prescription opiates (oxycodone, hydrocodone and methadone) and cocaine and its metabolite benzoylecgonine in raw influent wastewater acquired by LVI (1,800 μL)-LC/MS/MS conditions.

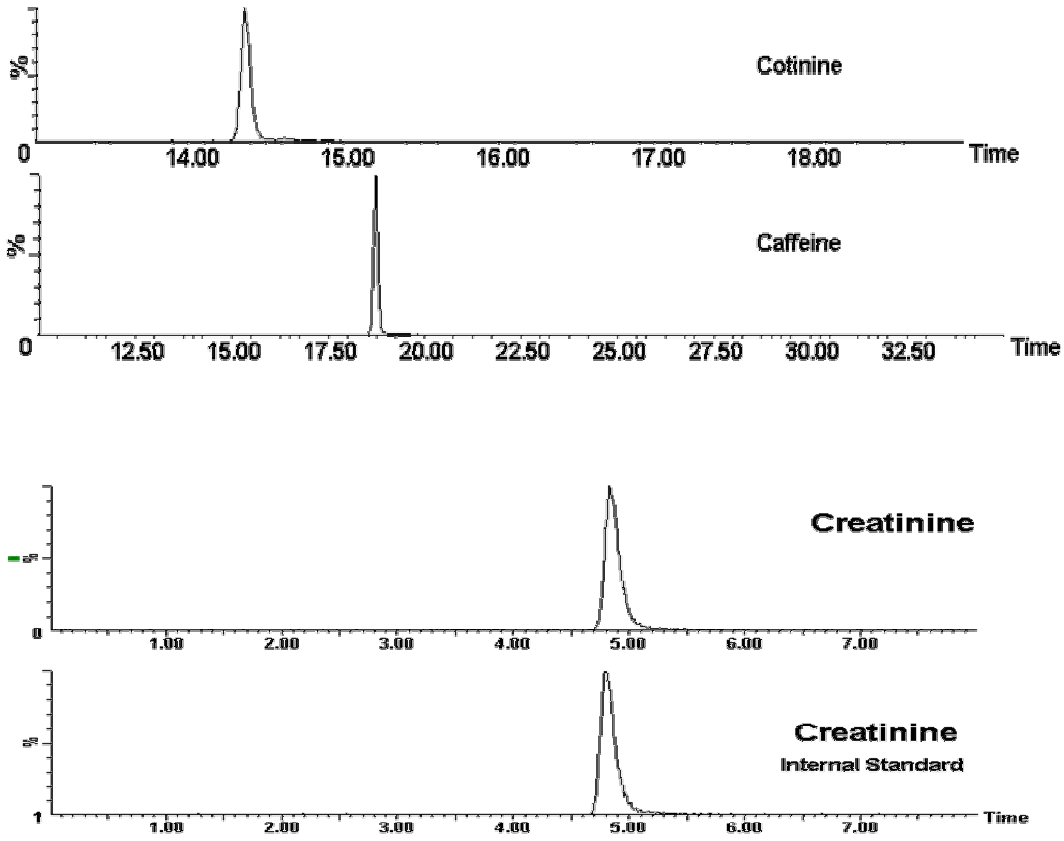


Figure 4. Chromatograms for human urinary biomarkers (cotinine, caffeine and creatinine) in raw influent wastewater acquired by LVI (1,800 µL)-LC/MS/MS conditions.

The molecular processes governing LVI are chemically-redundant with those occurring during SPE, which is why SPE can be removed as a sample preparation without causing deleterious effects on the chromatography or quantification of analytes in a matrix as complex as raw municipal influent. Narrow and symmetrical peaks are consistent with the small 5 µm particle size of the analytical column compared with 40 to 80 µm particle sizes associated with SPE sorbents. Given that the number of theoretical plates (*N*) will increase by 1.4 times for every half of the particle size *(42)*, LVI has an advantage over SPE due to the smaller particle size

employed as the sorbent phase. In addition, HPLC columns are packed under pressure and kept wet which results in a close packing arrangement with improved efficiency, rather than dry packed sorbents such as those used in SPE *(42)*.

In order to take advantage of the benefits of LVI, it is important to the control of the mobile phase during injection by correctly timing the rotation of the injection values. With the Agilent 1100 during sample withdrawal and temporary storage in the loop extension and seat capillary loop, the injection valve is in the 'by-pass' position so that the initial mobile phase (90% methanol with 0.1 % acetic acid (A) and 10% acetonitrile (B)) is not pumped through the injector but bypasses the injection by flowing directly into the column (Figure 5). Once the sample loops are loaded, the injection valve is switched to the 'main-pass' position so that the mobile phase passes through all sample loop extension and seat capillary tubing associated with the injector (Figure 6) and effectively pushes the sample onto the analytical column. However, the minimum time needed to transfer the sample onto the analytical column must be determined and be used as the time at which the injection valve should be turned back to the 'by-pass' position. Leaving the valve in 'main-pass' position would effectively increase the dwell time of the system, which is the delay between the time the gradient is started and the time the gradient reaches the column *(43)* and this would result in unnecessarily long run times. Therefore, in order to minimize the dwell time and total analysis time, the injection value was programmed to move from 'main-pass' position to 'by-pass' position at 9 min and the flow rate was increased to 500 μL/min over the more conventional flow rate of 200 μL/min. Once the flow rate was set, it was used to experimentally measure the

time required to transfer the 1,800 µL sample onto the analytical column (dwell time), which was 8 min. The dwell time was determined experimentally by removing the column and injecting a sample that was poorly retained, in this case acetone, and using its arrival time (8 min) at the detector as the time required to transfer the 1,800 µL injection volume.

The first attempts to use LVI with an 8 min dwell time to allow for sample transfer gave RSDs for n=5 replicate injections of a centrifuged raw wastewater sample that were greater than 30%. A wash step consisting of an additional 1 min of the injection value in 'main pass' mode reduced the average RSDs to 7%. The addition of this step is functionally equivalent to the 'wash step' commonly used in SPE in which solvent containing a low percent organic that rinses the column but does not elute the analytes .

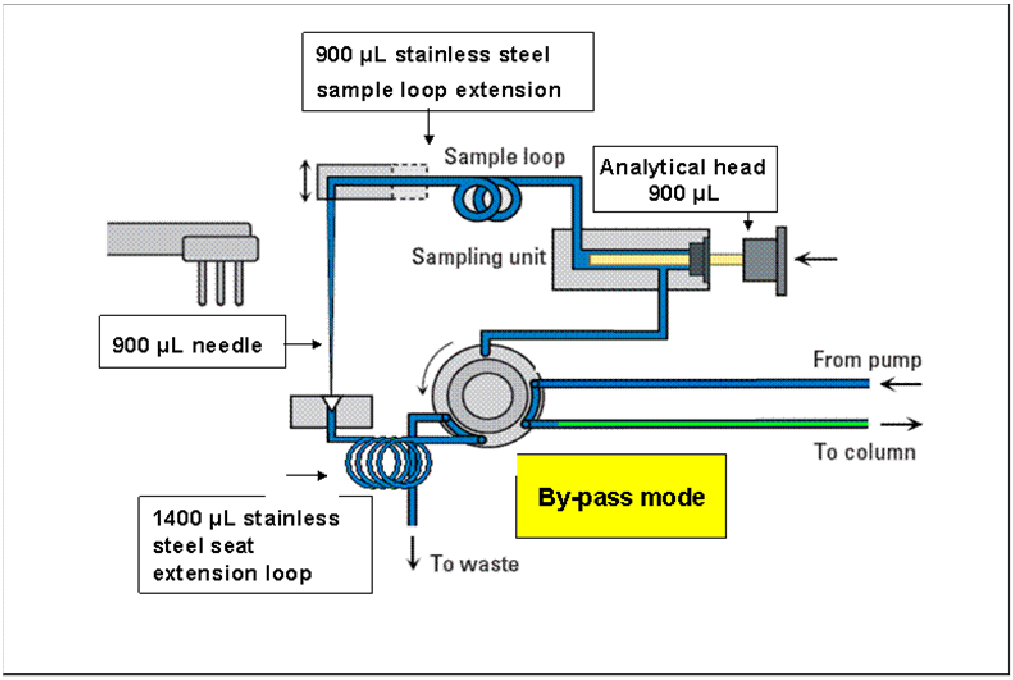


Figure 5. LVI injection valve in the 'by-pass' position at the beginning of the injection sequence when the mobile phase by-passes the injector when sample is

being withdrawn and placed in the 900 µL sample loop extension and 1,400 µL seat capillary.

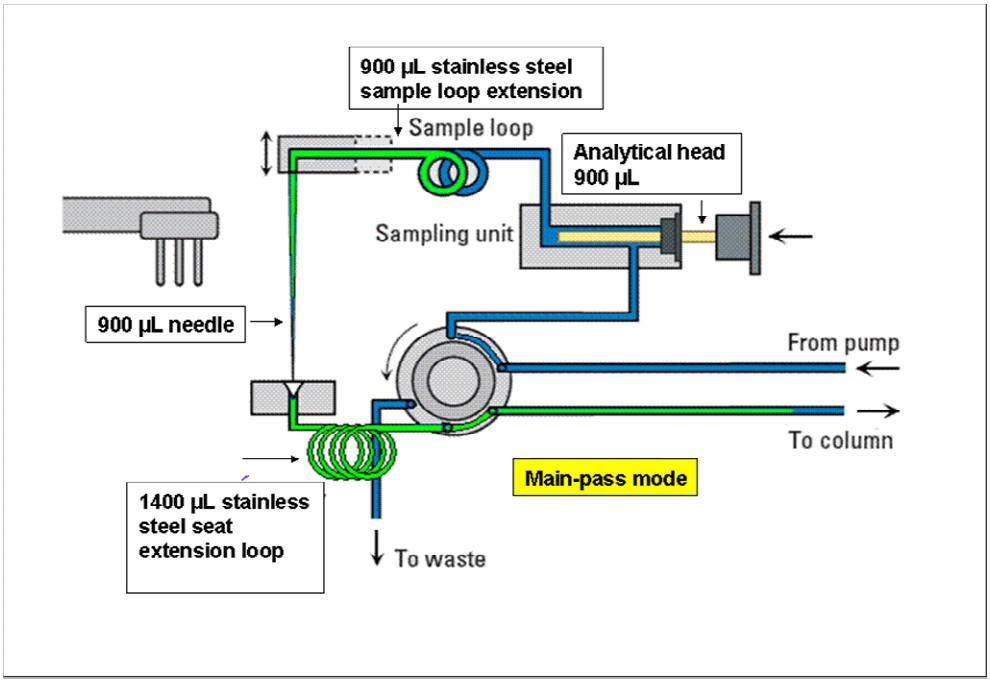


Figure 6. LVI injection valve in the 'main-pass' position when mobile phase is pumped through the 900µL sample loop extension and 1,400 µL seat capillary tubing in order to transfer the sample to the analytical column.

Due to the expected high concentrations of creatinine in wastewater, initial experiments to measure creatinine in raw wastewater began with injection volumes < 900 µL. During optimization, different injection volumes were tested and, due to the high water solubility and triply ionized nature of creatinine, breakthrough of creatinine occurred, which indicates a low k' value for creatinine under LVI conditions with a 900 µL injection volume. Decreasing the volume of injection from 900 µL down to 100 µL, resulted in narrow peaks for creatinine, which indicated good chromatographic focusing and a high k' for an injection volume of 100 µL (Figure 4).

Breakthrough is typically not a concept used in HPLC but it is often used to describe solute retention during SPE, which is a form of frontal chromatography *(20, 42)*. In frontal chromatography, a sample volume typically larger than the void volume of the sorbent system (in LVI it is the void volume of the analytical column) is continuously injected into a column to determine when/if breakthrough occurs *(42)*. Breakthrough is a function of an analyte's k', the void volume of the system, and the sample volume. Conditions need to be selected in all forms of frontal chromatography (LVI and SPE) to avoid analyte loss and band broadening, which results in a reduction in sensitivity *(22)*.

4.2. Accuracy and Precision

The first step was to verify that analytes were not lost during the centrifugation step. The potential for loss of analyte to suspended particulate matter during centrifugation was studied by spiking (all analytes) before (n=4) and after (n=4) centrifuging replicate aliquots of a single raw influent sample. The average analyte concentrations for the two groups were compared using the student's t test and no statistical difference at the 95% CI was found for aliquots spiked before or after centrifugation, which indicates that no significant loss of analyte occurs during centrifugation. For this reason, all wastewater samples were centrifuged prior to analysis and spiked with internal standards after the centrifugation step.

Average concentrations for un-spiked samples determined from solvent-based calibration curves were statistically equivalent at the 95 % CI to those determined by standard addition (Appendix A). This was true for all analytes with concentrations

ranging from 4 to 3,500,000 ng/L (Table 3). Accuracy determinations by standard addition indicated that the internal standards are able to compensate for matrix effects and that concentrations can be determined directly calibration standards prepared in DI water.

Table 3. Accuracy determined by standard addition indicated concentrations determined by standard addition were statistically equivalent to concentrations determined from solvent-based calibration curves at the 95%.

Name	Calculated Concentration at 95% CI ng/L	Concentration from Standard Addition at 95% CI ng/L
Methamphetamine	390 ± 20	400 ± 4
Amphetamine	?	?
Ephedrine	?	?
Cocaine	15 ± 1	15 ± 0
Benzoylecgonine	340 ± 5	350 ± 8
Norbenzoylecgonine	20 ± 10	20 ± 2
Norcocaine	ND	BD
Hydrocodone	50 ± 10	60 ± 2
Oxycodone	43 ± 3	45 ± 1
Methadone	24 ± 1	24 ± 0
MDA	4 ± 1	5 ± 1
MDMA	4 ± 1	6 ± 1
MBDB	ND	BD
MDEA	ND	BD
Ketamine	6 ± 1	7 ± 1
Norketamine	ND	ND
2-oxo-hydroxy-LSD	ND	BD
LSD	ND	BD
PCP	ND	BD
Flunitrazepam	ND	BD
Caffeine	4000 ± 500	3600 ± 100
Cotinine	580 ± 60	630 ± 20
Creatinine	3,500,000 ± 80,000	3,400,000 ± 60,000

ND (or < LOD) = Background concentrations of analyte in unspiked aliquots below detection.
BD = background concentrations determined by extrapolation of the standard addition data were below detection.

To determine recovery, plots of the mass added (pg) vs. % recovery were created using the standard addition data for each analyte (Appendix B). The slopes of the individual regressions and the standard deviations of the slopes were evaluated using the Student's t test to determine whether the slopes were statistically different than zero at the 95% CI. None of the slopes were statistically different from zero, which indicates that the percent recovery is independent of mass. For this reason, the percent recoveries were then averaged to compute an average recovery ± standard deviation for each analyte (Table 4). The percent recoveries ranged from 60 to 150 % and are similar to RSDs reported by other incorporating a SPE step into their analytical determinations for illicit drugs in wastewater that range from 71 to 173 % *(1-3, 5)*. However, unlike SPE, there is less opportunity for analyte loss due to breakthrough. The cause for the low apparent recovery of MDMA is not known; however, the standard addition data for MDMA indicates a high level of accuracy for MDMA determinations by the LVI method.

Table 4 The slopes of individual regression for the % recovery vs. mass for each analyte in standard addition experiments. Values of $t_{calculated}$ indicated by Equation 2 indicate the slope is not statistically different from zero at the 95% CI. The averaged recoveries from the standard addition data are reported ± standard deviation.

Name	Regression Slope	Slope SD	n	t_{table} 95% CI	$t_{calculated}$ 95% CI	Recovery ±SD (%)
Methamphetamine	0.005	0.004	4	3.182	2.284	100 ± 2
Amphetamine						
Ephedrine						
Cocaine	-0.752	0.493	4	3.182	3.050	120 ± 20
Benzoylecgonine	0.024	0.015	4	3.182	3.035	80 ± 10
Norbenzoylecgonine	-0.190	1.128	3	4.303	0.292	120 ± 10
Norcocaine	-0.268	0.552	4	3.182	0.971	150 ± 30
Hydrocodone	0.000	0.012	3	4.303	0.080	90 ± 8
Oxycodone	0.038	0.145	4	3.182	0.453	100 ± 10
Methadone	3.714	0.067	5	2.776	1.478	100 ± 3
MDA						
MDMA	0.066	0.042	4	3.182	3.121	60 ± 3
MDEA	-0.111	0.321	3	4.303	0.600	90 ± 3
MBDB	-1.139	0.732	3	4.303	2.693	90 ± 10
Ketamine	0.580	1.620	4	3.182	0.716	70 ± 30
Norketamine	0.556	1.091	3	4.303	0.882	60 ± 10
2-oxo-hydroxy-LSD						
LSD	-0.563	0.585	4	3.182	1.923	100 ± 10
PCP	-0.241	0.747	4	3.182	0.645	110 ± 10
Flunitrazepam	-0.217	0.276	4	3.182	1.570	90 ± 5
Caffeine	0.000	0.001	4	3.182	1.058	100 ± 5
Cotinine	-0.039	0.026	4	3.182	3.028	100 ± 10
Creatinine	0.000	0.002	4	3.182	0.471	100 ± 4

The instrument detection limits (IDL) ranged from 0.5 to 4 ng/L for the illicit and legal drugs and metabolites while human biomarkers ranged from 4.5 to 250 ng/L (Table 5). These IDL determined for the LVI method presented here are in the same range as methods that use either on-line or off-line SPE *(2)*. The high IDL values of

creatinine is due to the volume injected, which is 18 times smaller than the method use to determine the other analytes. The limits of quantification (LOQ) for illegal and legal drugs and metabolites ranged from 2.50 to 10 ng/L (Table 5). Due to high expected levels of some analytes like human urinary biomarkers in wastewater, the LOQ were set at 250 ng/L for caffeine and cotinine and at 50,000 ng/L (50 μg/L) for creatinine.

The intra-day and within day precision, as indicated by RSD, ranged from 2-14% with an average of 7 % (Table 5). The %RSD are similar to those reported for the analysis of drugs in wastewater (4 to 7%) using SPE-based technology at additional cost and time investment *(1, 2)*.

For larger studies in which the period of sample analysis is likely to span days to week, it is necessary to determine the uncertainty associate with analyses performed across multiple days. Of the few day-to-day precision values that are reported, few are applied to the resulting analytical data when interpreting differences between days. Day-to-day precision, as indicated by RSDs, for each analyte was computed with resulting values ranging from 3-32% with an average of 12% (Table 5) which are higher than the intra-day RSDs (Table 5). The highest RSDs of up to 32% correspond to norcocaine and 2-oxo-hydorxy-LSD, which do not have matched internal standards. Therefore, data for these two analytes should be treated as semi quantitative. Huerta-Fontela et al. *(2)* reported day-to-day RSDs that are higher than intra-day by 1%. The computed day-to-day RSDs were used to compute standard deviation associated with the nominal values reported for the demonstration part of the study in which single samples from 21 days were analyzed.

Table 5. Instrumental detection (IDL) and limits of quantification (LOQ) and whole method precision within a day (intra-day) and between days (day-to-day

Compounds	IDL ng/L	LOQ ng/L	Intra-day precision % RSD	Between day precision % RSD
Methamphetamine	1.50	10.0	7	7
Amphetamine	1.50	10.0	3	12
Ephedrine	2.50	10.0	5	7
Cocaine	2.00	2.50	6	12
Benzoylecgonine	1.00	10.0	6	14
Norcocaine	2.00	2.50	8	31
Norbenzoylecgonine	2.50	5.00	8	6
Hydrocodone	2.00	2.50	10	7
Oxycodone	2.00	2.50	4	7
Methadone	2.00	2.50	4	7
MDA	2.00	2.50	8	18
MDMA	1.00	2.50	8	13
MDEA	3.50	5.00	13	17
MBDB	4.00	5.00	14	13
Ketamine	4.00	5.00	10	17
Norketamine	3.50	5.00	11	10
2-Oxo-3-hydroxy-LSD	2.50	5.00	6	32
LSD	0.50	2.50	4	1
PCP	2.50	5.00	8	12
Flunitrazepam	1.50	2.50	3	4
Caffeine	6.00	250	6	3
Cotinine	4.50	250	7	7
Creatinine	250	50000	3	13

4.3. Temporal Trend in Psychoactive compounds, Opiates and Human Urinary Biomarkers in Wastewater

Raw influent to a single WWTP was collected and analyzed for a period of three weeks in order to quantify the temporal trends in concentration and loads for illicit and legal drugs, selected metabolites, and human urinary biomarkers (Table. 6). Loads were calculated using Equation (3) by multiplying the measured concentration (ng/L) by the measured average flow (L) (provided by WWTP) personnel and divided by the estimated population (50,500). The loads are reported in milligrams per person per day (mg/person/day) *(9, 44)*.

$$\text{Per capita load}\left(\frac{\text{mg drug}}{\text{person}\cdot\text{day}}\right)=\frac{\text{mg drug}}{\text{L}}\times\frac{\text{L flow}}{\text{day}}\times\frac{1}{\text{population}} \quad (3)$$

The population used for these calculations (50,000) is the stated population estimate of the municipality and does not take into account movements of individuals such as commuters.

Table. 6 List of illicit and legal drugs, metabolites, and human urinary biomarker names and classification

Drugs/Metabolites/Biomarkers	Class
Methamphetamine	illicit and prescription drug
Amphetamine	illicit and prescription drug
Ephedrine	precursor of meth and prescription drug
Cocaine	illicit drug
Benzoylecgonine	metabolite of cocaine
Norcocaine	metabolite of cocaine
Norbenzoylecgonine	metabolite of cocaine
2-oxo-3-hydroxy-LSD	LSD metabolite
LSD	illicit drug
MDMA	illicit drug- rave
MBDB	illicit drug- rave
MDEA	illicit drug- rave
MDA	illicit drug- rave
Ketamine	anesthetic and drug of abuse
Norketamine	Metabolite of ketamine
PCP	Veterinary tranquilizer and drug of abuse
Flunitrazepam	illicit drug- rave
Hydrocodone	prescription opiate
Oxycodone	prescription opiate
Methadone	prescription opiate
Cotinine	urinary biomarker/population indicator
Creatinine	urinary biomarker/population indicator
Caffeine	urinary biomarker/population indicator

Psychoactive Drugs

Methamphetamine (MA) was present in each sample collected at loads ranging from 0.13 to 0.23 mg/day/person (Figure 7), which indicates MA use within the community. MA concentrations (>121 ng/L) and loads are significantly different from other studies which report concentrations up to 20 ng/L *(1, 5, 6, 9)*. The large range in concentration/loads might be explained by the rapid expansion of methamphetamine use in recent years. The pattern in MA load results does not appear to be episodic when comparing weekdays (Monday through Thursday) to weekends (Friday to Sunday) (Figure 7) and therefore may indicate a chronic use within the community.

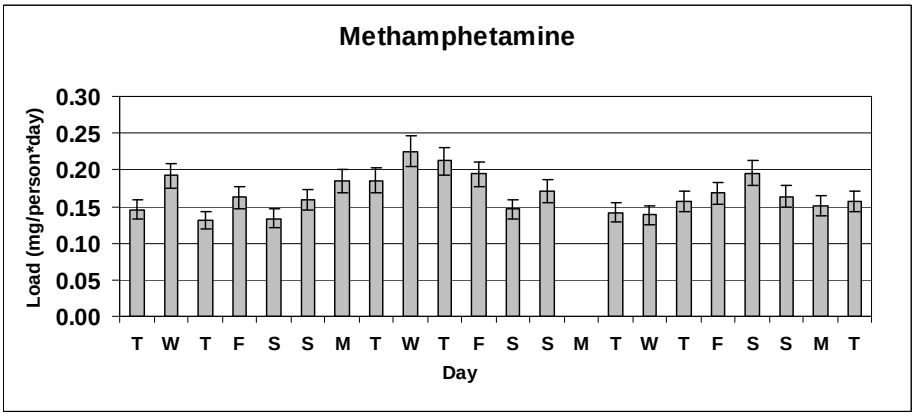


Figure 7. Methamphetamine loads ± standard deviation (mg/day/person).

Amphetamine (AM) loads ranged from 0.08-0.20 mg/person/day (Figure 8). The concentrations (>86 ng/L) and loads found are also greater than other studies which have reported levels up to 41 ng/L *(1, 2, 5, 6, 9)*. To be able to study the illegal

consumption of these two stimulants, ratios were computed, since AM is one of the major metabolites of MA (

Figure 9).

The visual correlation between AM and MA ratios during the three weeks of analysis could indicate be an indication that most of the AM analyzed comes from the MA intake.

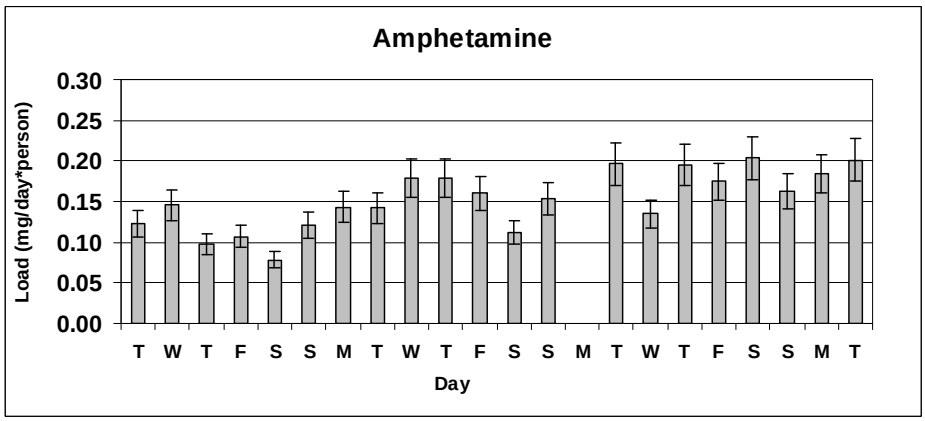


Figure 8. Amphetamine loads ± standard deviation (mg/day/person).

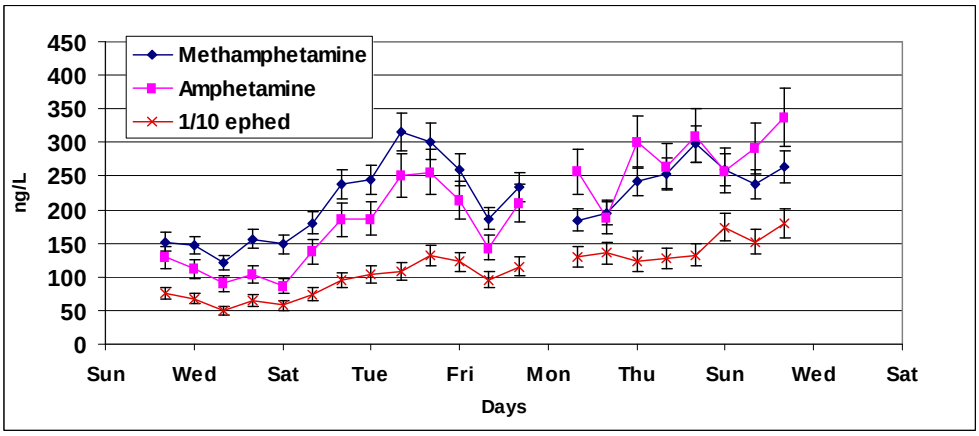


Figure 9. Methamphetamine, amphetamine and ephedrine concentrations ± standard deviation for the three week sampling period.

Ephedrine was found at loads ranging from 0.72 to 1.10 mg/person/day (Figure 10). Concentrations of ephedrine vary from 600 ng/L to 1800 ng/L. Postigo et al. *(5)* reports concentration around 600 ng/L in raw influent, which is similar to the low-level concentrations found in this study. The loads are greater because the population is 36 times bigger. The increase of ephedrine load over the three weeks of study (March-April) can be a contributed to allergy season. Ephedrine is used as a nasal decongestant and bronchodilator *(45)*. In addition, diet pills can be major contributor to ephedrine load. Ephedrine is also used as a precursor in the clandestine manufacture of methamphetamine; however, no correlation was found between the loads of MA and ephedrine (Figure 9).

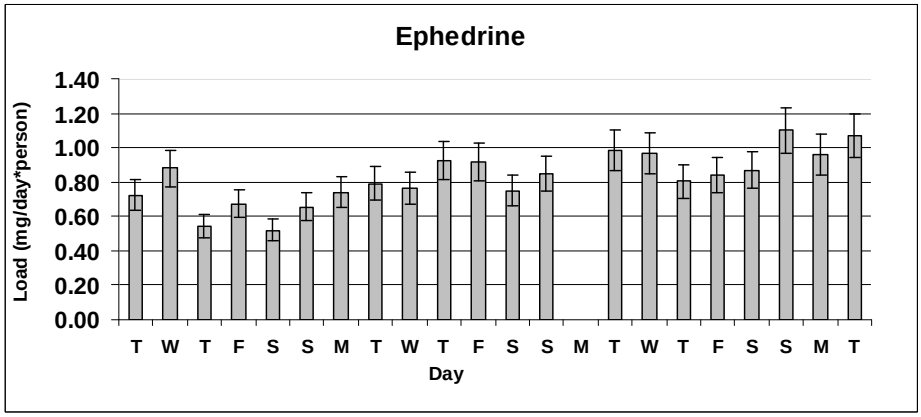


Figure 10. Ephedrine loads ± standard deviation (mg/day/person).

Rave and other illicit drugs

Of the rave drugs investigated, only MDMA and MDA were observed during the three week sampling period. Other drugs including MBDB, LSD, 2-oxo-hydroxy-LSD, PCP, and flunitrazepam were not detected in any of the samples and therefore are not discussed further. MDMA was the most frequent rave drug observed and

ranged in loads from 0.001 to 0.009 mg/day/person (3 to 14 ng/L). The temporal trend in MDMA should be interpreted with caution as MDMA appeared during midweek, Friday and Sunday in the first week, Thursday to Sunday in the second week and only on Saturday and Sunday of the third week of sampling (Figure 11). This might indicate that there is a relationship between MDMA consumption and college students, since more frequent and higher loads were found after the student population came back from Spring Break (First weekend). MDA was only detected in a single sample collected in the second Friday of the three weeks of analysis, and corresponded to a load of 0.004 mg/day/person (6 ng/L). This values is consistent with recent literature that reports MDMA concentration values from 3 to 14 ng/L and MDA from 4.6 to 6 ng/L *(1, 2, 5, 6, 9)*.

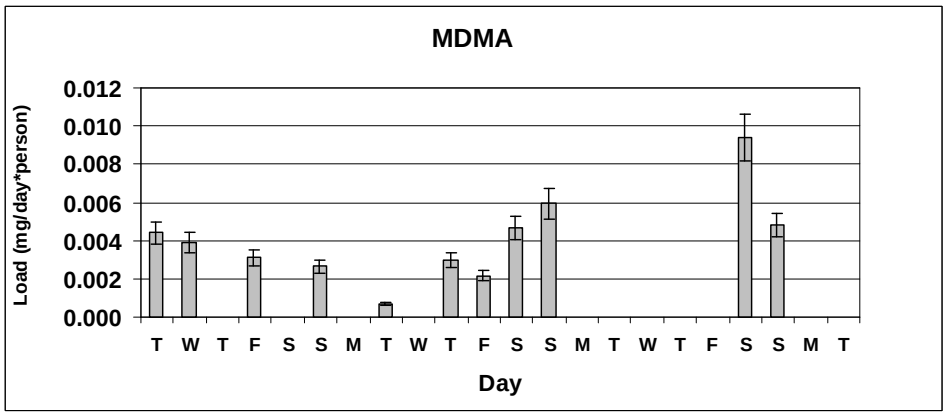


Figure 11. Loads of MDMA ± standard deviation (mg//day/person) for a three week period.

Ketamine was found in load ranges from 0.003 to 0.034 mg/day/person and was detected on only five days out of the three week period (Figure 12). Ketamine reflects infrequent use with no real trends during the three weeks of analysis. Norketamine, a main metabolite of ketamine, was not found in any of the samples.

However, because ketamine is not only a street drug, but also is used as an anesthetic in animals and humans, this method could potentially determine an increase in load ratios (Norketamine/ketamine) to investigate trends and use in future studies.

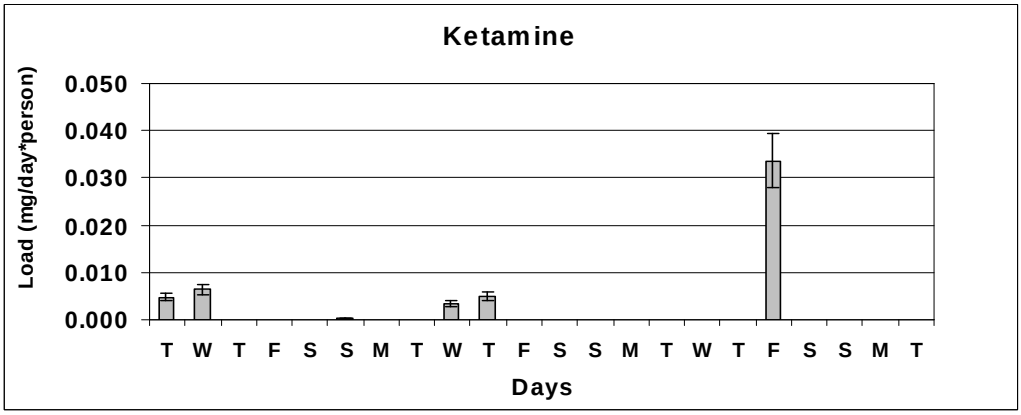


Figure 12. Ketamine loads ± standard deviation (mg/day/person) over a three week sampling period.

Prescription Opiates

Hydrocodone, which is the most frequently prescribed drug in the US and the most frequently-prescribed opiate, loads ranged from 0.01 to 0.03 mg/day/person (Figure 13). Oxycodone loads, which present similar loads as hydrocodone, ranged from 0.02 to 0.03 mg/day/person (

Figure 14). The loads of oxycodone appear consistent over the three week period (e.g., no episodic use), but there is an apparent increase in hydrocodone loads during the third week (Figure 13). The cause for the increase is not known; however, since the location of sampling is fixed and the total flow of wastewater does not vary significantly over the sampling period (Figure 15), the hypothesis is that the number of hydrocodone users increased in the third week relative to oxycodone users.

Hydrocodone concentrations and loads are not higher when they are compared with oxycodone loads, even though the number of hydrocodone prescriptions is greater than that of oxycodone. For these two target prescription opiates, further work needs to be done which includes an evaluation of the mass and typical purity of dose. In addition, metabolites along with the percent of drug excretion in unchanged form will be evaluated in order to rationalize the observed loads.

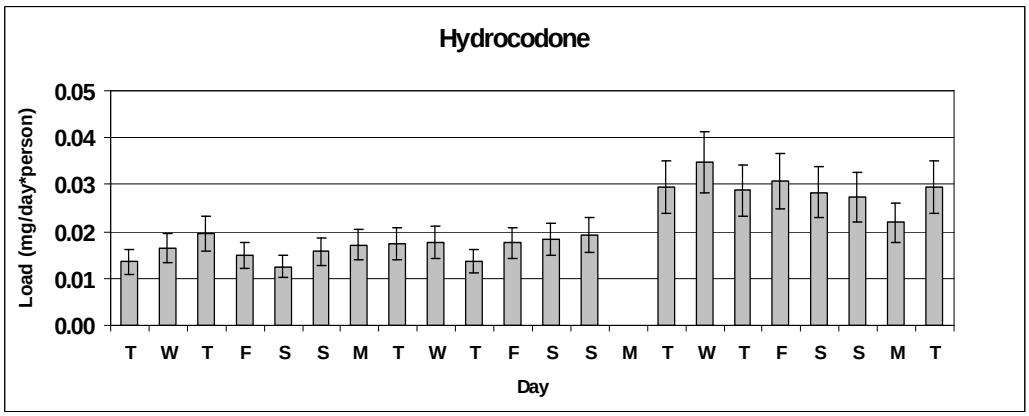


Figure 13. Hydrocodone loads ± standard deviation (mg/day/person) for the three week sampling period.

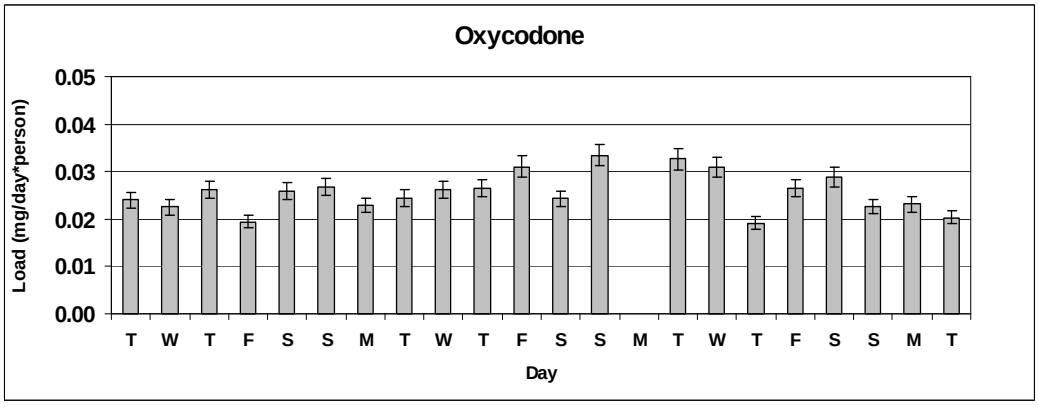


Figure 14. Oxycodone loads ± standard deviation (mg/day/person).

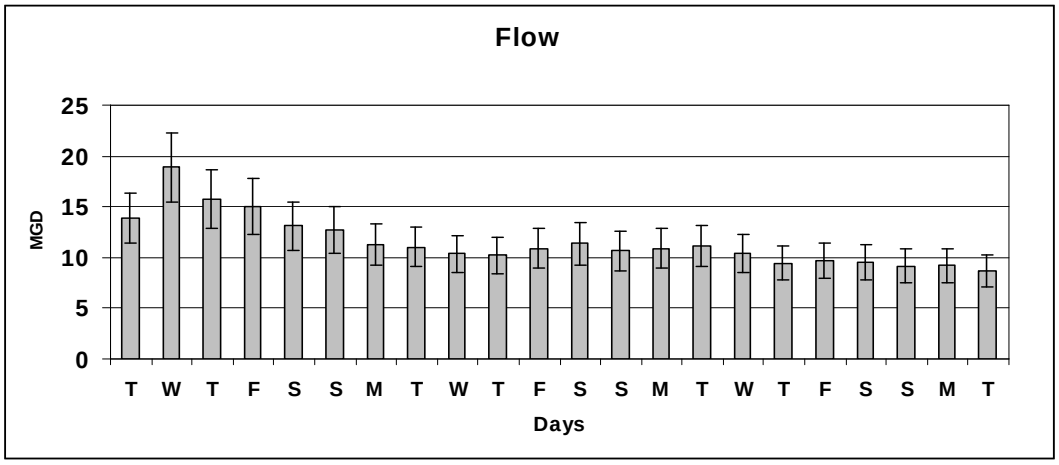


Figure 15. Flow ± standard deviation of wastewater over the sampling period
MGD=Million of gallons per day

Methadone, a prescription opiate used as a painkiller and in the treatment of addiction to heroin, was found at loads ranging from 0.01 to 0.02 mg/person/day (Figure 16). The concentrations and loads were higher than those reported by others ((*3, 4, 8)*. The data for methadone appears consistent over the three weeks and there is no apparent episodic use of methadone as weekday and weekend loads are similar.

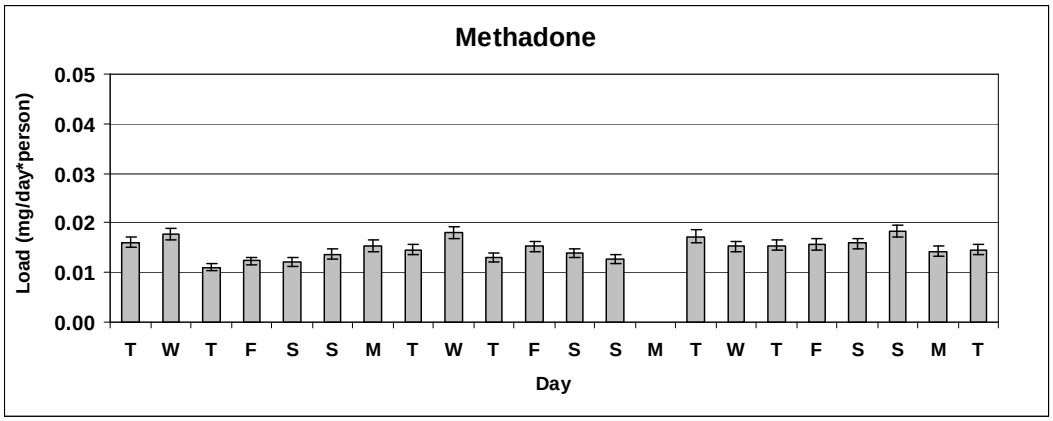


Figure 16. Methadone loads ± standard deviation (mg/day/person).

Cocaine

Cocaine and three of its metabolites, including benzoylecgonine (BE), norbenzoylecgonine, and norcocaine were investigated. Other major metabolites including ecgonine methyl ester and cocaethylene, known to occur in wastewater, were not part of this study, but would be incorporated in to future studies. Cocaine and its main metabolite, BE, were detected during the three week of sampling. Cocaine loads ranged from 0.004 to 0.01 mg/day/person (Figure 17), while BE loads were greater and ranged from 0.02 to 0.1 mg/day/person (Figure 18). With concentrations ranging from 4 to 18 ng/L for cocaine and 16 to 154 ng/L for BE, these values are similar to other studies that have similar population with concentrations ranging 14 to 225 ng/L for cocaine and 14-2307 ng/L for BE *(2)*. Other studies report finding greater concentrations up to 860 ng/L of cocaine and up to 4225 ng/L of benzoylecgonine *(5)*, but the populations were much larger than the population stated for this study. Norbenzoylecgonine was detected in some samples at concentrations lower than the LOQ and norcocaine was found in quantifiable amounts in only two samples at loads of 0.002 mg/day/person.

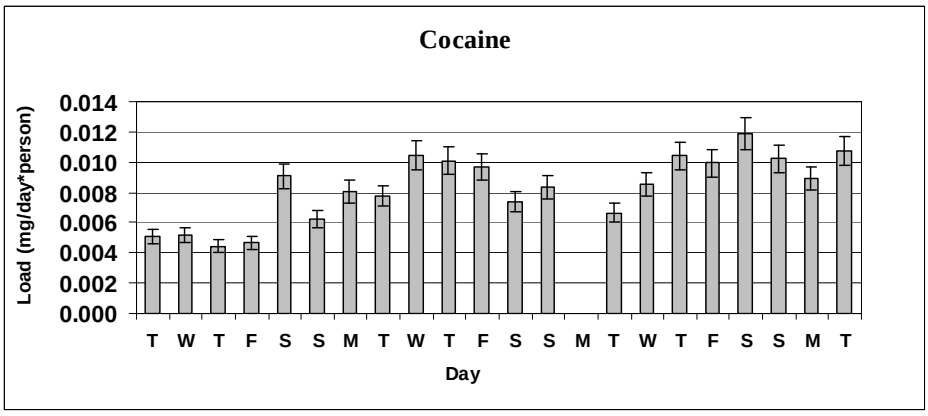


Figure 17. Cocaine loads ± standard deviation (mg/day/person).

The greater observed loads for BE than cocaine are consistent with reports that indicate that 1% -9% is excreted as the unchanged drug while 35%-54% is excreted as benzoylecgonine in the 24 hrs after administration *(45)*.

The loads of cocaine (Figure 17) and BE (Figure 18) vary during the sampling period, but neither-represents a clear trend over time. Although there might be a small increase in benzoylecgonine loads associated with weekends, it is not clear that a real trend exists. To explore a possible trend, the ratios of benzoylecgonine to cocaine concentration were plotted (Figure 19).

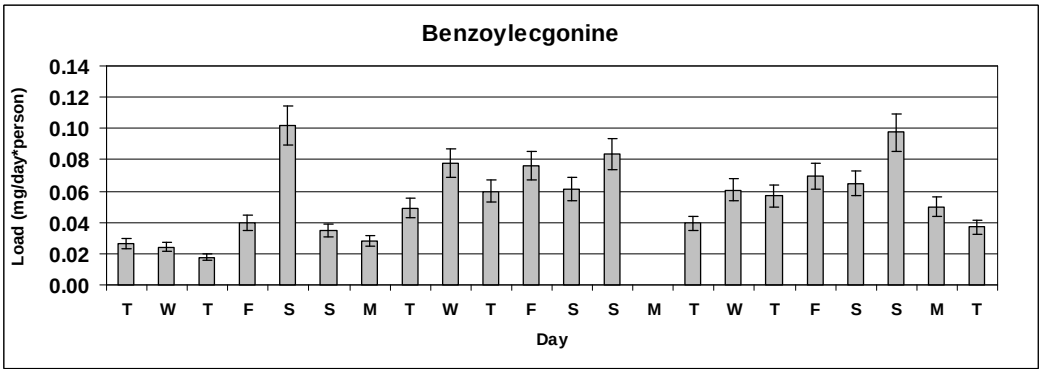


Figure 18. Benzoylecgonine loads ± standard deviation (mg/day/person).

Cocaine smoking and injection is the fastest route to the brain. Users with these behaviors excrete a higher percent of unchanged cocaine relative to BE. On the other hand, cocaine snorters tend to have a lower percent of unchanged cocaine relative to BE. The smoking and injection of cocaine is usually attributed to users that are likely to use it all week and not just on the weekend (Table 7) *(46)*.

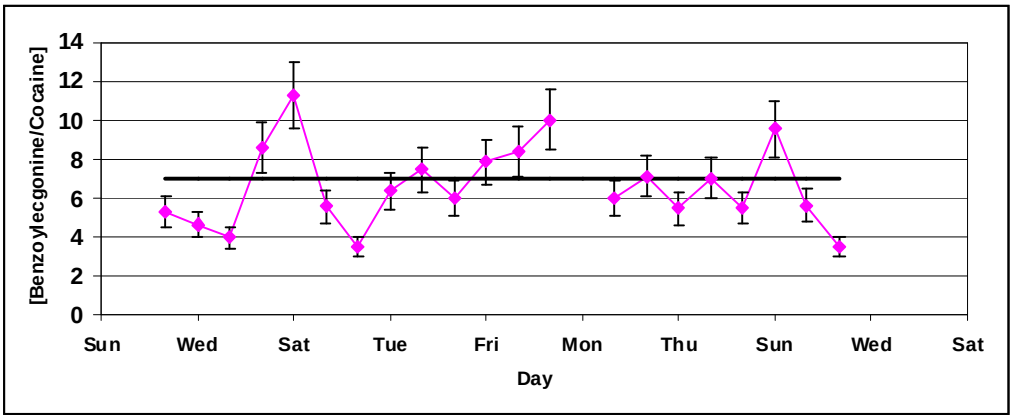
Table 7. Dose excreted in percentage of BE or cocaine when injected, smoked or snortes

Dose excreted as BE (%)		Dose excreted as cocaine (%)		Ratio BE/Cocaine	
Intravenous	39.2	Intravenous	1.0	Intravenous	39.2
Smoked	16.4	Smoked	0.5	Smoked	32.8
Intranasal	29.9	Intranasal	0.5	Intranasal	59.8

Ratio BE/Cocaine which range from 33 to 60 (Table 7) are different from the ratios shown in Figure 19. This could be because ratios in transit to the WWTP may change away from urine but transit time could be constant for this single municipality.

An average was compute for all the BE/Cocaine ratio. Ratios higher than average that are statistically significantly different, might be an indication of an episodical use since higher ratios BE/Cocaine are due to intranasal dose. Therefore, Friday and Saturday from the first week, and Sunday of the second and third week, could be an illustration of recreational use rather than chronic.

Figure 19. Ratios of benzoylecgonine to cocaine ± standard deviation for the three week sampling period. Line at ratio 7.0 represents the average of ratio for the three week period.



Human urinary biomarkers

Three human urinary biomarkers; cotinine, caffeine and creatinine were measured as potential population indicators. The measurement of human urinary biomarkers, hypothesized an alternative approach to treating population as a constant.

Cotinine, the main metabolite of nicotine, ranged in mass from 9 to 28 g (Figure 20). The mass of cotinine increases over the weeks, but when comparing between days, the week values are constant with the exception on the first Sunday of sampling. Cotinine has been measured by others *(2)* but data has not been treated. Future work is necessary to compare nicotine sales by zip code and pharmacokinetics of nicotine to rationalize observed mass.

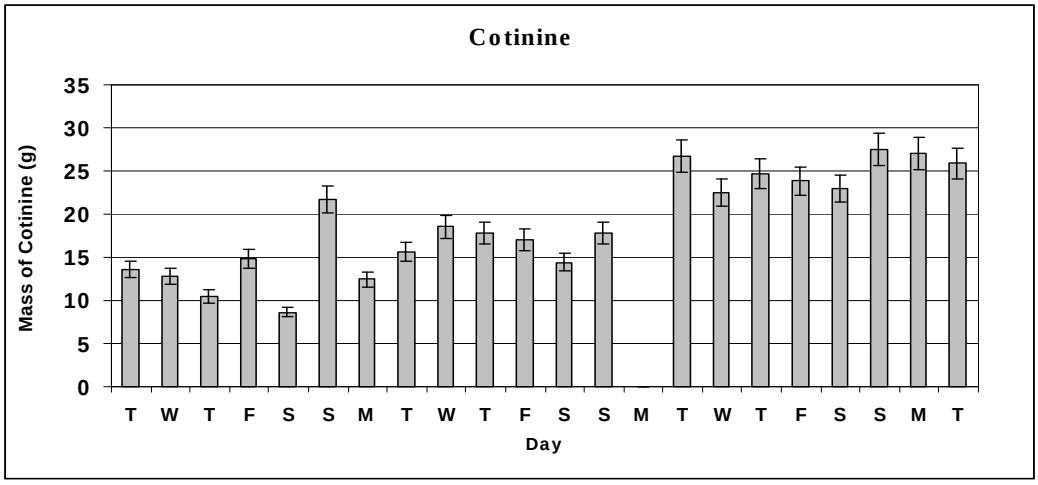


Figure 20. Mass of Cotinine (g) ± standard deviation

Caffeine, which enters wastewater via human excretion, was detected at masses ranging from 0.6 to 1.7 Kg (Figure 21). Others have measured, but not used, the data to understand possible correlation between populations and mass of caffeine consumption *(2, 11)*. Caffeine shows evenness in the second and third week, but the

first week has fluctuation, this might be due to the transit of people in the area. This transit population can have greater effect in smaller populations when large segments of population are gone (i.e., students in college towns).

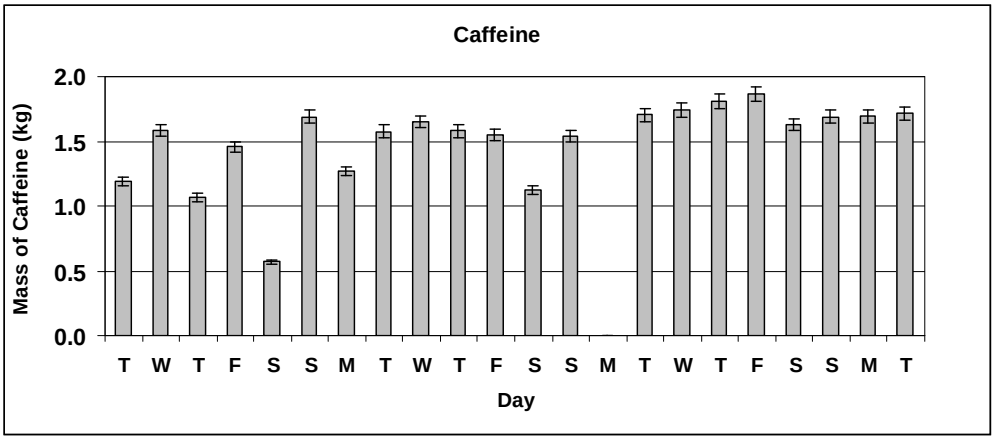


Figure 21. Mass of Caffeine (kg) ± standard deviation

The mass of creatinine ranged from 12 to 29 Kg (Figure 22). To the best of our knowledge, this is the first attempt to quantify creatinine in wastewater and to explore the use of creatinine as a more dynamic indicator of population. While creatinine concentrations in urine vary as a function of age, gender, and muscle mass *(17-19)*, creatinine is potentially a more inclusive biomarker than either cotinine or caffeine. A recent survey of 7,845 participants indicated excretion at 1.4g/L ± 0.8 g/L deviation (http://www.cdc.gov/nchs/about/major/nhanes/nhanes2007-2008/nhanes07_08.htm). Although the stability of creatinine once excreted is unknown, the samples for this study were all collected from the same WWTP, assuming that the degradation rates are similar, and the residences times are constant. The likely loss of creatinine during transit is steady and therefore may serve as an

index of population contributing to the WWTP. Creatinine vs flow day was plotted to test whether it was independent of flow (Figure 23). Results show that creatinine mass didn't follow the same trend as flow (L), therefore is assumed to be independent.

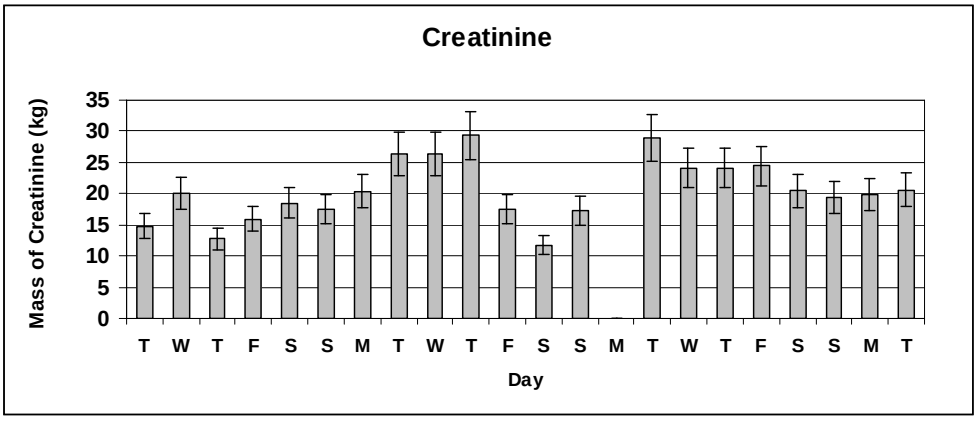


Figure 22. Mass of creatinine (kg) ± standard deviation

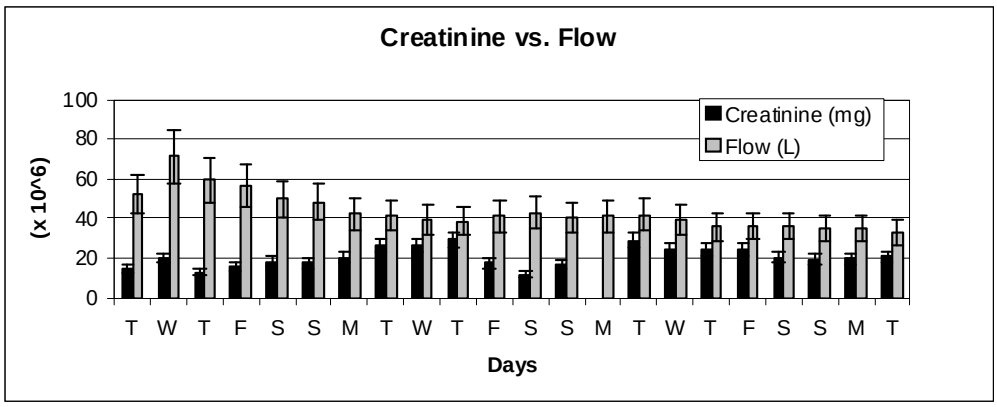


Figure 23. Creatinine (mg) vs Flow (L) ± standard deviation

BE/Creatinine ratio was plotted (Figure 24). Findings show that there is a clear trend of BE use on weekend vs. week day. This confirms previous findings which suggest the episodic used of this drug. Even though the rest of the data is not shown none of them experience real trends. Therefore, creatinine showed the potential to ratio analytes in the same way that is has done in serum.

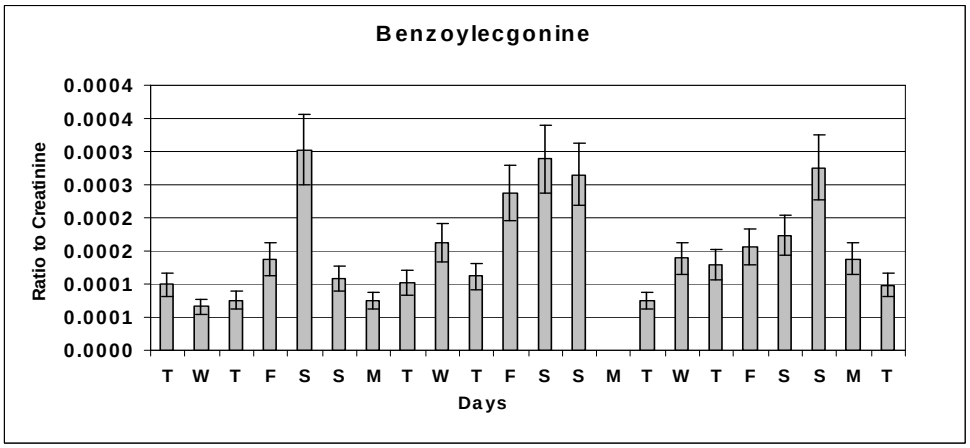


Figure 24. Ratios of benzoylecgonine to creatinine ± standard deviation for the three week sampling period

5. Conclusions

A LVI-LC-MS/MS method was developed for the analysis of psychoactive drugs, opiates, selected metabolites, and human urinary biomarkers in municipal wastewaters. The injection of large volumes (up to 1,800 μL) did not affect chromatographic behavior such as retention time and peaks. LVI demonstrated great reproducibility of retention times, peak symmetry, and good chromatographic focusing without pre-concentration or purification steps. The accuracy determination by standard addition reveals that internal standards are able to compensate for matrix effects and that concentrations can be determined directly from solvent based calibration curves. The percent recoveries of the method ranged from 60 to 150 %. IDL values ranged from 0.5 to 250 ng/L with LOQ values from f2.50 to 10 ng/L for psychoactive drugs and opiates. Human urinary biomarker LOQs ranged from 250

ng/L – 50,000 ng/L (50μL). The intra-day and within-day precision was determined to be 7 % and 12 % respectively.

The applicability application of the analytical method was evaluated by estimating community loads which are reported in mg/person/day from 24hrs. Flow – normalized composited from wastewater influent was collected for three consecutive weeks. The results show a high frequency of controlled psychoactive drugs and opiates. The results demonstrate that this method can be accurately and effectively applied to the analysis of wastewater samples and that it can be used to calculate loads and trends for a variety of psychoactive drugs, opiates and human urinary biomarkers.

6. Acknowledgments

This study was part of Oregon Health Sciences University Medical Research Fund. The authors want to thank Doug Martin from Agilent Technologies, Greg Witkop from Waters Corp., Guy Allen, Jeff Morre and Environmental Health Science Center Mass Spectrometry Facility. In addition, the authors greatly appreciated the help of Amanda Ackerman, Carl Isaacson, Carin Huset and Mindy Berge for all their aid through this research.

7. References

(1) Castiglioni, S.; Zuccato, E.; Crisci, E.; Chiabrando, C.; Fanelli, R.; Bagnati, R., Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. *Analytical Chemistry* **2006,** 78 (24), 8421 -8429.

(2) Huerta-Fontela, M.; Galceran, M. T.; Ventura, F., Ultraperformance liquid chromatography-tandem mass spectrometry analysis of stimulatory drugs of abuse in wastewater and surface waters. *Anal. Chem.* **2007,** 79, (10), 3821-3829.

(3) Hummel, D.; Loffler, D.; Fink, G.; Ternes, T. A., Simultaneous determination of psychoactive drugs and their metabolites in aqueous matrices by liquid chromatography mass spectrometry. *Environ. Sci. Technol.* **2006,** 40, (23), 7321-7328.

(4) Boleda, M. R.; Galceran, M. T.; Ventura, F., Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2007,** 1175, (1), 38-48.

(5) Postigo, C.; LopezdeAlda, M. J.; Barcelo, D., Fully Automated Determination in the Low Nanogram per Liter Level of Different Classes of Drugs of Abuse in Sewage Water by On-Line Solid-Phase Extraction-Liquid Chromatography-Electrospray-Tandem Mass Spectrometry. *Analytical Chemistry* **2008,** 80, (9), 3123-3134.

(6) Jones-Lepp, T. L.; Alvarez, D. A.; Petty, J. D.; Huckins, J. N., Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents. *Archives of Environmental Contamination and Toxicology* **2004,** 47, (4), 427-439.

(7) Kasprzyk-Hordern, B.; Dinsdale, R. M.; Guwy, A. J., Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* **2008,** 391, (4), 1293-1308.

(8) Zuccato, E.; Castiglioni, S.; Bagnati, R.; Chiabrando, C.; Grassi, P.; Fanelli, R., Illicit drugs, a novel group of environmental contaminants. *Water Research* **2008,** 42, (4-5), 961-968.

(9) Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Calamari, D.; Bagnati, R.; Schiarea, S.; Fanelli, R., Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environmental Health Perspectives* **2005,** 4, (1), 14.

(10) Bones, J.; Thomas, K. V.; Paull, B., Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals. *Journal of Environmental Monitoring* **2007,** 9, (7), 701-707.

(11) Standley, L. J.; Kaplan, L. A.; Smith, D., Molecular tracers of organic matter sources to surface water resources. *Environmental Science & Technology* **2000,** 34, (15), 3124-3130.

(12) Gardinali, P. R.; Zhao, X., Trace determination of caffeine in surface water samples by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS). *Environment International* **2002,** 28, (6), 521-528.

(13) Seiler, R. L.; Zaugg, S. D.; Thomas, J. M.; Howcroft, D. L., Caffeine and Pharmaceuticals as Indicators of Waste Water Contamination in Wells. *Ground Water* **1999,** 37, (3), 405-410.

(14) Buerge, I. J.; Poiger, T.; Mu?ller, M. D.; Buser, H. R., Combined sewer overflows to surface waters detected by the anthropogenic marker caffeine. *Environmental Science and Technology* **2006,** 40, (13), 4096-4102.

(15) Buerge, I. J.; Poiger, T.; Muller, M. D.; Buser, H. R., Combined sewer overflows to surface waters detected by the anthropogenic marker caffeine. *Environmental Science & Technology* **2006,** 40, (13), 4096-4102.

(16) Lehninger, A. L.; Cox, M. M., *Principles of Biochemistry*. W.H. Freeman: New York, 2004; p 1100.

(17) Carrieri, M.; Trevisan, A.; Bartolucci, G. B., Adjustment to concentration-dilution of spot urine samples: correlation between specific gravity and creatinine. *Int. Arch. Occup. Environ. Health* **2001,** 74, 63-67.

(18) Heavner, D. L.; Morgan, W. T.; Sears, S. B.; Richardson, J. D.; Byrd, G. D.; Ogden, M. W., Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines. *Journal of Pharmaceutical and Biomedical Analysis* **2006,** 40, (4), 928-942.

(19) Newman, D. J.; Pugia, M. J.; Lott, J. A.; Wallace, J. F.; Hiar, A. M., Urinary protein and albumin excretion corrected by creatinine and specific gravity. *Clinica Chimica Acta* **2000,** 294, (1-2), 139-155.

(20) Hennion, M. C.; Pichon, V., Solid-phase extraction of polar organic pollutants from water. *Environmental Science and Technology* **1994,** 28, (13), A576-A583.

(21) Schultz, M. M.; Barofsky, D. F.; Field, J. A., Quantitative determination of fluorinated alkyl substances by large-volume-injection liquid chromatography tandem mass spectrometry-characterization of municipal wastewaters. *Environmental Science and Technology* **2006,** 40, (1), 289-295.

(22) Broquaire, M.; Guinebault, P. R., Large Volume Injection of biological samples dissolved in a non-eluting solvent - a way to increase sensitivity and a means of automating drug determinations using HHPLC. *Journal of Liquid Chromatography* **1981,** 4, (11), 2039-2061.

(23) Guinebault, P.; Broquaire, M., Large-volume injection of samples dissolvedin a non-eluting solvent- application to the determination of antipyrene using normal-phase high-performance liquid chromatography *Journal of Chromatography* **1981,** 217, (NOV), 509-522.

(24) Cappiello, A.; Famiglini, G.; Berloni, A., Large volume injection of acidic pesticides by reversed-phase micro high-performance liquid chromatography. *Journal of Chromatography A* **1997,** 768, (2), 215-222.

(25) Silver, M. R.; Trosper, T. D.; Gould, M. R.; Dickinson, J. E.; Desotelle, G. A., The use of large volume injections for the isocratic separation of phenylthiohydantoin amino acids by microbore liquid chromatography. *Journal of Liquid Chromatography* **1984,** 7, 559-568.

(26) Mefford, I. N., Chromatographic approaches to signal and selectivity enhancement for determination of biogenic-amines by liquid chromatography with amperometric detection *Life Sciences* **1987,** 41, (7), 893-896.

(27) Hogenboom, A. C.; Hofman, M. P.; Kok, S. J.; Niessen, W. M. A.; Brinkman, U. A. T., Determination of pesticides in vegetables using large-volume injection column liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A* **2000,** 892, (1-2), 379-390.

(28) Rosales-Conrado, N.; Leon-Gonzalez, M. E.; Perez-Arribas, L. V.; Polo-Diez, L. M., Determination of chlorophenoxy acid herbicides and their esters in soil by capillary high performance liquid chromatography with ultraviolet detection, using large volume injection and temperature gradient. *Analytica Chimica Acta* **2002,** 470, (2), 147-154.

(29) Rybar, I.; Gora, R.; Hutta, M., Method of fast trace microanalysis of the chiral pesticides epoxiconazole and novaluron in soil samples using off-line flow-through extraction and on-column direct large volume injection in reversed-phase high performance liquid chromatography. *Journal of Separation Science* **2007,** 30, (18), 3164-3173.

(30) Chalanyova, M.; Paulechova, M.; Hutta, M., Method of analysis of a selected group of pyrethroids in soil samples using off-line flow-through extraction and on-column direct large-volume injection in reversed phase high performance liquid chromatography. *Journal of Separation Science* **2006,** 29, (14), 2149-2157.

(31) Parrilla Vázquez, P.; Gil García, M. D.; Barranco Martínez, D.; Martí Galera, M., Application of coupled-column liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection to the determination of pyrethroid insecticides in vegetable samples. *Analytical & Bioanalytical Chemistry* **2005,** 381, 1217-1225.

(32) Ingelse, B. A.; van Dam, R. C. J.; Vreeken, R. J.; Mol, H. G. J.; Steijger, O. M., Determination of polar organophosphorus pesticides in aqueous samples by direct injection using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2001,** 918, (1), 67-78.

(33) Kiso, Y.; Li, H.; Shigetoh, K.; Kitao, T.; Jinno, K., Pesticide analysis by high-performance liquid chromatography using the direct injection method. *Journal of Chromatography A* **1996,** 733, (1-2), 259-265.

(34) Hutta, M.; Rybár, I.; Chalányová, M., Liquid chromatographic method development for determination of fungicide epoxiconazole enantiomers by achiral and chiral column switching technique in water and soil. *Journal of Chromatography A* **2002,** 959, (1-2), 143-152.

(35) Sancho, J. V.; Hernandez, F.; Lopez, F. J.; Hogendoorn, E. A.; Dijkman, E.; vanZoonen, P., Rapid determination of glufosinate, glyphosate and aminomethylphosphonic acid in environmental water samples using precolumn fluorogenic labeling and coupled-column liquid chromatography. *Journal of Chromatography A* **1996,** 737, (1), 75-83.

(36) Hidalgo, C.; Sancho, J. V.; Hernandez, F., Trace determination of triazine herbicides by means of coupled-column liquid chromatography and large volume injection. *Analytica Chimica Acta* **1997,** 338, (3), 223-229.

(37) Hernandez, F.; Hidalgo, C.; Sancho, J. V.; Lopez, F. J., New method for the rapid determination of triazine herbicides and some of their main metabolites in water by using coupled-column liquid chromatography and large volume injection. *Journal of Chromatography A* **1997,** 778, (1-2), 171-181.

(38) Huset, A. A.; Chiaia, A.; Barofsky, D. F.; Jonkers, N.; Kohler, H.-P.; Ort, C.; Giger, W.; Field, J., Occurrence and mass flows of fluorochemicals in the Glatt Valley watershed, Switzerland. *Environ. Sci. Technol.* **ASAP**.

(39) Isaacson, C. W.; Usenko, C. Y.; Tanguay, R. L.; Field, J. A., Quantification of fullerenes by LC/ESI-MS and its application to in vivo toxicity assays. *Analytical Chemistry* **2007,** 79, (23), 9091-9097.

(40) Harris, D. C., *Quantitative Chemical Analysis*. W. Freeman and Co.: New York, 1991; p 782.

(41) Hutta, M.; Chalanyova, M.; Halko, R.; Gora, R.; Rybar, I.; Pajchl, M.; Dokupilova, S., New approach to large-volume injection in reversed-phase high performance liquid chromatography: Determination of atrazine and hydroxyatrazine in soil samples. *Journal of Separation Science* **2006,** 29, (13), 1977-1987.

(42) Thurman, E. M.; Mills, M. S., *Solid Phase Extraction: Principles and Practice*. John Wiley & Sons: Toronto, 1998; p 344.

(43) Miller, J. M., *Chromatography Concepts and Contrasts*. John Wiley & Sons: Hoboken, 2005; p 490.

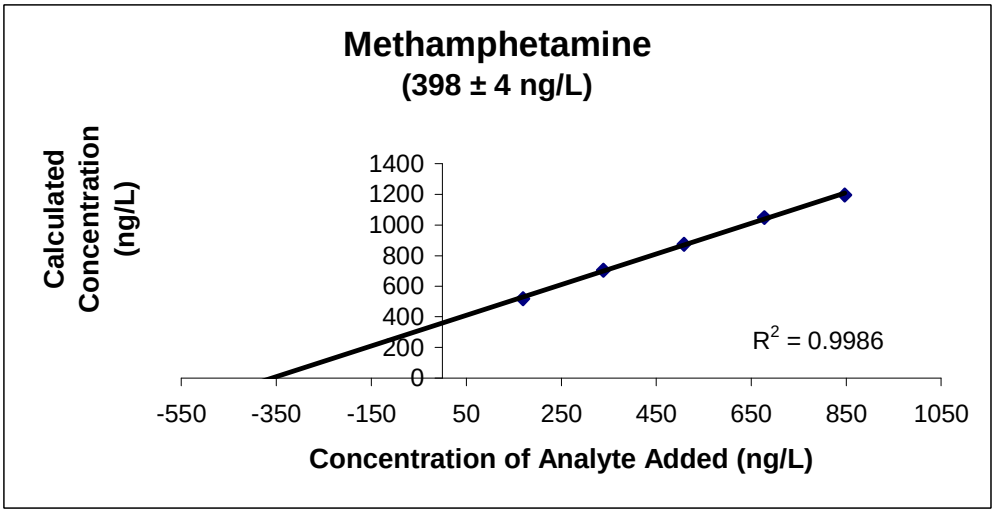
(44) Zuccato, E.; Castiglioni, S.; Fanelli, R., Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. *Journal of Hazardous Materials* **2005,** 122, (3), 205-209.

(45) Baselt, R. C., *Disposition of Toxic Drugs and Chemicals in Man*. Biomedical Publications: Davis, CA, 1982.

(46) Cone, E.; Sampson-Cone, A.; Darwin, W.; Huestis, M.; Oyler, J., Urine testing for cocaine abuse: metabolic and excretion patterns following different routes of administration and methods for detection of false-negative results. *Journal of Analytical Toxicology* **2003,** 27, (7), 386-401.

Appendices

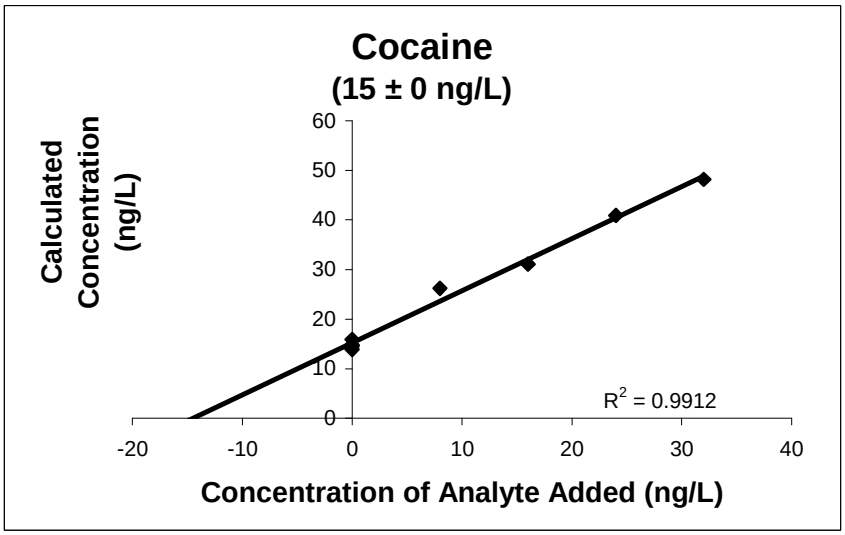
Appendix A: Standard Addition Figures for Large Volume (1,800 μL) Injection HPLC/MS/MS for the Quantitative Determination of Illicit Drugs and Human Urinary Biomarkers in Municipal Wastewater



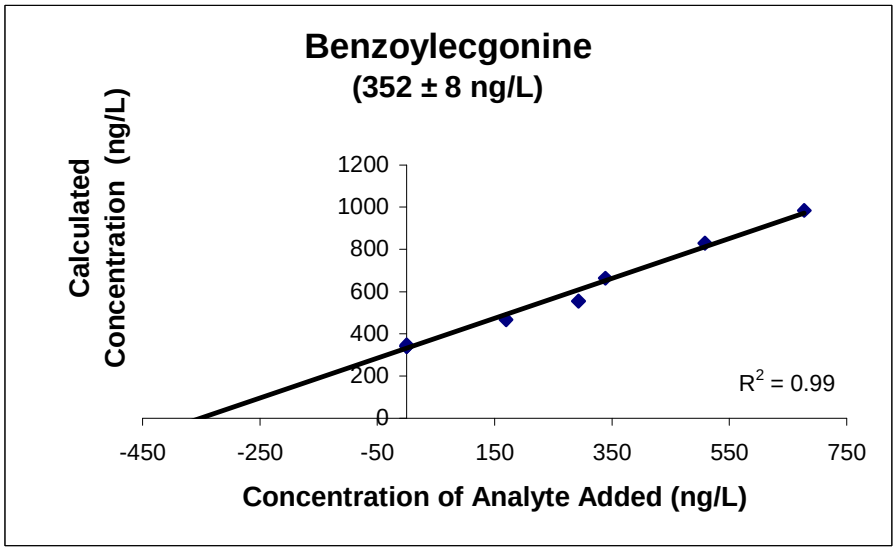
Appendix A Figure 1 Methamphetamine

Appendix A Figure 2 Amphetamine

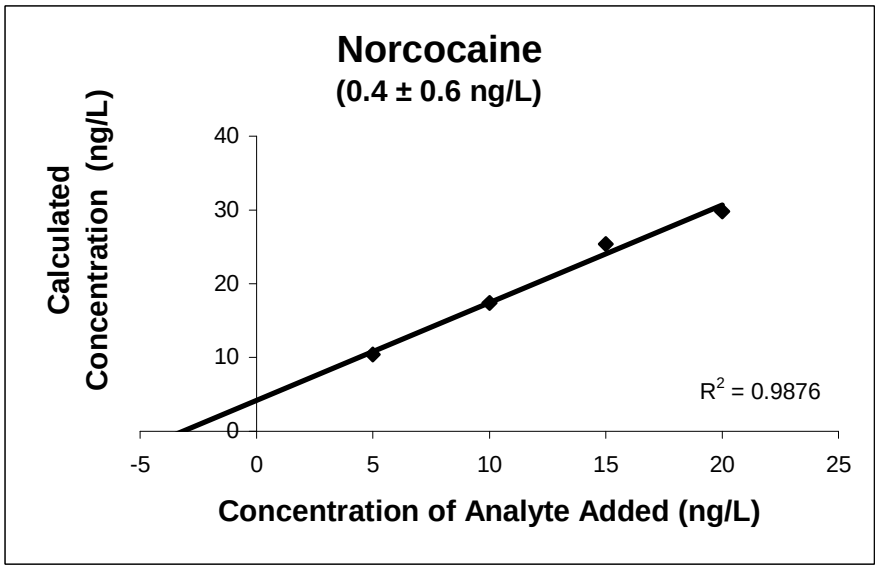
Appendix A Figure 3 Ephedrine



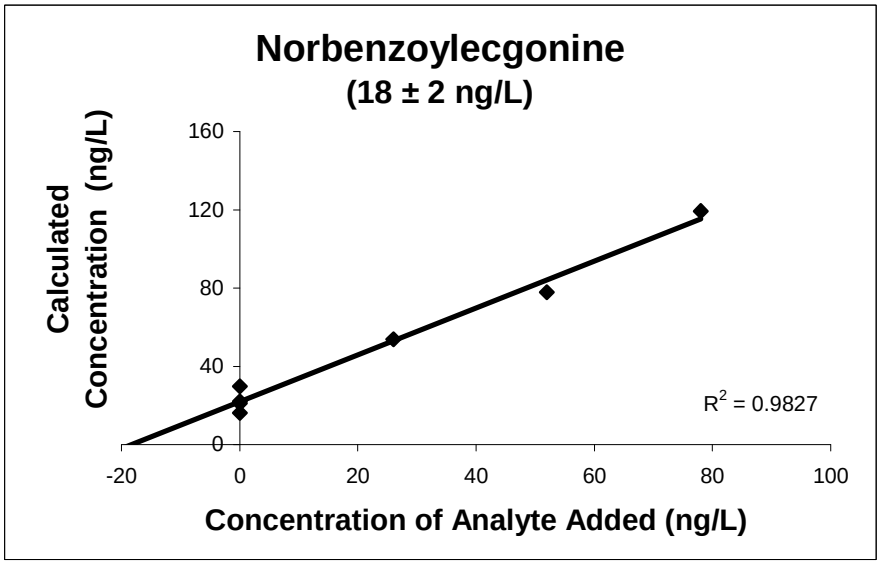
Appendix A Figure 4 Cocaine



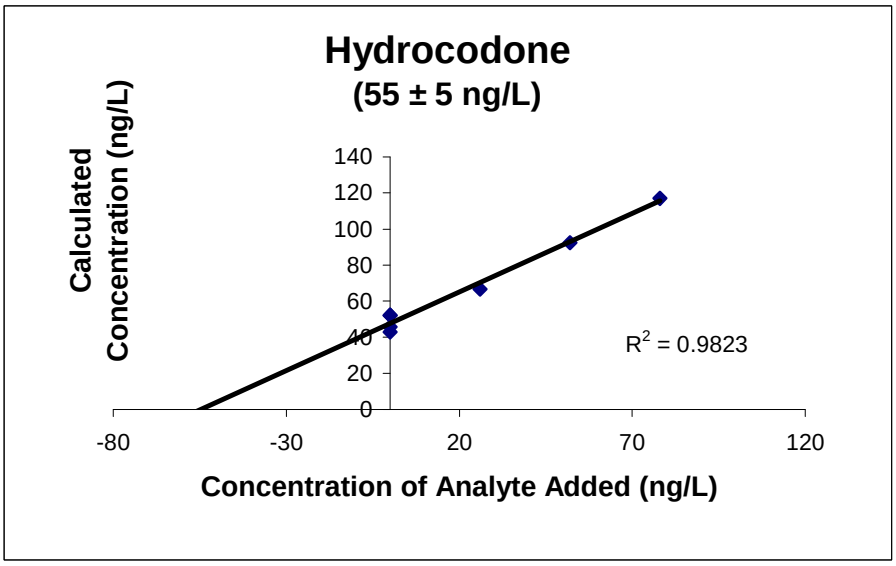
Appendix A Figure 5 Benzoylecgonine



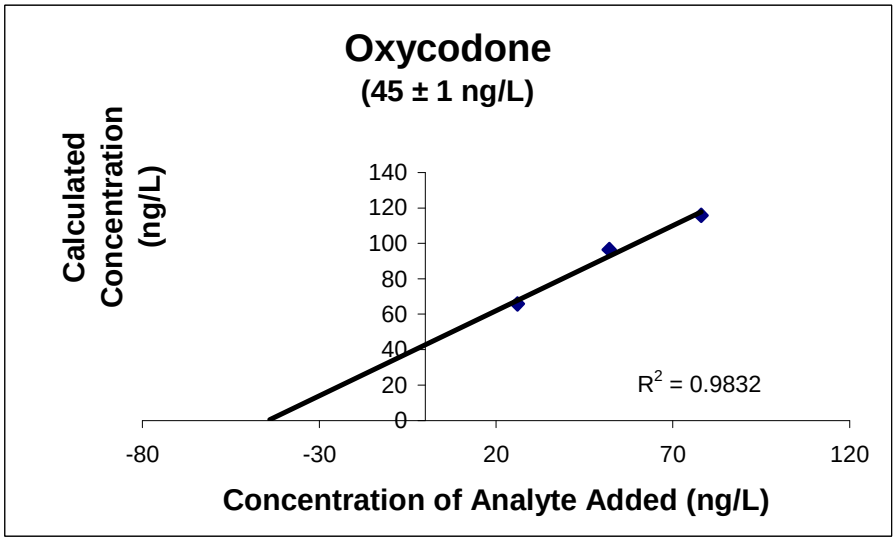
Appendix A Figure 6 Norcocaine



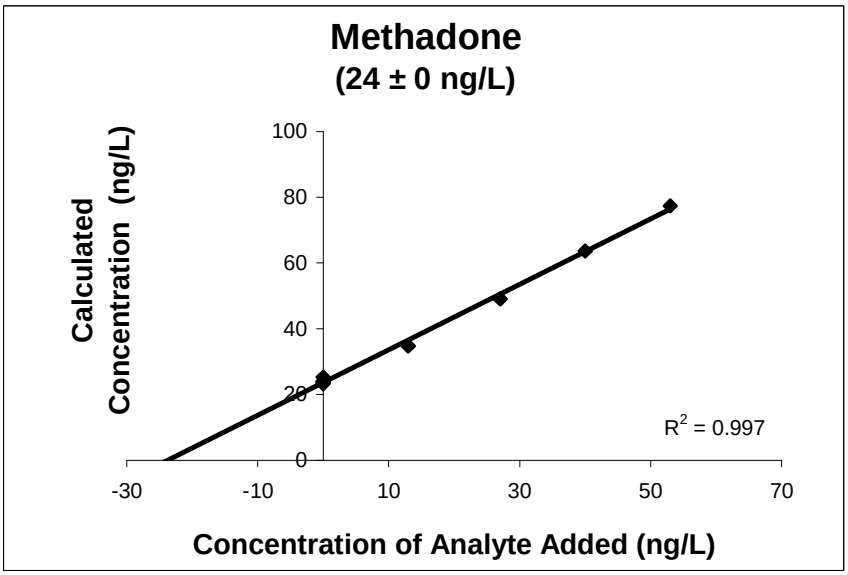
Appendix A Figure 7 Norbenzoylecgonine



Appendix A Figure 8 Hydrocodone

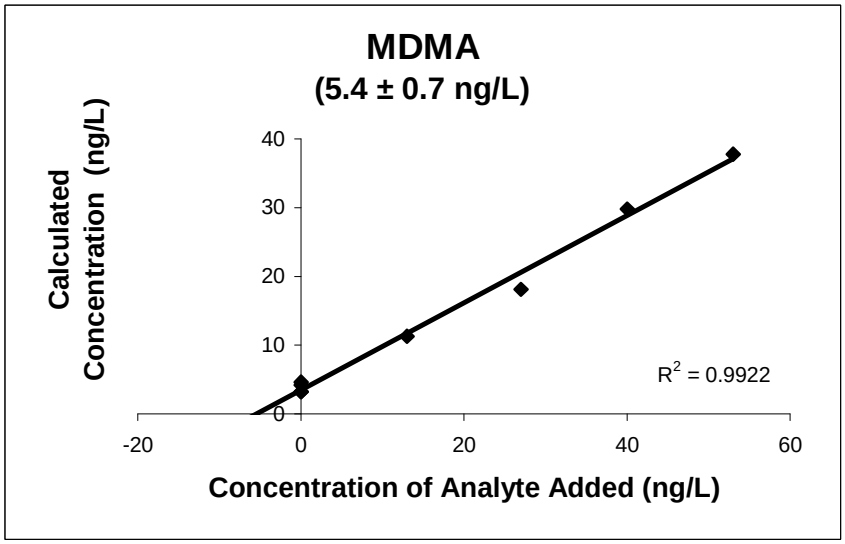


Appendix A Figure 9 Oxycodone

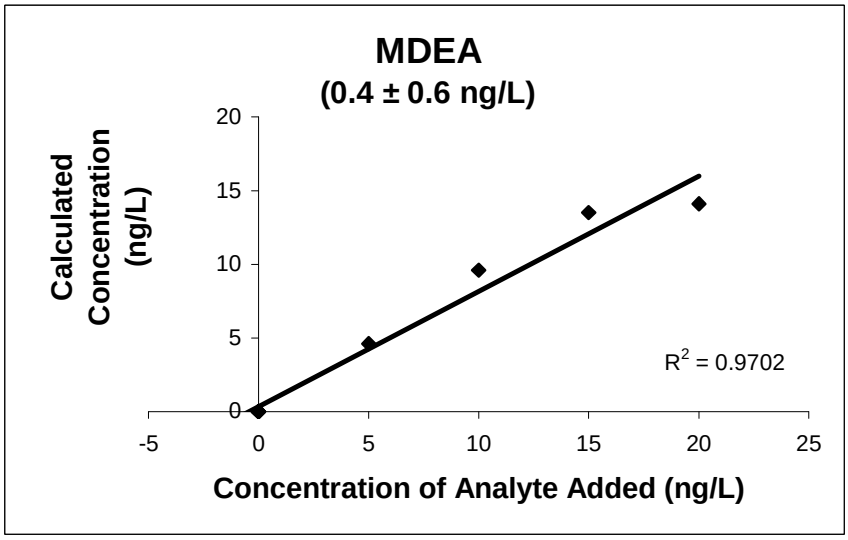


Appendix A Figure 10 Methadone

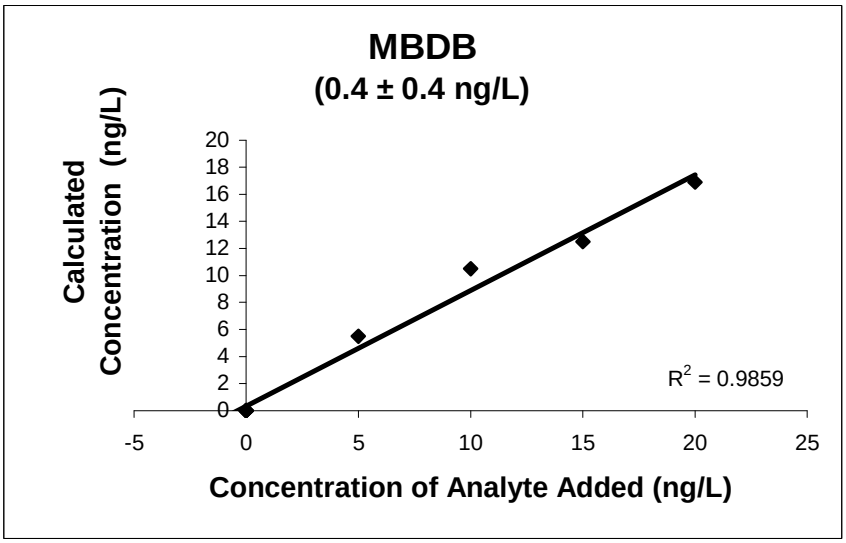
Appendix A Figure 11 MDA



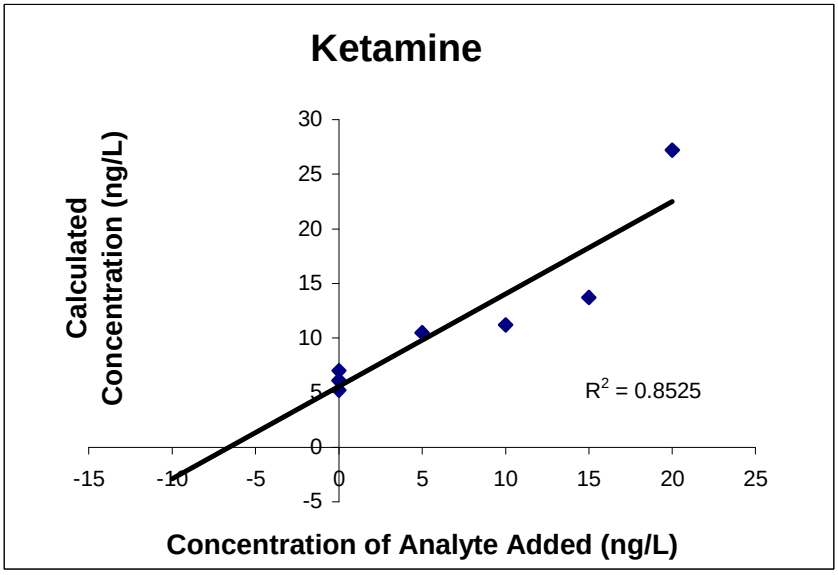
Appendix A Figure 12 MDMA



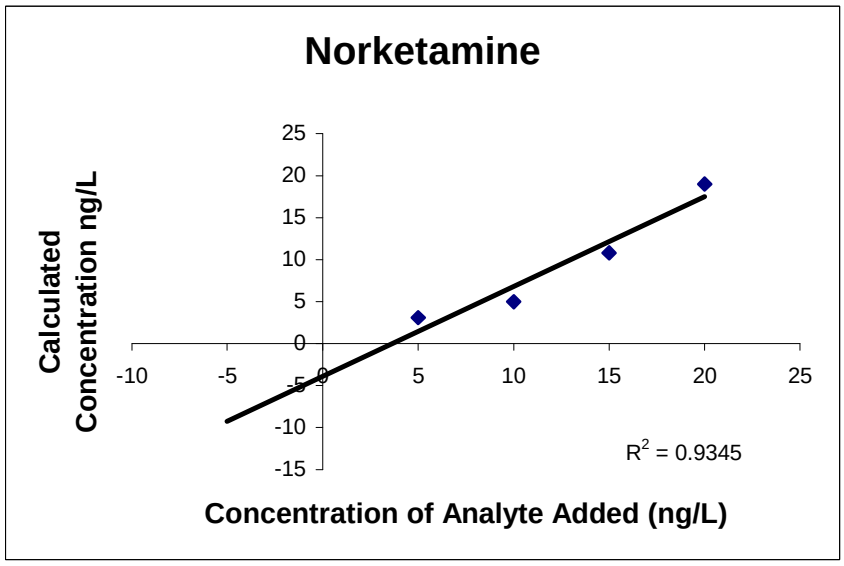
Appendix A Figure 13 MDEA



Appendix A Figure 14 MBDB

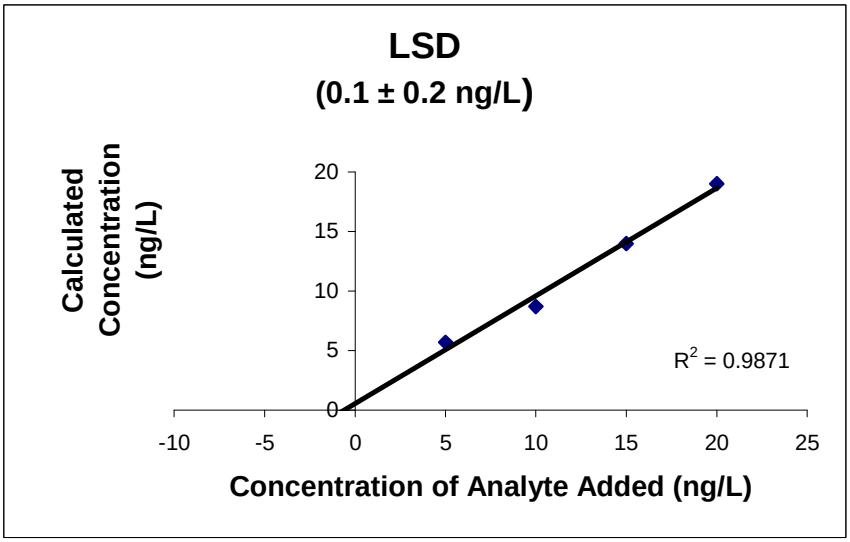


Appendix A Figure 15 Ketamine

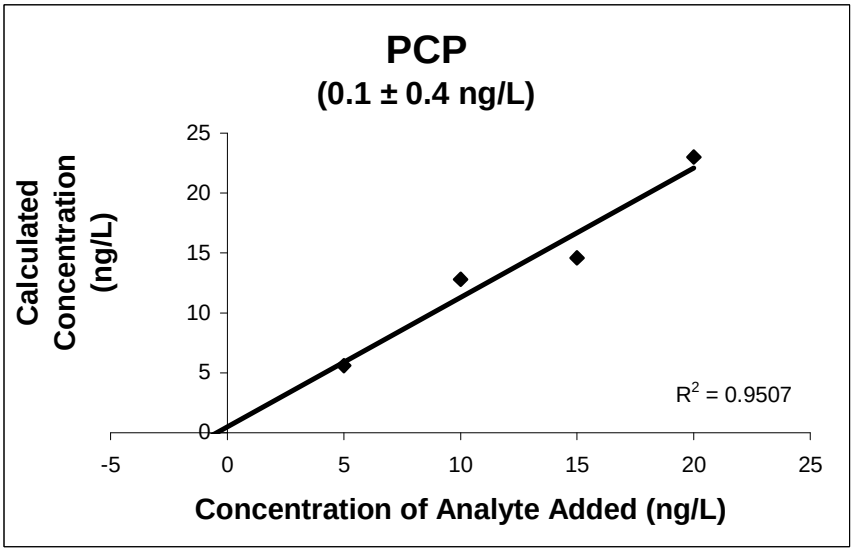


Appendix A Figure 16 Norketamine

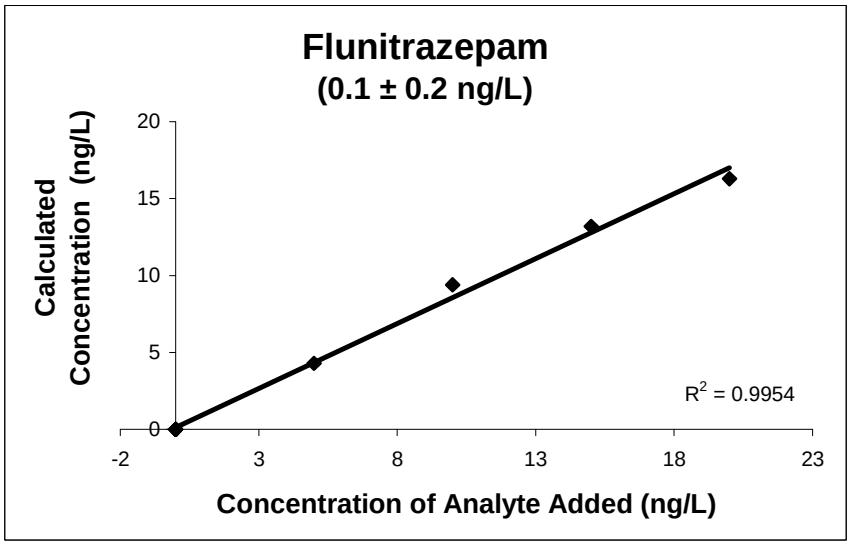
Appendix A Figure 17 2-oxo-3-hydroxy-LSD



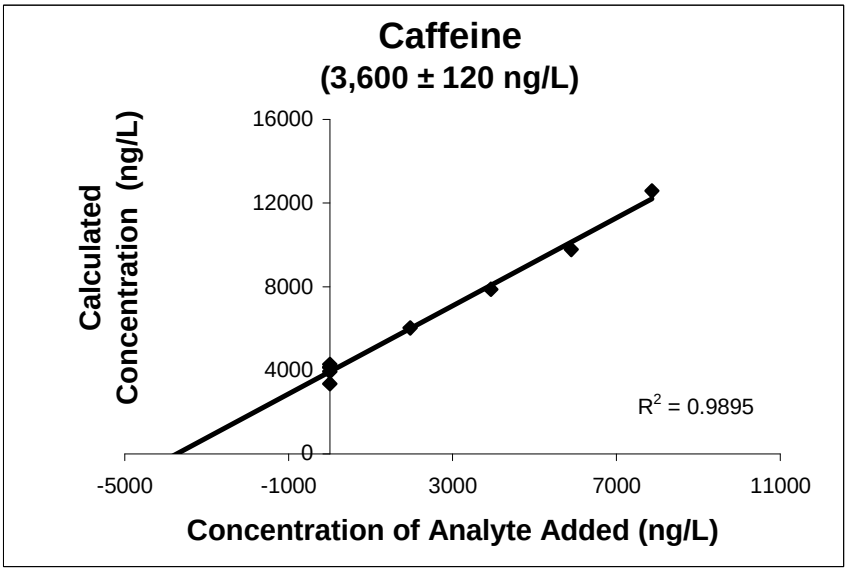
Appendix A Figure 18 LSD



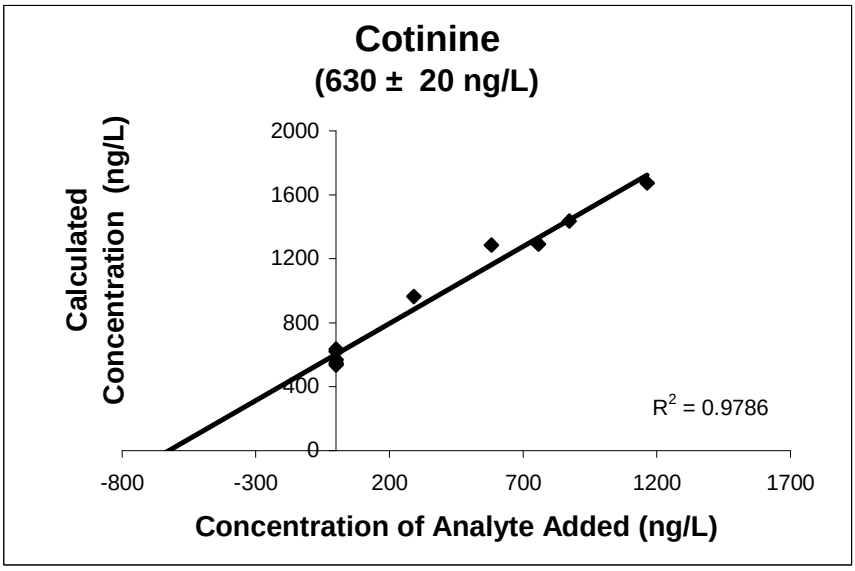
Appendix A Figure 19 PCP



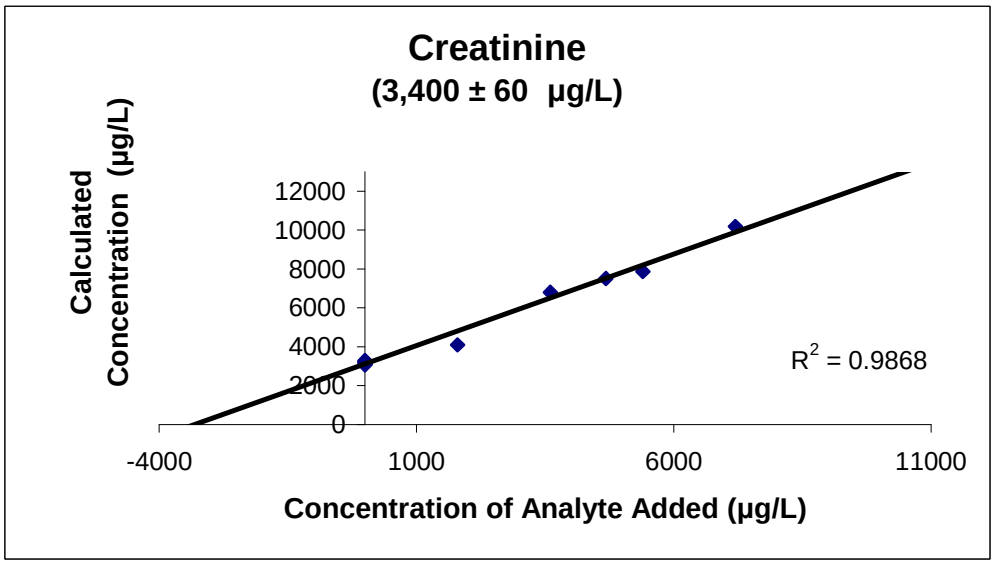
Appendix A Figure 20 Flunitrazepam



Appendix A Figure 21 Caffeine

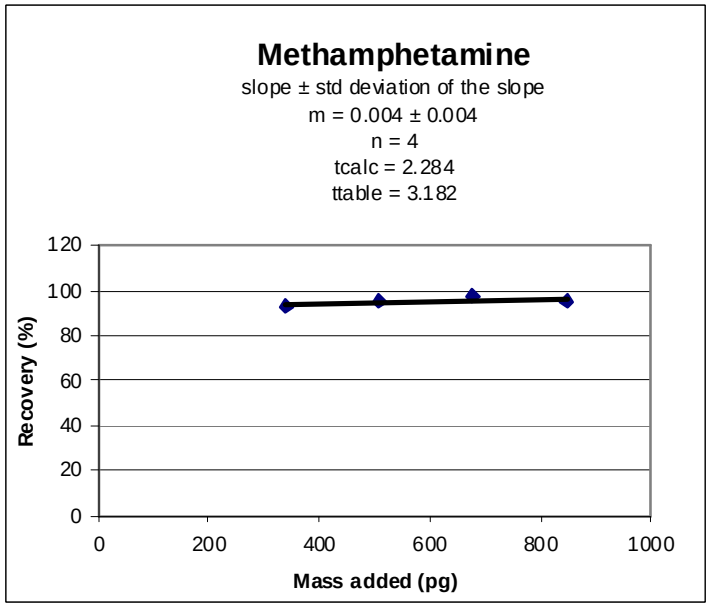


Appendix A Figure 22 Cotinine



Appendix A Figure 23 Creatinine

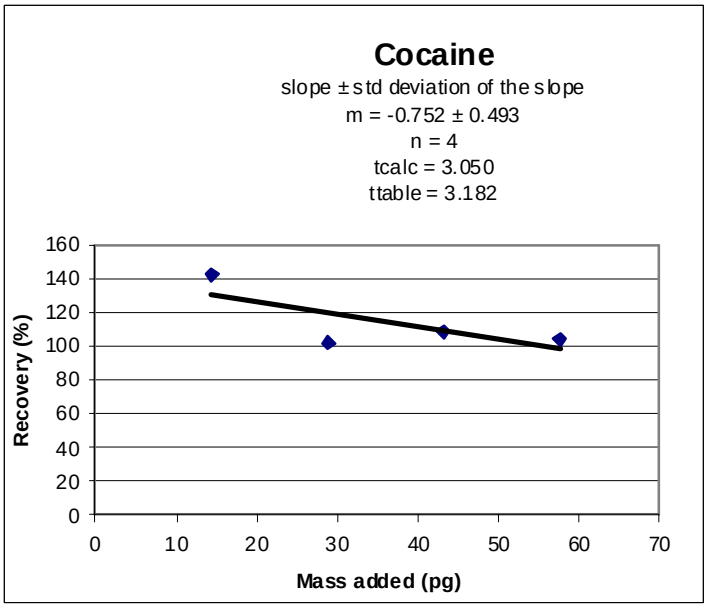
Appendix B: Percent Recoveries Figures for Large Volume (1,800 μL) Injection HPLC/MS/MS for the Quantitative Determination of Illicit Drugs and Human Urinary Biomarkers in Municipal Wastewater



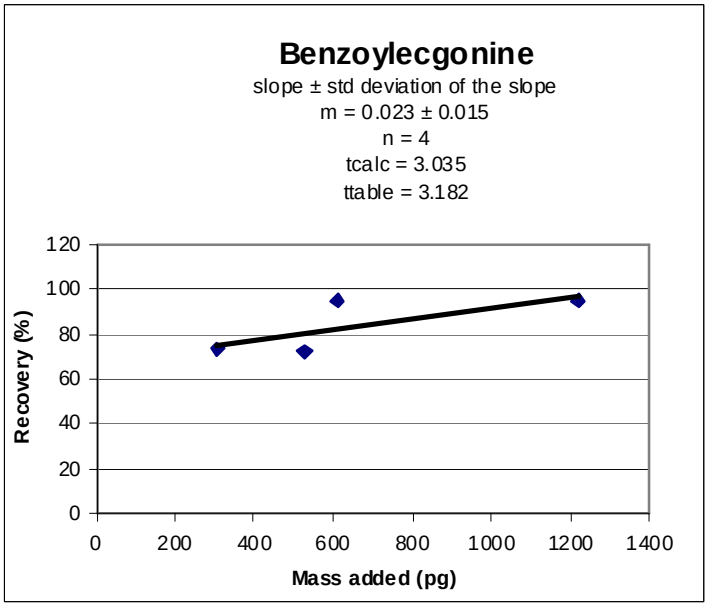
Appendix B Figure 1 Methamphetamine

Appendix B Figure 2 Amphetamine

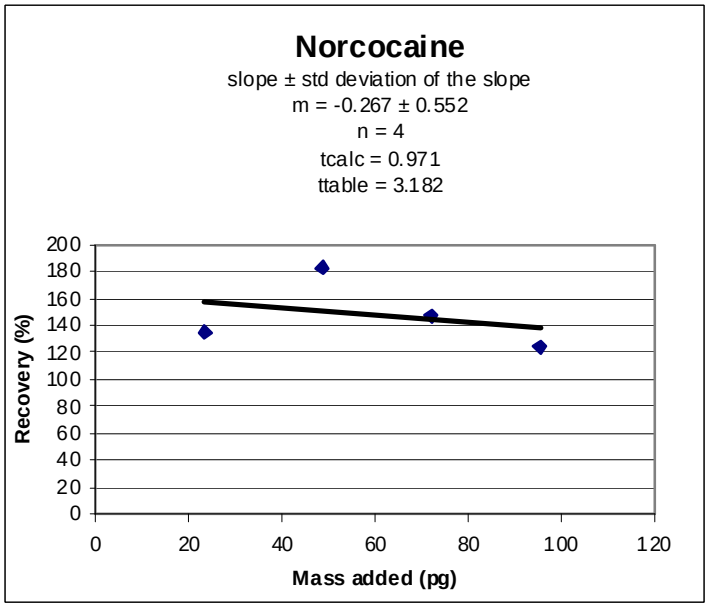
Appendix B Figure 3 Ephedrine



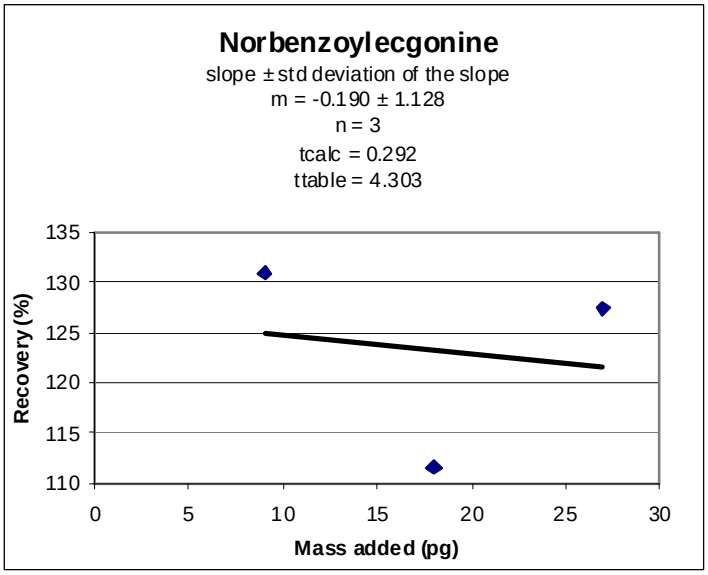
Appendix B Figure 4 Cocaine



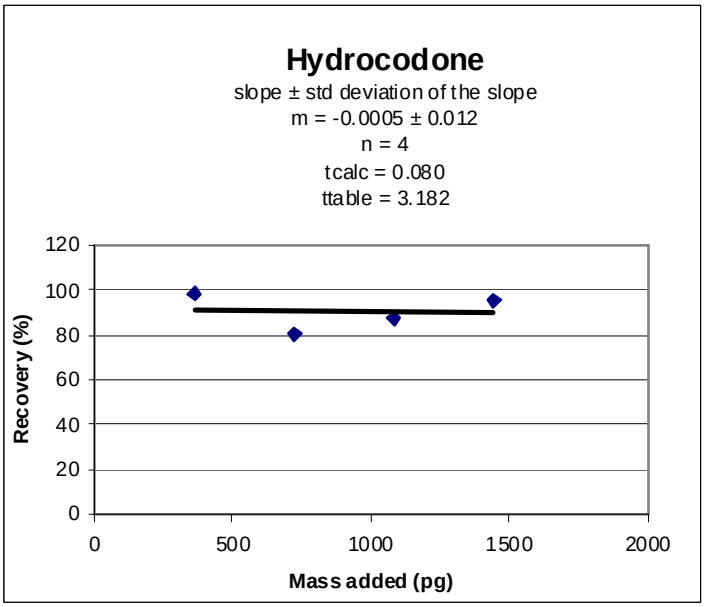
Appendix B Figure 5 Benzoylecgonine



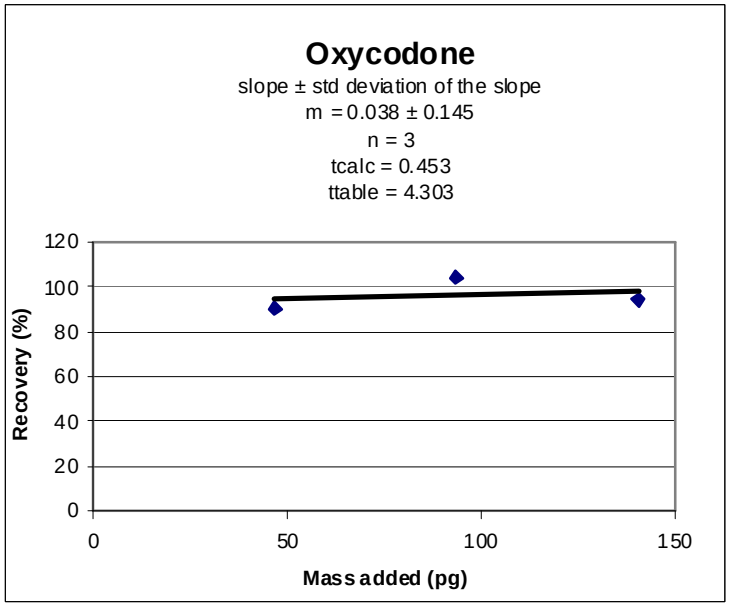
Appendix B Figure 6 Norcocaine



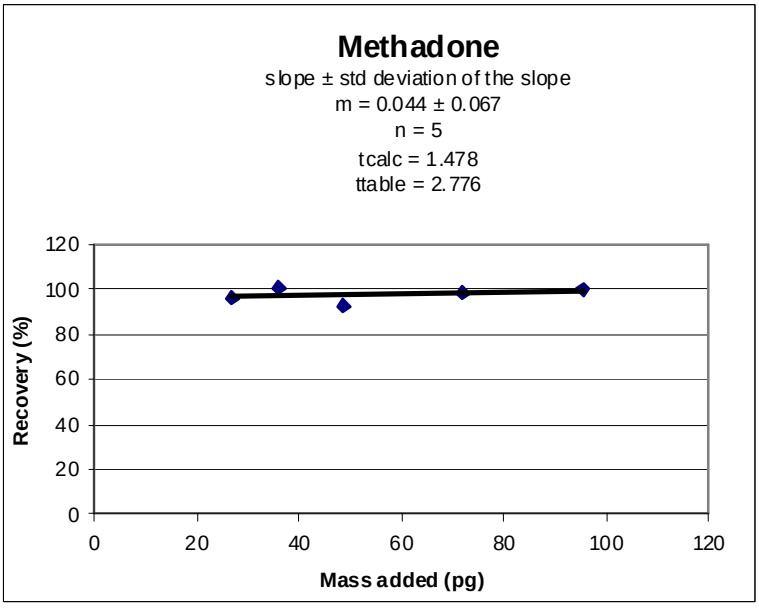
Appendix B Figure 7 Norbenzoylecgonine



Appendix B Figure 8 Hydrocodone

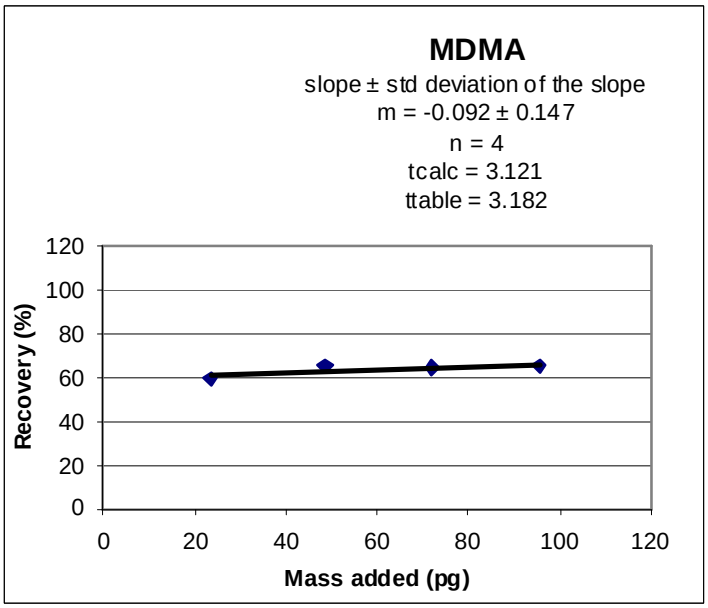


Appendix B Figure 9 Oxycodone

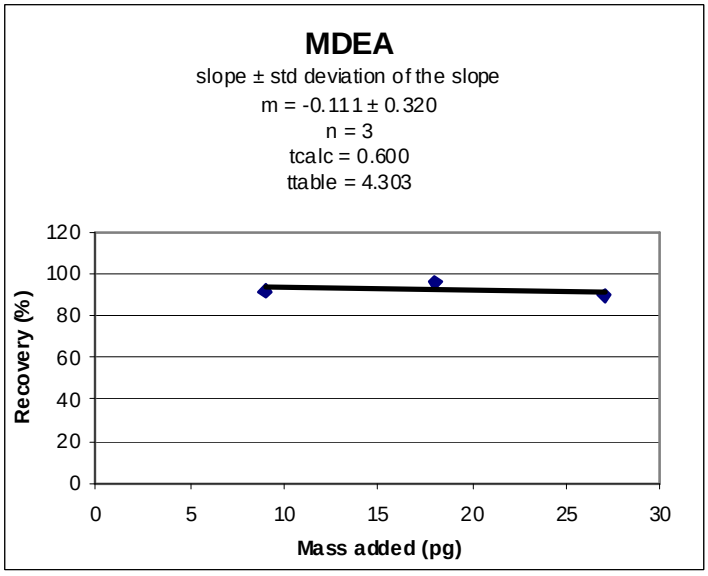


Appendix B Figure 10 Methadone

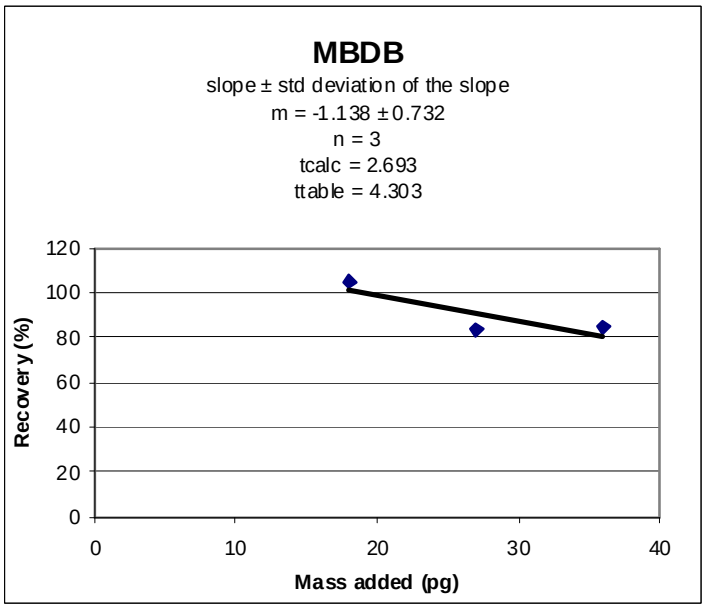
Appendix B Figure 11 MDA



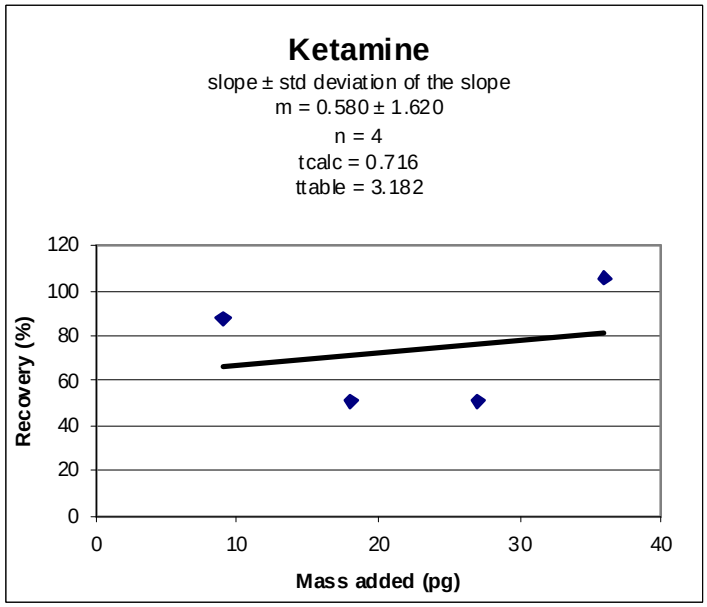
Appendix B Figure 12 MDMA



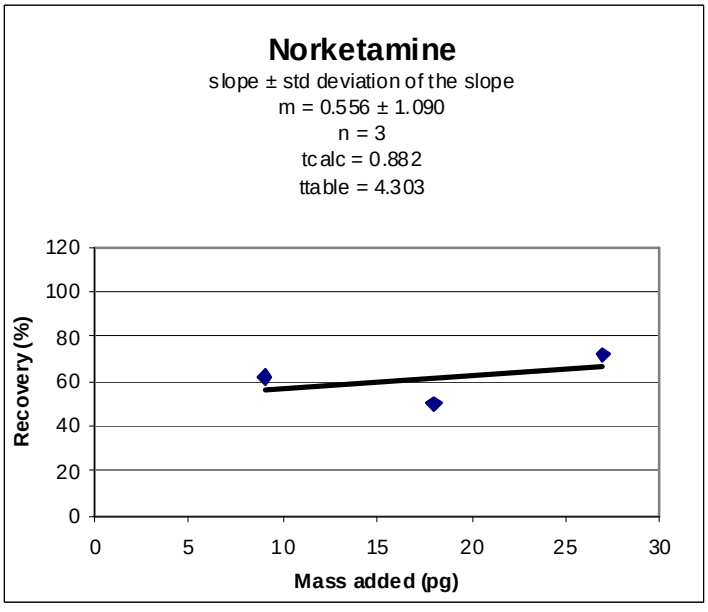
Appendix B Figure 13 MDEA



Appendix B Figure 14 MBDB

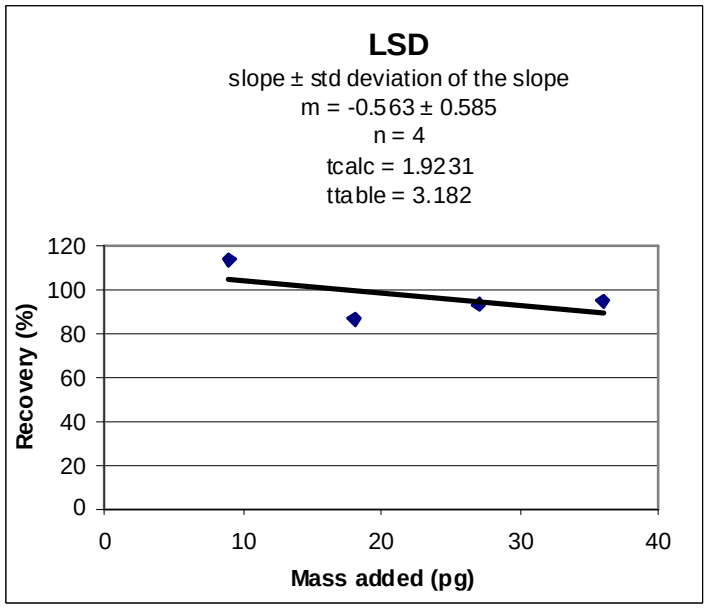


Appendix B Figure 15 Ketamine

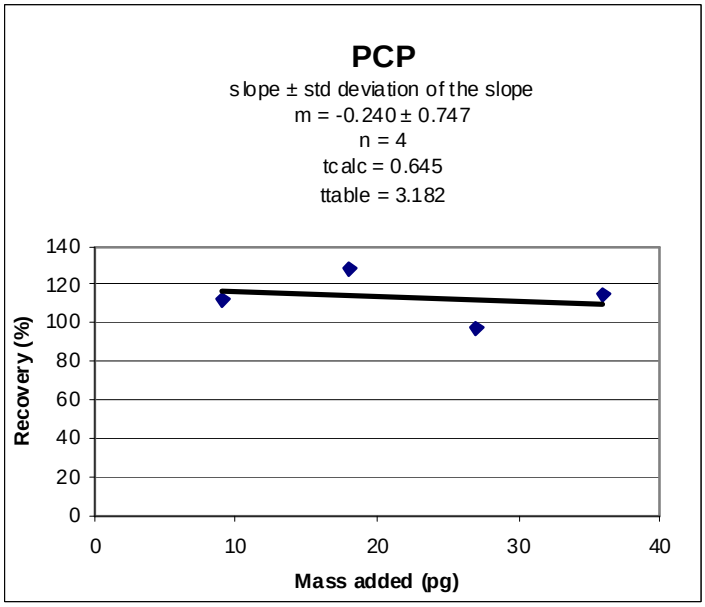


Appendix B Figure 16 Norketamine

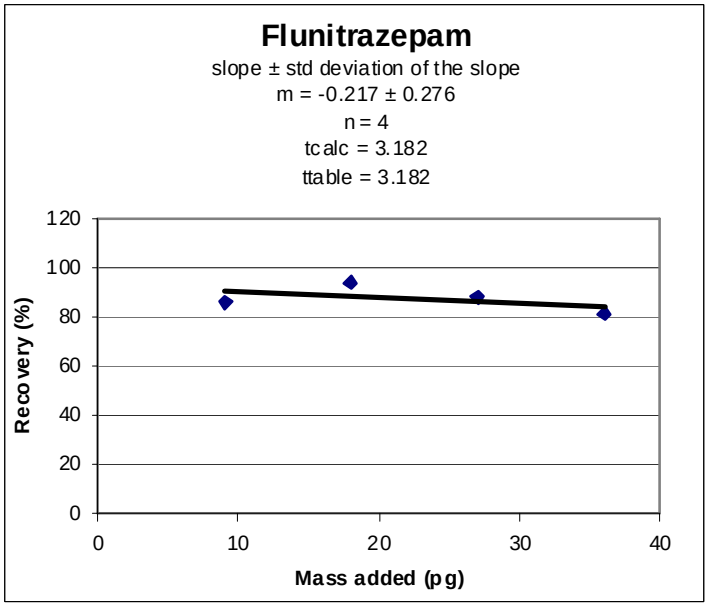
Appendix B Figure 17 2-Oxo-hydroxy- LSD



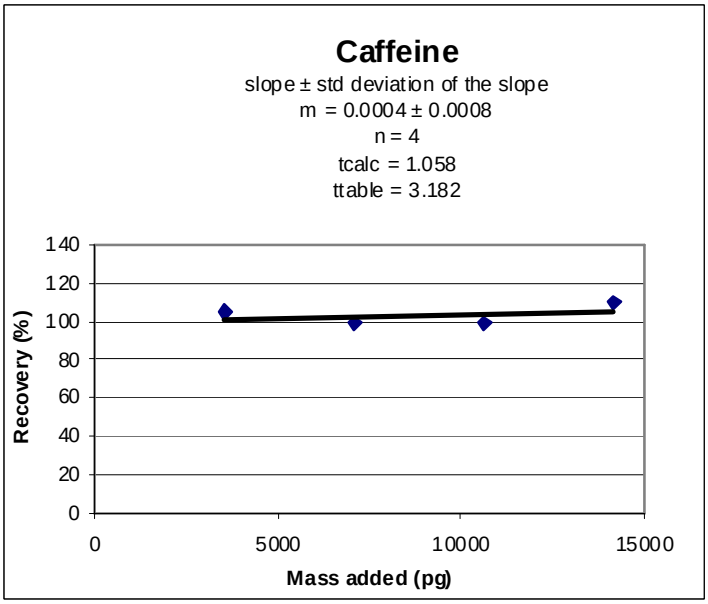
Appendix B Figure 18 LSD



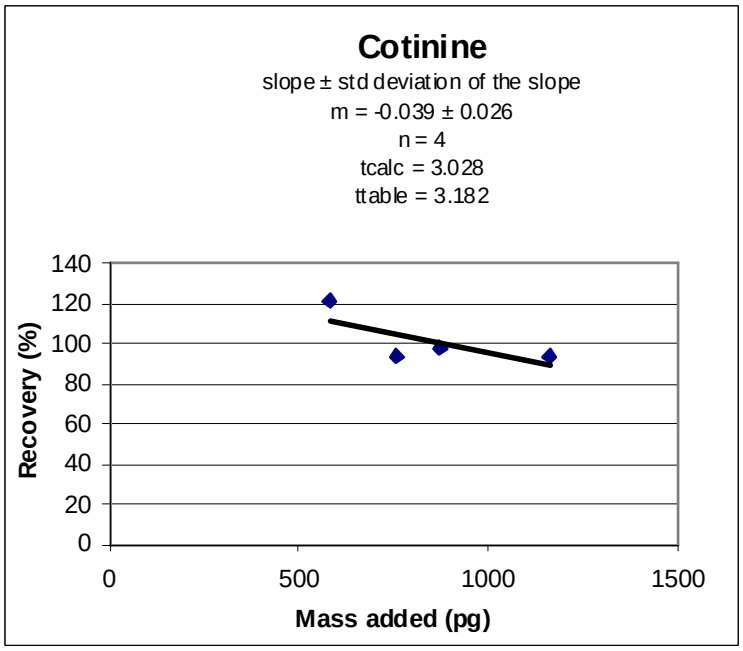
Appendix B Figure 19 PCP



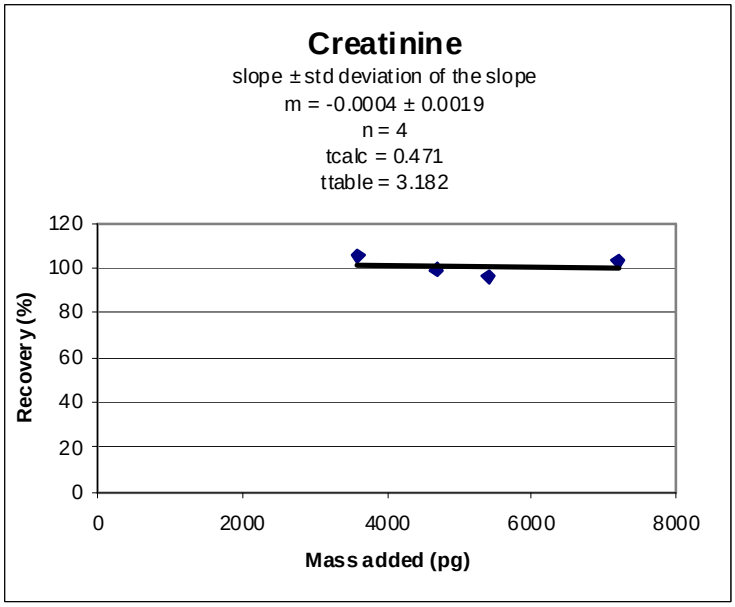
Appendix B Figure 20 Flunitrazepam



Appendix B Figure 21 Caffeine



Appendix B Figure 22 Cotinine



Appendix B Figure 23 Creatinine