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ANALYSIS OF ILLICIT COMPOUNDS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY IN WASTEWATER

BY

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I Hendrik Jacobus Viviers declare that the dissertation, which I hereby submit for the degree M.Sc Analytical Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date:

SUMMARY

The occurrence in wastewater of pharmaceuticals, drugs of abuse, and the metabolites produced through the use of such compounds, is becoming a question of concern. The recreational abuse of illicit compounds in South Africa has increased tremendously over the past years and is currently a serious problem among the youth. Data on the use of illicit drugs and the amounts consumed is limited to information coming from law enforcement agencies, rehabilitation centres, and self-reported cases. Thus, an objective means of measuring the actual drug use of a specific geographical area needs to be developed. In this study, a method was developed to measure trace amounts of drug metabolites, including 11-nor-9-carboxy-Δ9-THC, benzoylecgonine, codeine, morphine, and 6 monoacetyl morphine in wastewater. The Daspoort treatment plant—a facility serving the Pretoria West and the Hatfield areas—was selected. An appropriate sampling procedure and sample pre-treatment was employed to yield the best resolution and separation possible for the selected analytes. Sample pre-treatment involved the derivatization of analytes with methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA), pentafluoropropanol (PFPOH) and propionic anhydride (PA), respectively. The samples were prepared in accordance with the method of standard addition. A GCxGC/TOF-MS analysis was performed and a weekly concentration profile for each analyte was produced. The measured concentration obtained for each analyte was corrected for flow to ensure accurate estimation of the total weight of parent compound per day. The total parent compound weight measured confirms that the abuse of illegal substances is occurring in the population. The results obtained in the study provide further evidence for the fact that drug abuse in the Daspoort area must be urgently addressed.

OPSOMMING

Die voorkoms van medisyne, dwelms en hul metaboliete in afvalwater is steeds kommerwekkend. Die misbruik van onwettige middels in Suid-Afrika het geweldig toegeneem oor die afgelope paar jaar en is 'n ernstige probleem onder die jeug in 'n sosiale omgewing. Inligting oor die gebruik van onwettige dwelms is sterk afhanklik van wetstoepassingsagentskappe, inligting van rehabilitasiesentrums en self-gerapporteerde gevalle. Metodes word benodig om ‘n objektiewe opname van die werklike dwelmgebruik van ‘n geografiese area te kan doen. Tydens hierdie studie is 'n metode ontwikkel om spoorvlakke van dwelm-metaboliete in afvalwater te meet. Hierdie metaboliete sluit in: 11-nor-9-karboksie-Δ9-THC, bensoïelecgonine, kodeïen, morfien en 6 monoasetielmorfien. Die Daspoort Waterwerke Fasiliteit was gekies en bedien die Pretoria-Wes en die Hatfield metropolitaanse areas. Die mees toepaslike monsterneming asook monster-behandeling vir die geselekteerde analiete, is uitgevoer om die beste instrumentele analise moontlik te lewer. Monster behandeling behels die derivatisering van analiete met Metiel-N-tert-butieldimetielsilieltrifluoroasetamied (MTBSTFA), Pentafluoropropanol (PFPOH) en propionsuuranhidried (PA). Die monsters is geanaliseer in ooreenstemming met die metode van standaard addissie. 'n GCxGC / TOF-MS analise is uitgevoer en 'n weeklikse konsentrasie-profiel vir elke analiet is gevind. Die konsentrasie vir elke analiet is aangepas vir vloei om sodoende ‘n akkurate skatting van die totale massa van elke dwelm per dag te bekom. As daar na die resultate gekyk word, is die misbruik van onwettige middels kommerwekkend. Hierdie studie bevestig die erns waarmee die misbruik van dwelms in die Daspoort-area aangespreek moet word.

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LIST OF ABBREVIATIONS

%RSD - Percentage relative standard deviation
Δ^9-THC - Delta 9 Tetrahydrocannabinol
6MAM - 6 monoacetyl morphine
AC - Alternating current
Acn - Acetonitrile
ANOVA - Analysis of variance
ARV - Antiretroviral
CE1 - Carboxyl esterase 1
DC - Direct current
DCM - Dichloromethane
DMAP - Dimethylaminopyridine
EI - Electron impact
Eq - Equation
EtAc - Ethyl acetate
eV - Electron Volt
GC - Gas chromatography
GCxGC/TOF-MS – 2-dimensional gas chromatography coupled with time-of-flight mass spectrometry
HCl - Hydrochloric acid
Hex - Hexane
Isoprop - Isopropanol
IUPAC - International Union of Pure and Applied Chemistry
K_a - Acid dissociation constant
kHz - Kilohertz
LLE - Liquid-liquid extraction
LLOQ - Lower limit of quantification
LOD - Limit of detection
LSD – Least Significant Difference
m/z - Mass-to-charge ratio
MAD - Microwave assisted derivatization
MS - Mass spectrometer
MTBSTFA - Methyl-N-tert-butyldimethylsilyltrifluoroacetamide
NAD^+ - Nicotinamide adenine dinucleotide
NADH - Protonated nicotinamide adenine dinucleotide
NH_4OH - Ammonium hydroxide
PA - Propionic anhydride
PFPA - Pentafluoropropionic anhydride
PFPOH - Pentafluoropropanol
PS-DVB - Polystyrene divinylbenzene

rpm - revolutions per minute
S/N - Signal-to-noise ratio
SACENDU - South African Community Epidemiology Network on Drug Use
SIM - Single ion monitoring
SPE - Solid-phase extraction
TBDMS - Tert-butyldimethylsilyl
TIAFT - The International Association of Forensic Toxicologists
TMS - Trimethylsilyl

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1. INTRODUCTION

1.1 INTRODUCTION: EPIDEMIOLOGY OF DRUG USE IN SOUTH AFRICA AND ENVIRONMENTAL IMPACT ASSESSMENT

1.1.1 INTRODUCTION

The occurrence in wastewater of pharmaceuticals, drugs of abuse, and the metabolites produced by the use of such compounds, is becoming cause for concern. The impact of these substances may have adverse effects on animal, plant and ultimately, human life. [1,2,3] Pharmacologically active substances are increasingly being prescribed by physicians and used by the community. [3,4] Hence it can be deduced that an increased amount of these compounds and their metabolites will be prevalent in the wastewater.

The abuse of illicit compounds in South Africa has increased tremendously over the past years, and is a serious problem among the youth in a recreational setting. In South Africa, data on the use of illicit drugs and the amounts consumed is largely limited to information provided by law enforcement agencies, rehabilitation centres, and self-reported cases. The South African Community Epidemiology Network on Drug Use (SACENDU) [5], reports statistics for illicit compound use on a six-month basis in South Africa. The number of patients admitted to rehabilitation centres in the years 2007-2009 are shown in Table 1.1.

TABLE 1.1 THE NUMBER OF PATIENTS ADMITTED TO REHABILITATION CENTERS LOCATED AROUND SOUTH AFRICA ON A SIX-MONTH BASIS

	Jul-Dec 2007	Jan-Jun 2008	Jul-Dec 2008	Jan-Jun 2009
Cape Town	3058	2637	2807	3667
Gauteng	3053	3053	3158	2822
Northern region	600	556	729	809
Port Elizabeth	472	424	457	690
East London	136	126	155	188
Kwa-zulu Natal	954	1531	1542	1575
Total	8273	8327	8848	9751
Average patients	2363	2379	2528	2786

It is suspected that the accuracy of the usage statistics from the sources above may be questionable, due to the following reasons:

- Limited analytical data are made available by law enforcement agencies concerning the substances available on the street. According to South African legislation, only the South African police service is allowed to perform testing on seized drugs. Therefore, the composition of illicit preparations available on the street market cannot be obtained.
- A second factor that complicates the estimation of drug use is the variation in the purity of the products on the street market. Some street products may contain extremely high and lethal concentrations of active constituents, while others contain only small amounts of the active constituents.
- A small percentage of active drug abusers are referred to rehabilitation centres.
- It is difficult to estimate the real magnitude of illicit drug use, as self-reported information is sometimes subjective and the data therefore compromised. [1,6]

In view of the above mentioned difficulties and the concern regarding illicit compound abuse, a different approach is required to evaluate the extent of drug abuse in a defined population, especially that of young people in a recreational environment. Two possible approaches may be followed:

1. Gather information on the presence of drugs of abuse directly from the subjects. Hair analysis of a statistically representative sample from a specific population would be a possibility, since hair may contain a record of a person's drug-abuse pattern. [7] This approach would be complicated, however, by the fact that hair sampling can be regarded as an invasion of human privacy, and will therefore require ethical permission as well as informed consent from the subject. [8] Body fluid sampling (urine, blood, saliva etc.) will be even more invasive and will not provide the integrated information on a person's drug abuse pattern that hair analysis offers. [8]
2. Analyse the effluent/wastewater of a specific area for illicit compounds, in an effort to monitor patterns and trends of a community, as first performed by Zucatto. [6] This approach requires analytical measurements at extremely low concentration levels (ng/L-µg/L). [9,10,11,12]

1.1.2 AIM OF THE PROJECT

The aim of this project was to develop a method that would be able to estimate the patterns or trends of drug use in a population. In the study, this trend will be studied over a period of one week. The concentration of drug metabolites in the wastewater is a direct indicator of drug use in a population, as wastewater analysis gives a more accurate representation of drug use than rehabilitation centre figures. After consumption of an illegal drug, the metabolites created through its use are eventually excreted and will end up in the wastewater supply. These metabolites will ultimately arrive at the wastewater treatment plant, where samples are then collected for analysis. In this process, drug users remain anonymous, as it would be impossible to trace metabolites back to any person, thus decreasing the invasiveness of sampling and allowing the ethical disclosure of results.

These types of analyses can determine drug use patterns for entire metropolitan areas as well as smaller areas, by sampling from smaller sections of the underground wastewater grid. Each treatment plant has a designated metropolitan area it serves. Sampling at a treatment plant will enable observation of drug use trends for the given area. A more focused approach can also be followed, where it would be possible to sample at the main sewage line of a building block, acquiring only the block's drug use pattern. It would not, however, be possible to identify a single drug user, since the data reflect the profile of all the residents in the area where the sampling was done. There are numerous applications for this type of wastewater analysis:

1. By measuring a population's drug use patterns, an estimate of the number of drug users can be obtained.
2. Sampling at different wastewater grid locations can further narrow down the areas of interest where drug use is significantly increased.
3. By employing population statistics and epidemiology, areas showing increased drug use can be targeted by law enforcement agencies.

In this study, wastewater samples were collected every hour for a period of seven days, to observe a trend in population drug use. Samples were then processed and filtered until metabolites were ready to be extracted by means of solid-phase extraction. After extraction, the metabolites were derivatized and the samples were analysed by 2-

dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC/TOF-MS). Data was then analysed and trend analysis conducted.

1.2 TOXICOLOGICAL PRINCIPLES, METABOLISM AND EXCRETION OF DRUGS OF ABUSE

1.2.1 INTRODUCTION

Toxicology can be defined as the study of the absorption, distribution, metabolism, and excretion of foreign chemical substances interacting with a biological system.[13] This interaction may be therapeutic or pathogenic depending on the substance in question and its dose administration. A living organism or biological system copes with external variability by keeping its internal environment constant. This can be described as homeostasis (*homeo,* similar + *stasis,* condition).[14] If one of the body's internal processes is disrupted, it tries to compensate to re-establish equilibrium; if this is accomplished, a state of homeostasis is achieved. Conversely, if compensation fails, a state of disease or illness results. Any foreign chemical substance may have the ability to disrupt homeostasis and cause a state of disease or illness. The ability of a substance to alter homeostasis depends entirely on the dose.[13,15]

At a sufficient dose, drugs of abuse are chemical substances that disrupt the homeostasis of a biological system. Therefore, it is important to restrict or fully eliminate the use of illegal substances. In order to detect whether an illegal drug has entered the body, an appropriate marker metabolite of the parent drug needs to be assayed. The parent drugs are metabolized by the liver through different reactions and enzymes. Different metabolites can be formed for each parent drug. Deciding which metabolite to assay for, would depend on the major pathway of metabolism.

1.2.2 METABOLISM

Metabolism refers to the chemical reactions that take place within a biological system.[14] These may be further subdivided into catabolic reactions (reactions that mainly produce energy) and anabolic reactions (reactions that use energy to synthesize large biomolecules).[14] Metabolism or alteration of xenobiotics or drugs of abuse (*xeno,* foreign) occurs by altering the xenobiotic molecules to produce a less active form. Enzymes are protein molecules that facilitate the transformation of xenobiotics into less active or less toxic substitutes.[14] It should be noted that detoxification does not always occur with enzymatic

reactions, in many cases a more active metabolite is produced. [15] Conversion of a foreign chemical substance to a more potent one is termed metabolic activation. An example of such metabolic activation can be found in the design of pro-drugs, in which the inactive parent drug only becomes metabolically active after it has been metabolized. [16] Examples of these pro-drugs include codeine, which is metabolized into a more active form, morphine. The metabolism of drugs has been described in detail by Gibson *et al.* [17]

Many of the enzymes that metabolize drugs of abuse are, in fact, enzymes that are principally involved in endogenous enzymatic reactions. Exogenous drugs of abuse are metabolized into molecules resembling or mimicking the structure of the body's own endogenous molecules. [17] A detailed description of Phase I and II reactions has been given by Campbell *et al.* [13]

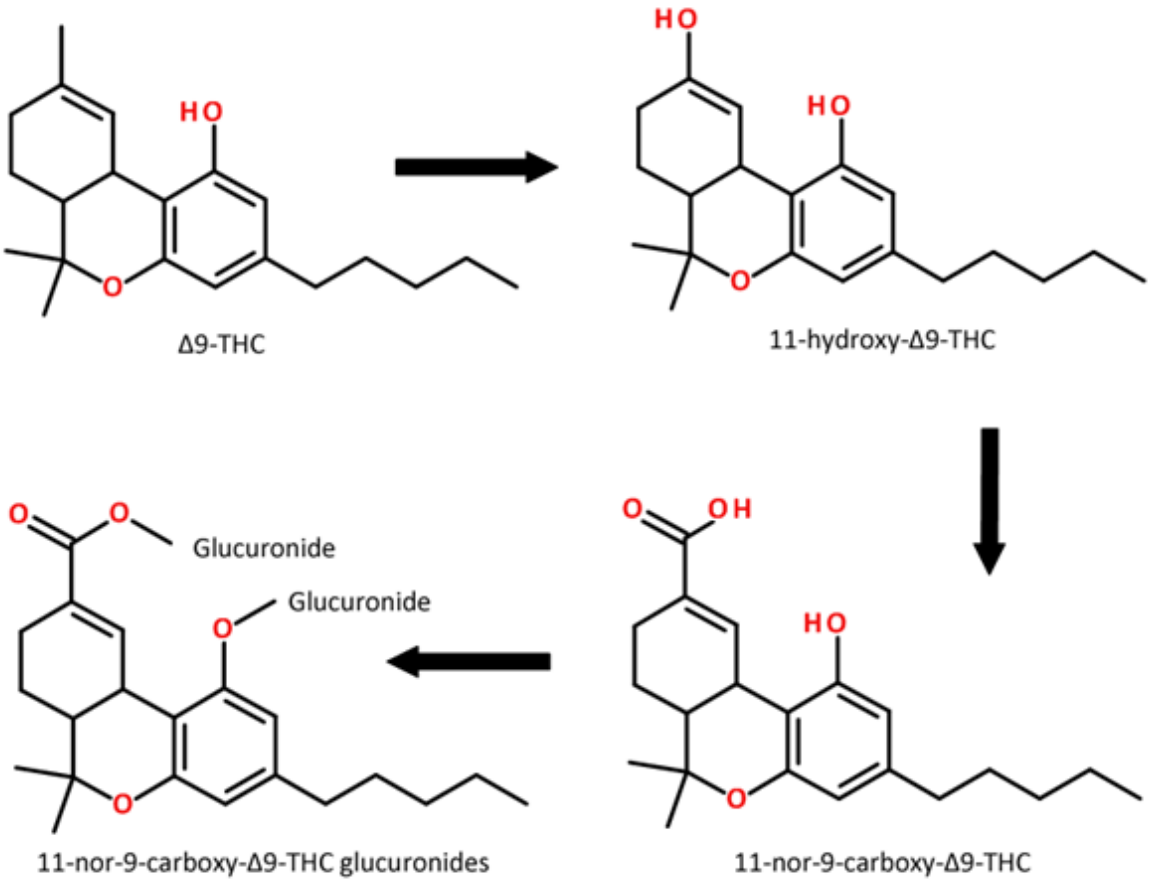

FIGURE 1.1 METABOLISM OF Δ9-THC TO ITS RESPECTIVE METABOLITES

1.2.2.1 METABOLISM OF Δ^9-TETRAHYDROCANNABINOL

The principle psychoactive constituent contained in the cannabis plant is Δ^9-Tetrahydrocannabinol (Δ^9-THC) (Figure 1.1). This compound is metabolized to 11-hydroxy-Δ^9-THC and then further to 11-nor-9-carboxy-Δ^9-THC (Figure 1.1). The reactions occurring in the metabolism of Δ^9-THC are aromatic hydroxylation involving the cytochrome p450 enzyme and oxidation involving alcohol dehydrogenase and aldehyde oxidation. [17] Δ^9-THC is metabolized to 11-hydroxy-Δ^9-THC by means of aromatic hydroxylation, a very common reaction in xenobiotics containing an aromatic ring. Following this conversion, alcohol dehydrogenase is utilized to form an aldehyde intermediate of 11-hydroxy-Δ^9-THC. This enzyme is prevalent in the liver and kidney and uses nicotinamide adenine dinucleotide (NAD^+) as a cofactor instead of protonated nicotinamide adenine dinucleotide (NADH). Aldehyde oxidation converts the aldehyde to a carboxyl functional group, forming 11-carboxy-Δ^9-THC. The cannabinoid metabolite that was analysed in this study is 11-nor-9-carboxy-Δ^9-THC. Human excretion of this metabolite indicates the use of cannabis. The glucuronide metabolites will be discussed in Section 1.2.3.

1.2.2.2 METABOLISM OF COCAINE

Cocaine is primarily metabolized by ester hydrolysis into benzoylecgonine. Hydrolysis of an ester can take place in the plasma via non-specific esterase enzyme (acetylcholine esterases, choline esterases and pseudocholinesterases) or in the liver via group-specific esterase enzymes. [17] In the case of benzoylecgonine, the liver enzyme carboxyl esterase 1 (CE1) facilitates the conversion of cocaine to its primary metabolite, benzoylecgonine. Other metabolites that are also formed are ecgonine and ecgoninemethylester. The cocaine marker metabolite in this study is benzoylecgonine, since cocaine is primarily metabolized to benzoylecgonine. The metabolic pathways for cocaine are shown in Figure 1.2. Typically, 35% to 55% of the cocaine administered will be metabolized to benzoylecgonine. [18]

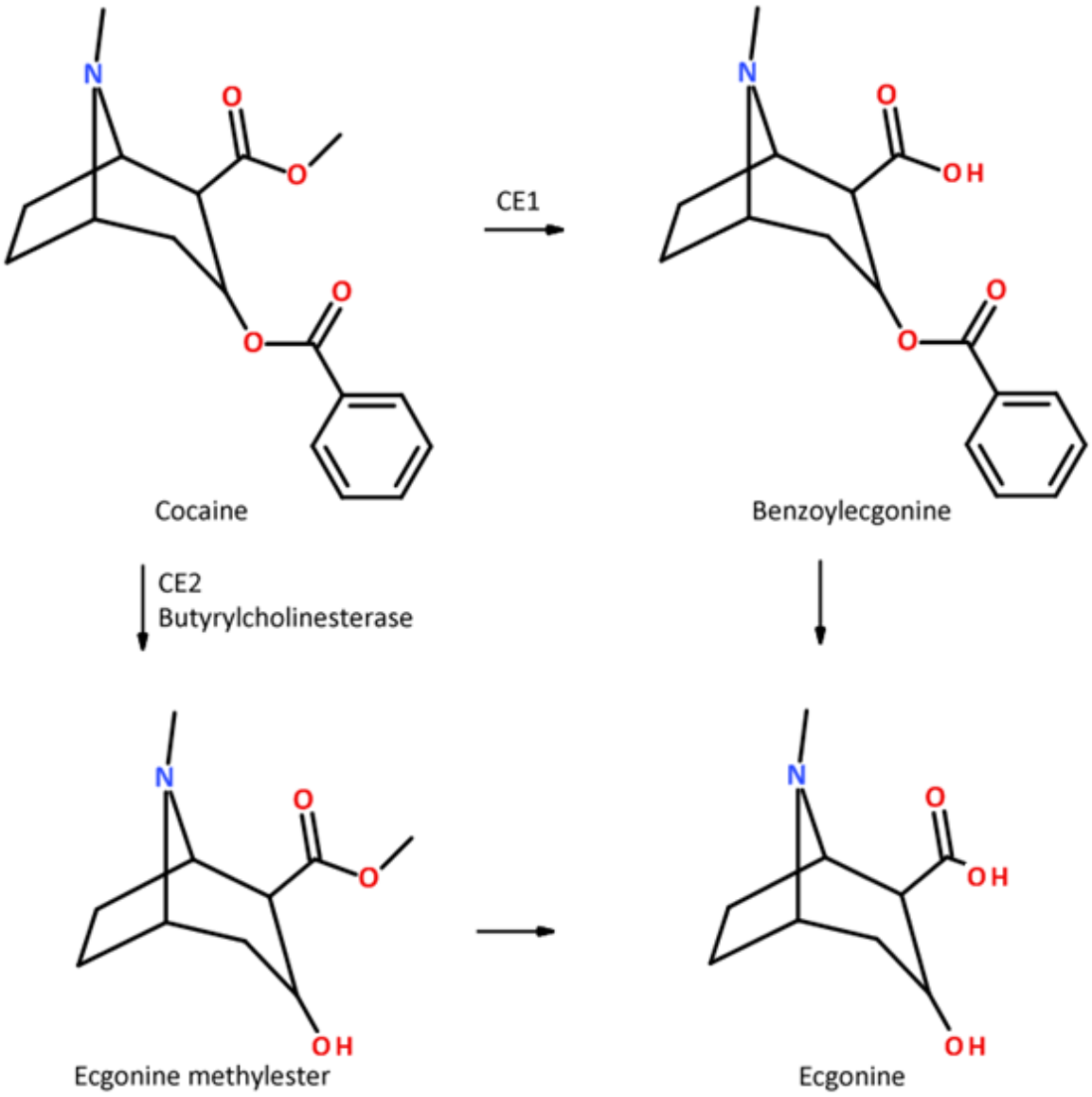

FIGURE 1.2 ENZYMATIC REACTIONS OF COCAINE METABOLISM

1.2.2.3 METABOLISM OF OPIOIDS

The three opioid compounds that were measured in this study were codeine, morphine and 6 monoacetyl morphine (6MAM). The structures of these opioids are very similar, as can be seen in Figure 1.3. [19] Codeine is designed as a pro-drug, as mentioned in Section 1.2.2, meaning that after the inactive form of the drug is administered, enzymatic conversion alters the chemical structure and produces its active form, morphine. [16] It should be noted that this conversion is only a minor metabolic pathway, with only 2%-3% of the codeine transformed to morphine. [20] The rest of the codeine is excreted as the parent drug and

consequently, codeine is the metabolite that will be measured. In the case of heroin, the 3-acetoxy group is hydrolysed to a phenol by the enzyme carboxyl esterase (CE1), forming 6MAM. This metabolite is indicative of heroin use since both codeine and heroin are ultimately metabolized to morphine. [19] The 6 acetoxy group of 6MAM is hydrolysed to a phenol, forming morphine, and the conversion is driven by the same CE1 enzyme. Hence, both morphine and 6MAM will be excreted as a result of heroin use. The short 10-20 minute half life of heroin facilitates quick elimination of the compound from the blood stream. [21] As time passes following dosage administration, it is inevitable that decreasing amounts of 6MAM will be excreted, compared with increasing amounts morphine. It is thus important to take note of the ratio of these two metabolites to one another, as well as the time period after administration. These metabolic pathways reveal the importance of measuring codeine, morphine, and 6MAM to ensure an accurate prediction of opiate abuse.

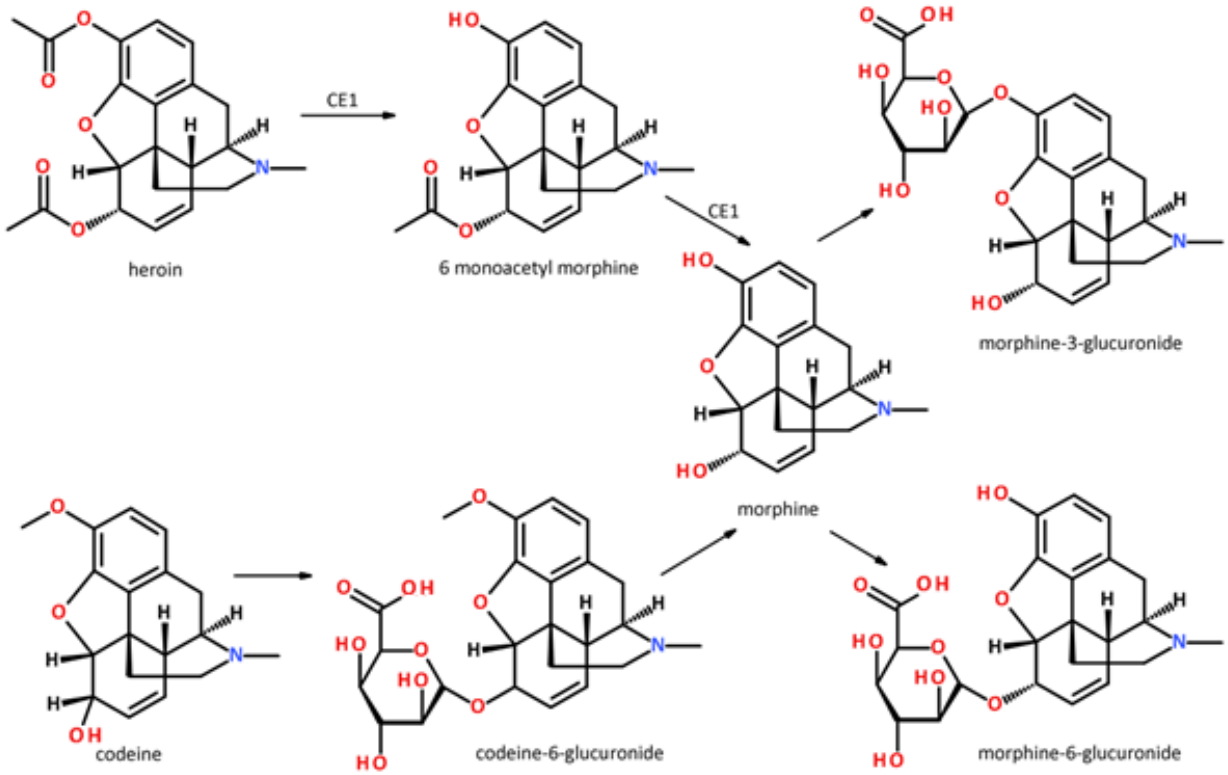

FIGURE 1.3 METABOLIC PATHWAY OF CODEINE, HEROIN AND MORPHINE

Codeine is a molecule found primarily in pharmaceuticals designed to attenuate sensed pain. Due to the close structural similarity between morphine and codeine, it is easily seen that these molecules act on the same receptor in the human body, both decreasing perceived pain. Due to differences in structure, morphine is classified as a more potent pain

attenuator. With the high dosage required as well as metabolism of the liver, codeine is primarily excreted as the parent compound glucuronide. Since bacteria cleave the glucuronide to use the glucose as an energy source, the remaining codeine molecule is the compound measured. [1]

1.2.3 Phase II reactions

Phase II reactions, also known as conjugation reactions, involve a diverse group of enzymes often requiring an active cofactor or catalyst. [17] These reactions generally lead to a water-soluble product that is excreted either in bile or urine. [17] In Phase I reactions, functional groups are uncovered or added, preparing the substrate so that Phase II enzymes can subsequently bind. Of the Phase II reactions, only glucuronidation will be discussed in detail, as this is the only reaction that relates to the analytes measured in this study.

1.2.3.1 Glucuronidation

The major route for sugar conjugation is glucuronidation, a reaction involving the conjugation of glucuronic acid to the substrate of interest. Glucuronidation is the most prevalent of the conjugation reactions due to the copiousness of the reaction co-factor (Uridine diphosphate-Glucuronic acid) and enzyme (Uridine diphosphate-Glucuronosyltransferase). Glucuronidation is the most important type of conjugation for endogenous compounds as well as drugs containing one of the following functional groups: alcohol, hydroxyl, carboxyl, amine or sulphonamide. Glucuronides form from carboxyls, phenols, and hydroxyl functional groups to produce an ester or ether bond. Glucuronidation is the most significant reaction in this study. [17]

The aim of glucuronidation is to increase the hydrophillicity of a compound and increase its excretion rate by way of the renal system. The metabolites for cannabis and opioids, excluding benzoylecgonine, undergo glucuronidation. Glucuronidation of 11-nor-9-carboxy-Δ^9-THC occurs on the 11-carboxy functional group, as well as the 9-hydroxyl group, forming glucuronides as seen in Figure 1.1. All three of the opioid metabolites are able to form glucuronide conjugates. Codeine glucuronidation can only occur on position six, forming codeine-6-glucuronide. In contrast to codeine, morphine has two functional groups at which glucuronidation can occur. Both morphine-3-glucuronide and morphine-6-glucuronide can

be created. Lastly, 6MAM contains only one functional group at which glucuronidation can occur to form 6MAM-3-glucuronide. The opioid conjugation reactions can be seen in Figure 1.3.

With the high dosage required as well as metabolism of the liver, codeine is primarily excreted as codeine glucuronide. Since bacteria cleave the glucuronide to use the glucose as an energy source, the remaining codeine molecule is measured.

1.2.4 METABOLISM INVOLVING THE EXTERNAL ENVIRONMENT

'External environment' is a term that describes the region outside of a biological system. Since the intestinal tract can be characterized as an external environment, metabolism occurring in the intestinal tract can be termed external metabolism. The glucuronides that are released into the gut by way of bile excretion can be broken down to form β-glucuronides. [17] This sugar can be reabsorbed by the gut, depending on the energy needs of the body's bio-systems. The same reaction occurs outside of the gut in wastewater. [1] Wastewater contains faecal bacteria such as E-coli, also present in the gut, which are capable of metabolizing and deconjugating glucose-containing molecules for energy expenditure. Since excreted metabolites contain glucuronides, the faecal bacteria present in wastewater deconjugates glucuronides to their respective Phase I metabolites. [1] Taking faecal bacteria deconjugation into account, it would not be wise to measure the glucuronide conjugates in view of the fact that these glucuronides spend ample time in contact with wastewater bacteria, upon which deconjugation can occur.

We can therefore conclude that only the Phase I metabolites should be targeted for analysis. The analytes measured in this study include: 11-nor-9-carboxy-Δ^9-THC, benzoylecgonine, codeine, morphine, and 6 MAM.

1.3 QUANTITATIVE ANALYTICAL MEASUREMENT AND INSTRUMENTATION.

1.3.1 INTRODUCTION

The ability to detect analytes quantitatively in a sample matrix is one of the major challenges scientists face. The IUPAC Gold Book defines matrix effects as "the combined effect of all components of the sample other than the analyte on the measurement of the quantity." It then goes on to state that "if a specific component can be identified as causing an effect then this is referred to as interference". [22] Wastewater can be considered as one of the most difficult matrices for target analysis, the reason being the wide array of compounds present in wastewater. These compounds range from commercial and industrial waste to any compound that can be discarded via a household drainage system. The immense dilution factor resulting in extremely low concentrations further aggravates the difficulty of measurement. The average person in a medium- to high-population area uses, on average, 130 litres of water per day. [23] The average human urinary output is 0.8-2 litres. [24] Thus, if the average person contributes 130 litres of wastewater to the public sewage system per day, and this figure is divided by the higher limit of the average urine excretion (2 litres), a dilution factor of 65 is achieved. Taking into account the fact that normal screening for drugs of abuse takes place in unaltered urine, a dilution factor of 65 needs to be considered in order to measure the same analyte in wastewater. Thus, the appropriate instrumental technique needs to be considered carefully. An instrument capable of separating and detecting individual analytes in complex mixtures at exceedingly low detection limits is required.

Any results should also include the statistical uncertainty associated with it. Quantitative analyses are defined by the IUPAC as "*analyses in which the amount or concentration of an analyte may be determined (estimated) and expressed as a numerical value in appropriate units. Qualitative analysis may take place without quantitative analysis, but quantitative analysis requires the identification (qualification) of the analytes for which numerical estimates are given*". [22] In the standard addition method of quantitative analysis a calibration curve needs to be plotted in order to calculate the unknown concentration of analyte present in a sample. To create this curve, increasing amounts of certified analyte

standard is spiked into wastewater samples. The responses of increasing standards are then plotted on a Cartesian plane, with relative response (dependent variable) on the y-axis and concentration of spiked standard (independent variable) on the x-axis. A regression line is fitted through the acquired data points. The unknown concentration of analyte is acquired by back extrapolation, or by calculating the x intercept of the regression line.

Sample preparation is another significant step that should be considered in analytical measurement. Depending on the instrumental technique selected, sample preparation can be one of the most important steps in the analytical process. Pre-treatment prepares the sample for the instrumental analysis. This preparation may include chemical derivatization, liquid-liquid extraction or solid-phase extraction, and other clean-up steps. Sometimes chemical alteration of the analyte is a necessity to be able to obtain an accurate and reliable response from the instrument.

1.3.2 Instrumentation

The instrumental techniques involved in preparation, detection, and separation of analytes will be discussed in the following sections of this chapter.

1.3.2.1 Mass spectrometry

Mass spectrometry is the process of separating analytes and their mass fragments by means of atomic mass or mass-to-charge ratio (m/z), as well as providing the concentration of an analyte present in a sample. [25] Mass spectrometric analysis entails the following steps:

- Ionisation
- Focusing a fraction of ions into a beam
- Separating the ions based on their mass-to-charge ratios (m/z)
- Measuring the ion current produced when ions strike a transducer.

The mass-to-charge ratio of an ion is the unit-less ratio of its mass number (m) to the number of fundamental charges on the ion (z). For a single charged ion, it would be m/1; for a dual charged ion, it would be m/2. As the result of most of the ions being singly charged in mass spectrometry, mass-to-charge ratio is often shortened into a more convenient term "*mass*". [25]

A mass spectrometer is an instrument that produces ions and measures the mass-to-charge ratio of those generated ions. [25]

A brief description of the process follows: First, the inlet is used to introduce a micro amount of sample into the instrument. [25] In gas chromatography, the inlet also vaporizes the liquid samples. The ion source produces ions from the sample by different means. For example, electron bombardment, or electron impact, uses a filament whereby electrons are accelerated and then collide with the incoming sample stream. Electrons are separated from their orbitals by the impact and a positively charged fragment is formed. The bombardment of electrons may also fragment the molecules present in the sample in a unique and reproducible manner. The term 'EI source' originates from electron impact. When an EI ion source is used, the output is a stream of positively charged ions. [25] It should be noted that analytes with a mass larger than 10^3 Daltons cannot be measured using an EI source. As mentioned, due to the reproducibility of the EI source, the fragment spectrum of analytes will always be identical for a certain set electron voltage (eV) value. An abundance of spectral libraries are available for mass spectrum comparison. These spectra are always identical for a certain analyte at a certain eV setting. The function of the mass analyser is to separate the ions in this positive ion stream according to their mass-to-charge ratio. An example of an EI ion source is shown in Figure 1.4

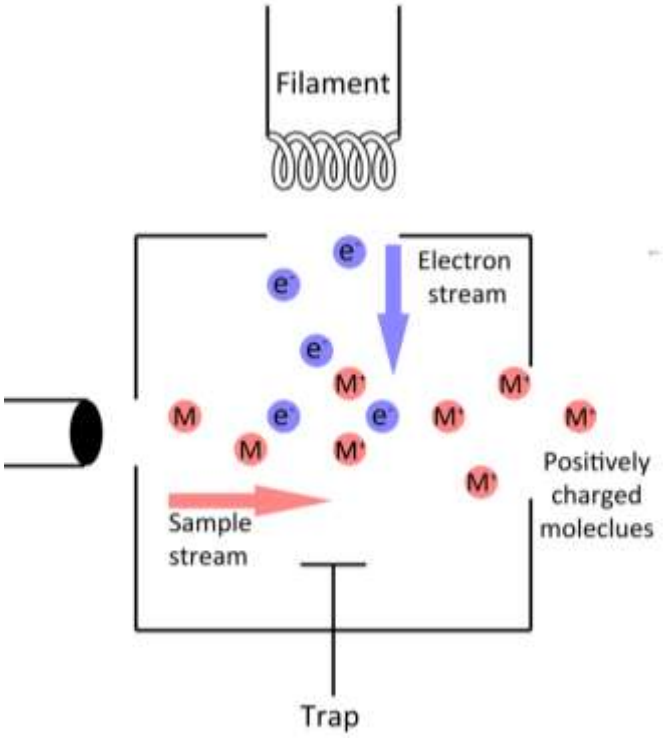

FIGURE 1.4 EI MASS SPECTROMETER ION SOURCE. [25]

A variety of mass analysers are available and the separation of a stream of ions can also take place by a range of different mechanisms. One example would be a quadropole mass analyser. A quadropole mass analyser has four cylindrical rods that serve as electrodes. [25] Each opposite pair of rods are connected electronically. A variable DC voltage is applied across the rods, one pair attached to the positive terminal and the other attached to the negative terminal of the DC voltage source. Out-of-phase variable radio frequency AC voltages are also applied to each pair of rods. [25] To obtain a mass spectrum, ions are accelerated into the space between the rods, whilst both DC and AC voltages are increased. [25] Only ions with a certain mass to charge ratio will have a stable trajectory for a given ratio of DC and AC voltages. If these conditions are not met the ions will have unstable trajectories and collide with the quadropole rods. When ions have stable trajectories and pass through the rods, they strike a transducer and produce a signal. Voltages are increased from minimum to a maximum completing a cycle every 100 ms, producing a full spectral scan 10 times per second. [25]

Another example of a mass separation technique is a Magnetic Sector mass analyzer. Produced ions are accelerated into a flight tube where ions are separated according to their

m/z ratio. The separation occurs by means of a magnetic field. As the produced ions enter a magnetic field the ion is deflected into its own unique radius perpendicular to the direction of the applied magnetic field. The unique radius can be determined if the magnitude of the magnetic field as well as the voltage difference for the region of acceleration are known. In this way ions with certain m/z ratios can be separated. [25] A different mass separation technique, time-of-flight mass spectrometry, separates ions in space and time and will be discussed in detail in the following paragraph.

The time-of-flight ion source works on the same mechanical principle as a quadropole electron impact source. Positive ions are produced periodically by colliding accelerated electrons with the sample. [25] A 10 kHz-50 kHz frequency is employed to produce the pulsed collisions. [25] The molecular ion fragments that are generated usually have a lifetime of 25 milliseconds. [25] The ions produced are then accelerated using an oscillating electric field at the same frequency as the bombardment electron pulses. [25] As these ions enter a free field drift tube, their kinetic energies are essentially equal, but differential velocities will be obtained for each ion due to its individual mass. [25] The lighter ions will arrive at the transducer first, followed by the heavier ions. The free field flight tube is only 1 meter long but the flight path of the ions may exceed 2 meters, the reason being that the ions are reflected back in the same tube before the transducer is struck.

The next step in the process is the conversion of the separated ions into electrical signals that can be measured. This is accomplished by means of a transducer that feeds its signal to a signal processor. [25] The signal processor is a computer containing software to interface with the mass analyser. The whole system, including the ion source, mass analyser, and ion transducer, is operated under high vacuum. This low pressure environment ensures that as few collisions as possible occur between the ions in the free field drift tube and the atmospheric gas molecules. Thus, this high vacuum is needed to prevent kinetic energy loss due to collisions that will negatively influence the results. The transducer for a time-of-flight mass spectrometer is usually an electron multiplier. The mass spectrum acquisition rate for a time-of-flight mass spectrometer is 50 times greater than that of a quadropole, reaching 500 spectra per second versus the latter, at 10 spectra per second. This is especially important when two dimensional gas chromatography is coupled with mass spectrometry. [25] Each chromatographic peak eluting in the 1st dimension needs to be separated in 2nd

dimension as well. For separation to occur in the 2nd dimension, very fast acquisition rates are required. A representation of a 2 dimensional gas chromatograph coupled with time-of-flight mass spectrometer can be seen in Figure 1.5. The Figure was supplied by LECO corporation. [26]

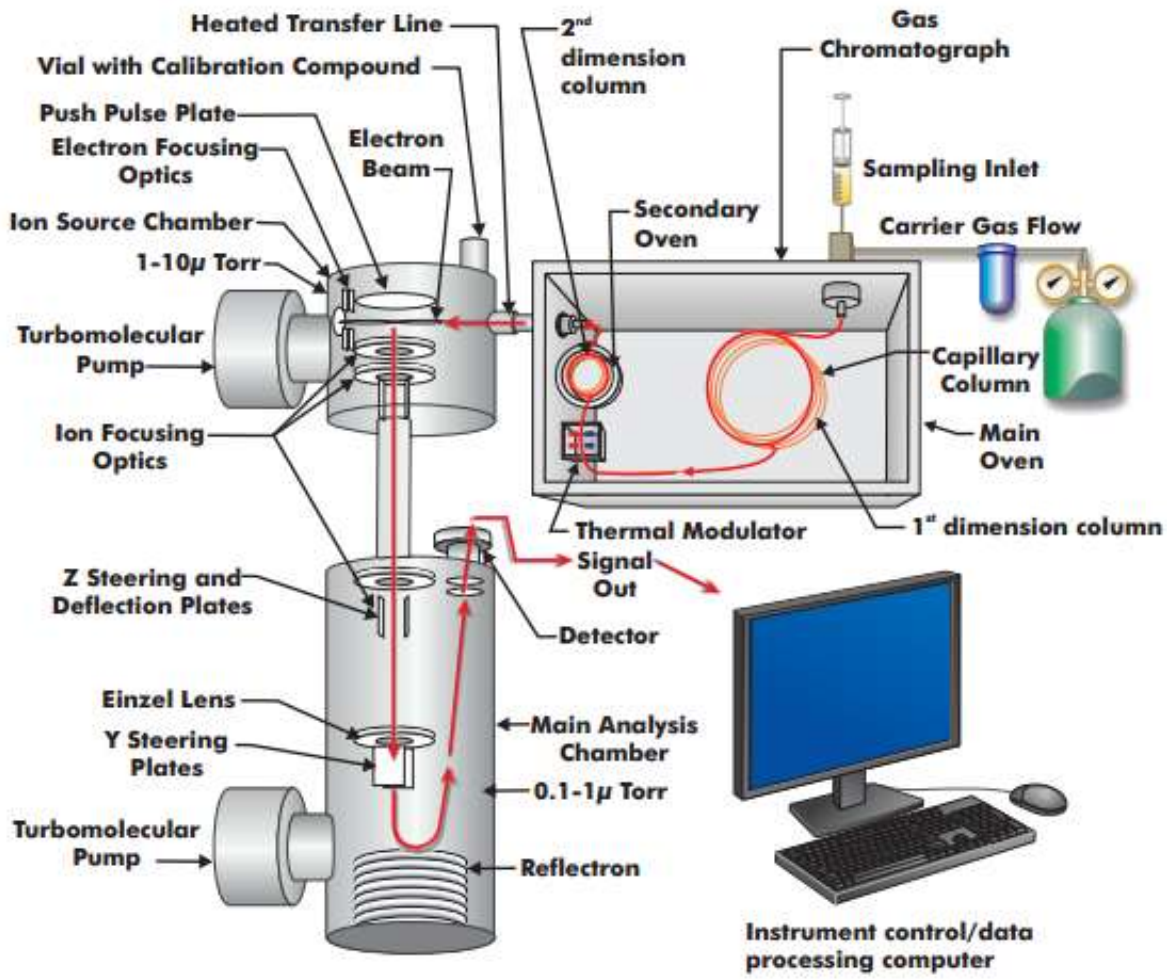

FIGURE 1.5 A REPRESENTATION OF A 2 DIMENSIONAL GAS CHROMATOGRAPH COUPLED WITH TIME-OF-FLIGHT MASS SPECTROMETER AS PRODUCED BY LECO CORPORATION [26]

Mass spectral data is usually presented on a bar graph containing the mass-to-charge ratio m/z on the x-axis and relative abundance on the y-axis. The molecules of interest are bombarded by electrons to form positive molecular ions. The bombardment leaves the positive molecule in highly excited rotational and vibrational states. [25] When the molecule decays to a less excited state, fragmentation occurs, producing a large number of positive ion fragments called daughter ions. [25] A detailed explanation of the fundamentals of mass spectrometry can be found in "*Principles of Instrumental Analysis*" by Skoog *et al*. [25,27]

One aspect of the quadropole mass analyser that should be noted is its ability to either scan the full spectra of masses or to select only a specific set of masses to analyse. This scan option allows the researcher to scan all the masses and match an experimentally obtained mass spectrum to a library. If the single ion monitoring (SIM) option is employed, only the selected ions will be detected and no library matches can be obtained. It should be mentioned that SIM is significantly more sensitive than scan modes. If appropriate ions and internal standards are selected there is no need for a mass spectral library match. The time-of-flight mass spectrometer has higher sensitivity collecting full mass spectra than a quadropole mass analyser. [28] A representative mass spectrum of cocaine can be seen in Figure 1.6 below.

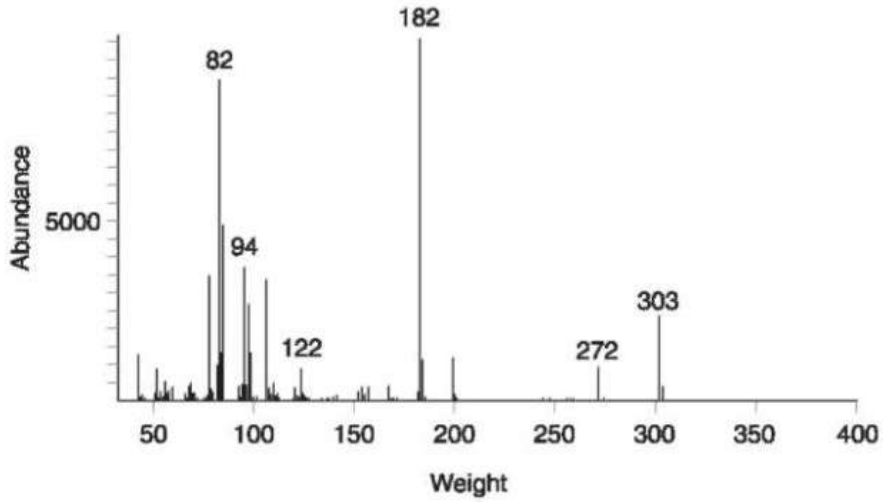

FIGURE 1.6 EI MASS SPECTRUM OF COCAINE. [18]

Certified deuterated analyte standards contain deuterium atoms instead of hydrogen atoms at certain locations on the analyte molecule. These deuterated standards will react identically and elute at approximately the same retention time as the analyte. They will also have the exact same qualifier ion ratios, with the exception of an increased m/z ratio for each ion as a result of the heavier deuterium atoms present on the molecule. Thus the deuterated internal standard can be resolved from the analyte by means of mass spectrometry. A deuterated standard is an internal standard that is added to the sample itself. The ratio of analyte peak area to deuterated internal standard peak area is used to determine a relative analyte response. Because the deuterated internal standard reacts in a similar manner as the analyte, between sample preparation errors can be minimized. For example when the derivatization yield for a specific analyte in a specific sample is low, the

derivatization yield for the deuterated internal standard will be equally low as result of both reacting chemically similar. The relative analyte response between samples will have much less variation because the deuterated internal standard compensates for analyte response variations. With external standards that are not added to the sample and measured separate from the analyte samples, the recovery or loss of analyte during sample preparation needs to determined in order calibrate analyte concentration response effectively. Since derivatization yields may vary between samples, the error also needs to be taken into account. Decreased sample preparation variance is obtained by the use of internal standards compared to external standards, because the error of analyte recovery for each sample is counteracted by calculating the response factor or ratio instead of measuring absolute recoveries.

Qualifier ion ratios of a known, certified, standard solution of an analyte can also be measured and compared to the analytes present in the sample. This way, certainty in measurement and quantification of the correct peak can be guaranteed. According to The International Association of Forensic Toxicologists (TIAFT), a chromatographic peak retention time may only have a 1% variation if relative retention time is employed, as is the case when deuterated internal standards are used. [29,30]

TIAFT also addresses qualifier- as well as quantifier mass spectrometric ion selection and guidelines. It states that at least 3 qualifier ions should be used for targeted analysis. Guidelines for the accepted variance of ion abundances are shown in Table 1.2. [29] These guidelines refer to the relative abundance of the 3 ions to one another, and are not absolute quantities. For example, an ion peak abundance larger than 50% of the base peak should only have a variance of ±10% between successive runs. It should also be noted that the proper choice of qualifier and quantifier ions needs to be considered, as certain ions will have a higher baseline, as well as increased matrix interference, compared to others.

TABLE 1.2 GUIDELINES FOR ACCEPTED VARIANCE OF ION ABUNDANCE OF RELATIVE QUALIFIER MASS SPECTROMETRIC ION RATIOS [29]

Relative Intensity (% of base peak)	EI-GC-MS (Relative)
>50%	±10%
>20% to 50%	±20%
>10% to 20%	±20%
≤10%	±50%

1.3.2.2 CHROMATOGRAPHY

Chromatography refers to a diverse collection of methods that allow for the separation, identification, and determination of compounds within a complex mixture of similar compounds. [25] In gas chromatography (GC), the components of a gaseous sample are separated as a result of them being portioned between an inert gas mobile phase and a solid or liquid stationary phase immobilized on the inside of a capillary column. Elution occurs as a consequence of the flow of the gas through the column. In contrast with other chromatographic separations, in gas chromatography the mobile phase does not interact with the analyte and only acts as a transport medium. [25,31] The molecules that strongly interact with the stationary phase have a decreased mass proportion present within the mobile phase at a given time; hence, they will elute at a later time on the chromatograph. [25] A detector is placed at the end of the capillary column and the response is plotted as a function of time. As each component elutes at its respective time, the detector measures this response and a series of peaks are obtained. Such a plot is called a chromatogram. The position of the peaks on the time axis of the chromatogram can be used to identify compounds qualitatively, and the area under the curve is used to quantitatively determine the concentration of the analyte. If the column used does not achieve proper separation, two components may co-elute and form one visible chromatographic peak when the detector at the GC outlet is a Mass spectrometer. The analytes eluted at that time will contain the mass spectral ions for both components, thereby distinguishing co-eluting analytes based on mass separation.

The effectiveness of a capillary column to separate compounds depend on the diffusion of analytes in the mobile phase, the diffusion coefficient of eluting particles as well as the mass transfer of the analyte molecules in and out of the stationary phase. [25] These three factors

also influence peak width of eluting compounds. Movement of a compound down a chromatographic capillary column only occurs when the compound is in the mobile phase. [25] Certain molecules travel faster due to their random inclusion in the mobile phase, while others travel slower due to their incorporation into the stationary phase. [25] The result is a symmetric spread of molecule velocities around a mean value comparable to that of a Gaussian distribution. [25] Thus the peak width of an analyte increases as the residence time of the molecule in the capillary column increases, an effect termed band broadening. For the diffusion of analytes in the mobile phase the peak width is directly related to the residence time in the column and inversely related to the mobile phase pressure. [25] Contrary to increasing mobile phase flow rates to decrease peak width, mass transfer of analyte in and out of the stationary phase will cause band broadening for a certain set of conditions. If the analyte molecules are interacting with the stationary phase, and mobile phase flow rates are too fast, the analyte molecules contained in the mobile phase will move down the column before interacting stationary phase analytes are randomly included in the mobile phase. This will in turn cause peak broadening. An optimal flow rate needs to be determined in order to counteract both diffusion of analyte in the mobile phase as well as providing enough time for mass transfer of the analyte out of the stationary phase. Chromatographic column efficiency can be described using *plate height H*, *number of theoretical plates N,* and length of the column *L as N=L/H.* A detailed explanation of column efficiency, as well as applicable equations, can be found in "*Principles of Instrumental Analysis"* by Skoog *et al*. [25]

Minimum plate height with decreased peak base width will result in an increase in efficiency. The number of theoretical plates (N) and plate height (H) can be quite useful values in comparing the efficiency and separating power of different column manufacturers.

Resolution is a term that describes the effectiveness of the separation of two peaks. It is a measure of the ability of a column to separate two analytes. [25] Resolution can be described relative to the base peak width of both analytes or to the number of theoretical plates in the column, the selectivity factor and the retention factors of the two analytes. Resolution is denoted by the following formula:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha\text{-}1}{\alpha}\right)^2\left(\frac{1+k_B}{k_B}\right)^2 \qquad \textbf{...Eq 1.1}$$

N is the number of theoretical planes. The term k_B is the retention factor of the slower moving species and α the selectivity factor. The term $\alpha=k_B/k_A$ where k_B and k_A are the retention factors for analytes A and B. [25] With an increase in capillary column length the number of theoretical plates will also increase. Doubling the column length will also double the theoretical number of plates (N). Using Equation 1.1 it can be seen that dividing the column length in two will only decrease the column resolution by 30 %. The logic of buying a 30-meter column and cutting it in half to obtain two 15-meter columns is explained by the aforementioned formula, since the resolution of a 15-meter column is 30 % less compared to a 30 meter column, whereas the savings in cost amounts to significantly more.

To ensure minimal band broadening, a sample needs to be introduced onto a capillary column in a narrow discrete band. Modern gas chromatographic instruments are equipped with an auto injection manifold. [25,32] Here, a calibrated micro syringe is used to inject a small volume of liquid sample into the inlet. The inlet consists of a number of components. The individual components of a GC inlet can be seen in Figure 1.7A. A narrow plug of sample vapour is introduced through the septum into the inlet and carried onto the GC column.

Three basic types of injections can be performed:

1. Splitless injection is used when trace analysis is employed. The whole sample is injected onto the column. No dilution of the sample occurs since the split-vent/inlet purge is closed at the time of injection. After a set period of time, the split-vent, or inlet purge, is opened to discard any residual sample remnants not injected onto the column. This technique will load the entire injected sample onto the column, thus more sensitivity will be obtained compared to split injection.
2. Split injection is used when concentrated samples are injected and splitting or dilution of the sample is required. In this type of injection, the split-vent/inlet purge is opened at the start of injection. The split-ratio is adjusted to facilitate the amount of sample that is discarded through the inlet purge vent at the time of injection. If a high split ratio (50:1) is selected, an increased amount of sample (e.g. 50 parts) is discarded through the inlet purge vent and a decreased amount of sample (e.g. 1 part) is loaded onto the column. When splitless injection overloads the column this type of injection may be used to decrease the amount of sample loaded onto column.

3. Pulsed splitless injections were used in this study and will thus be explained in more detail. Pulsed splitless injection is used when a large amount of sample is introduced onto the column, and the sensitivity of trace analysis is required. The split-vent/inlet purge is in a closed position during pulsed splitless injection (Figure 1.7B). When a large volume of sample is introduced into the inlet, the sample is vaporized and expands. Because of the small volume of the inlet and the large volume of gaseous sample, the expanded sample gas may escape through the septum purge vent (Figure 1.7A). [32] Pulsed splitless injection makes use of a time program at which a higher inlet pressure set point or carrier gas flow rate is maintained for a desired amount of time, after which the flow rate of the carrier gas is returned to a constant 2 ml/min flow rate. For large volume sample injections (e.g. 3 µl-6 µl), pulsed splitless mode is favoured. Because of the high initial pressure that can be maintained, a large sample volume is loaded onto the column in a narrow plug, instead of escaping through the septum purge vent (Figure 1.7B). The high initial pressure of the pulsed injection counteracts the rapid expansion and forces the vaporized sample to the bottom of the inlet liner. After the sample has been loaded onto the column the pressure is normalized to provide a 2 ml/min carrier gas flow rate. This type of injection is used when normal splitless injection doesn't provide adequate sensitivity, and an increased amount of sample needs to be loaded onto the column to obtain the desired sensitivity for the specific analyte. A depiction of pulsed splitless injection can be viewed in Figure 1.7B. [33]

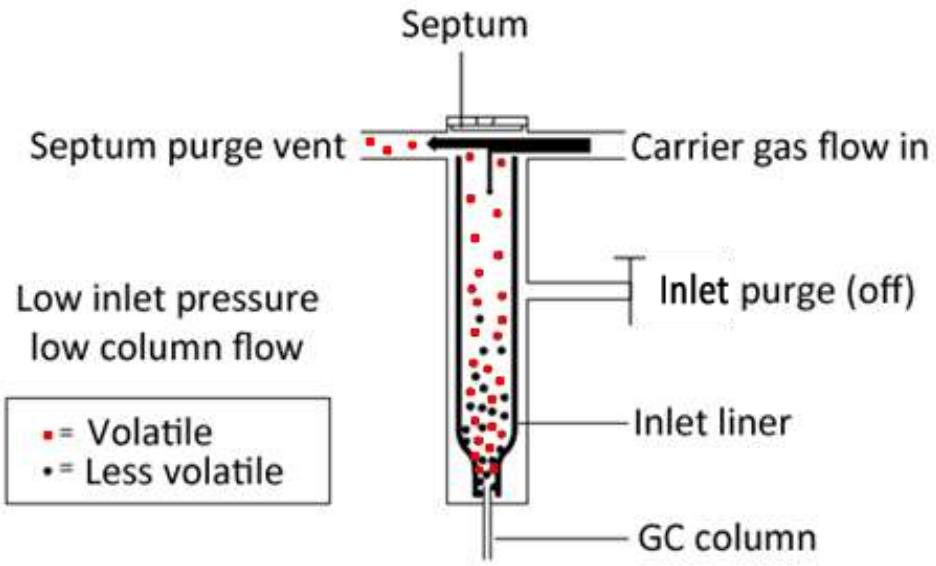

FIGURE 1.7A HIGH VOLUME SPLITLESS INJECTION WITHOUT PULSED PRESSURE. [33]

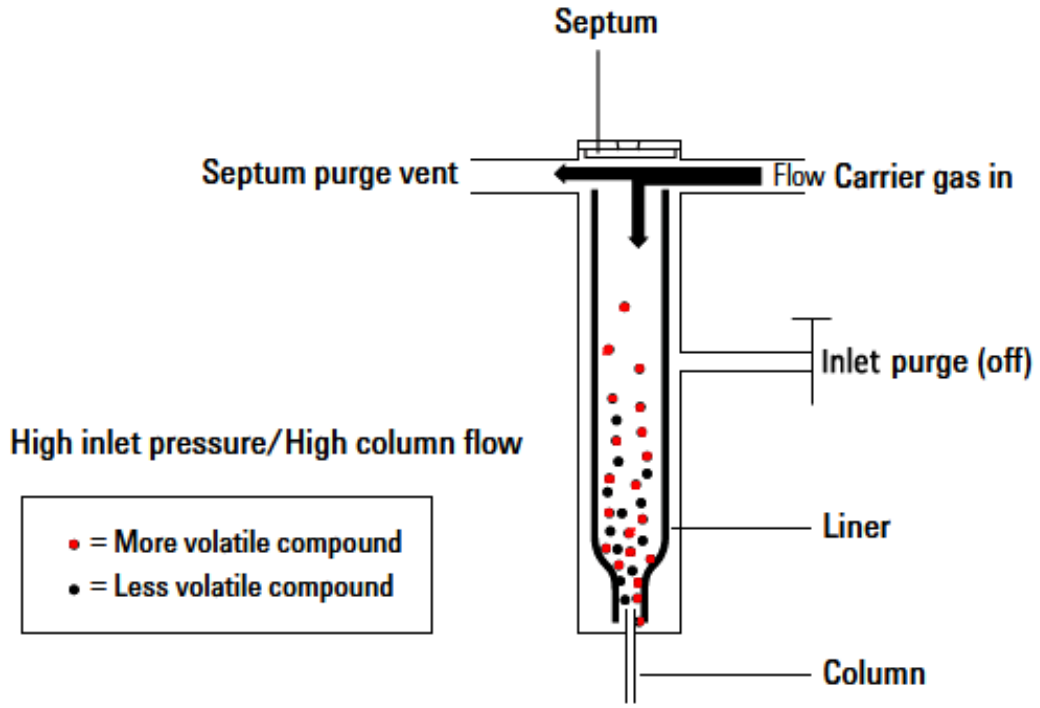

FIGURE 1.7B HIGH VOLUME PULSED SPLITLESS INJECTION. [33]

Next GCxGC/TOF-MS analysis will be discussed. The first part of this method's name indicates that two gas chromatographic separations take place, also known as 2-dimensional gas chromatography. Two capillary columns are coupled in series and connected to a detector of choice, in this case a time-of-flight mass spectrometer. When two columns are used, the 2nd dimension column is usually, but not necessarily, a column with a different polarity or stationary phase than that of the 1st dimension column. After separation takes place in the 1st dimension, separation continues in the 2nd dimension. [34,28] In this study, the front column used (1st dimension) was a 30 meter column, with a shorter, 2 meter column at the back (2nd dimension).

When two dimensional GC is employed, the use of a modulator is necessary. A cryogenic modulator is an interface that is placed between the two GC columns to trap compounds eluting off the first column by means of liquid nitrogen jets. [28] As the jets cool the capillary column, compounds are retained on the stationary phase of the 2nd dimension column. When jets are disengaged, the secondary oven temperature thermally desorbs the analytes, allowing chromatography to continue on the 2nd dimension column. This process of

cryogenically trapping compounds on the GC column is repeated at set intervals, usually every one to five seconds. Reference timing is applied to the modulator to enable the detector to categorize analyte chromatographic bands received from the 2nd dimension column. These analyte bands are combined to form a two dimensional visual representation of the chromatographic separation that took place. [35] A graphical representation of a two dimensional GC instrument can be seen in Figure 1.8.

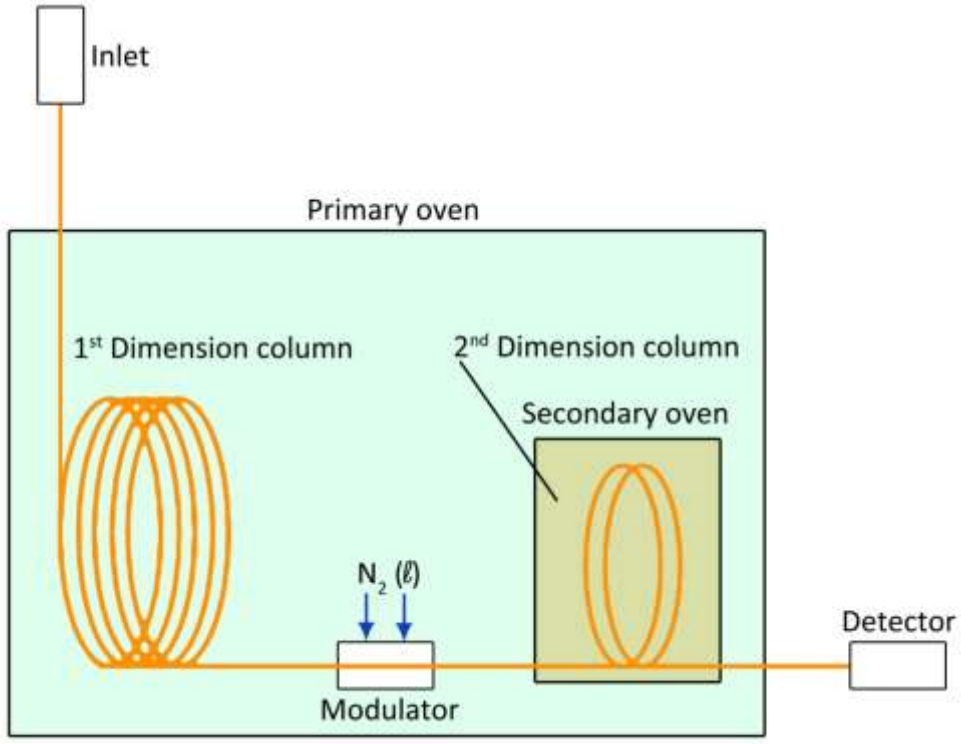

FIGURE 1.8 TWO DIMENSIONAL GAS CHROMATOGRAPHY.

The major advantage of using an extra dimension of separation over conventional GC-MS is the separating power achieved, which is especially necessary for complex wastewater samples. As a result of the separation in the two dimensions being independent, the peak capacity of a multidimensional system is the product of its individual parts or peak capacities (n_c). [35]

$$(n_{c1} \times n_{c2} \times n_{c3} \times \ldots\ldots \times n_{cn}) \qquad \ldots \textbf{\textit{Eq 1.2}}$$

Even though a shorter column is usually implemented in the second dimension, it may have a proliferative effect on the total separation power of the multidimensional system. [35]

As the sample elutes from the 1st dimension, the 2nd dimension column should be capable of further separating the eluted peak into its constituents. The 2nd dimension separation takes place much faster than the 1st dimension separation; therefore, a mass spectrometer capable of very fast acquisition speeds is needed. During two dimensional gas chromatography, the 1st dimension separation provides the 2nd dimension with substituent sample parts that are relatively simple, as well as substances of comparable volatility. (35) The separations performed by a two dimensional gas chromatography system , can be considered independent from one another even if the stationary phases of both columns are identical and an orthogonal chromatogram is observed. (35) A peak on the first dimensional plane is separated into its constituents, seen as several peak slices in the second dimensional plane. To obtain an integral peak response, the individual peak area slices from the 2nd dimension need to be added together. In layman's terms, this means that the 2nd dimension peak slices with identical mass spectrums are added. (35) A sample obtained from the instrument software of a two dimensional chromatogram can be seen in Figure 1.9.

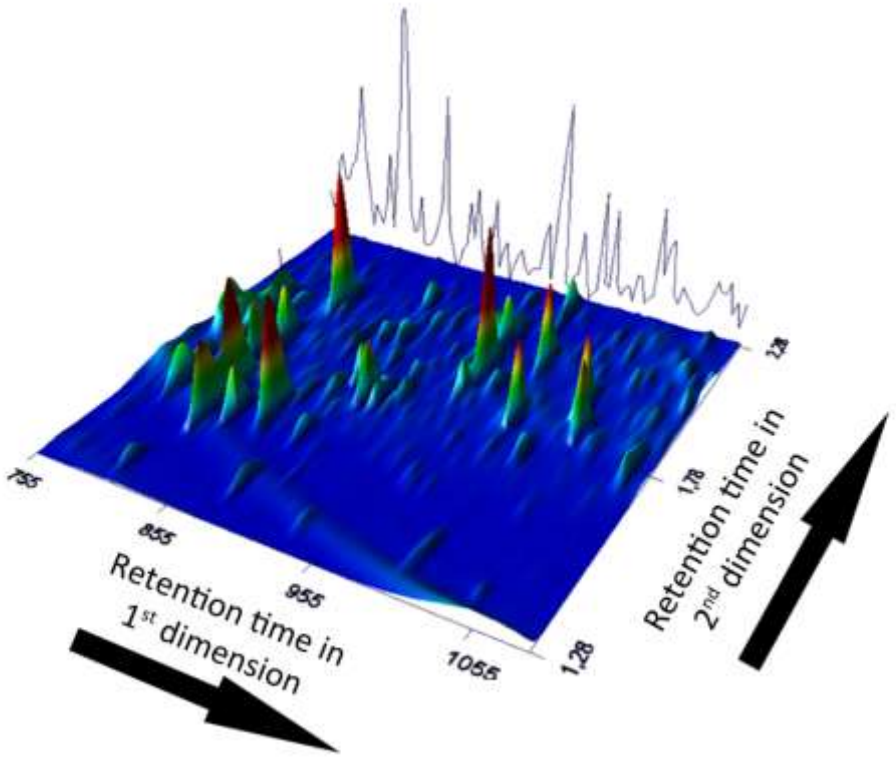

FIGURE 1.9 A TWO DIMENSIONAL CHROMATOGRAM REPRESENTING BOTH TIME AXES AS WELL AS DETECTOR RESPONSE.

The increased separating power as well as fingerprint identification mass spectrometry offered by GC×GC/TOF-MS is excellent for separating the analytes present in the complex wastewater matrix.

1.3.3 Quantitative analytical measurement

For an analytical measurement to be regarded as acceptable, a multitude of factors needs to be considered before a final unknown analyte concentration can be determined. When using GC-MS or GC×GC/TOF-MS instrumentation, multiple sample work-up steps are required before analyses can be performed. Analytical methods, even if reproduced from the literature, need to be validated for adequate accuracy, precision, robustness and linearity before they can be acknowledged as reliable. Method validation is the only objective measure available to demonstrate whether an analytical method is fit for the purpose it is used for. The developed method needs to fulfil minimum established acceptance criteria. This will ensure that results generated from the method can be conferred with the minimum amount of uncertainty. Method validation requires evaluation of the following parameters: selectivity, linearity, precision, accuracy, lower limit of quantification, and limit of detection. [36]

1.3.3.1 Selectivity

Selectivity (qualitative) is defined by IUPAC as: *"The extent to which other substances interfere with the determination of a substance according to a given procedure"*. [22] In short, it is the ability of a method to differentiate between analytes in the presence of matrix compounds capable of interfering with the analysis. Specificity and selectivity should not be used interchangeably, where the former refers to the measurement of a single analyte and the latter to a measurement consisting of multiple analyte compounds. [15] The absence of the desired metabolite response in a blank matrix is regarded as the evidence for the selectivity of a given method. In the case of wastewater measurement, it would be impossible to prove selectivity since no blank wastewater matrix can be collected. Since wastewater influent may contain the analytes of interest, and population drug use cannot be controlled, there is no way to evaluate whether the wastewater will contain analytes or not. A helpful measure would be comparing gas chromatography retention times as well as the

mass spectrum ion ratios of both the analyte and the added deuterated internal standards. The retention times of both the analyte and the internal standard, as well as their mass spectrum ratios, should match closely. If there is proper baseline separation of peaks, and no matrix compounds contribute to the selected qualifier and quantifier mass ions, it can be assumed with confidence that acceptable selectivity criteria have been met. [36]

1.3.3.2 LINEARITY

Linearity is described by a mathematical model that compares detector response against increasing concentrations of the analyte. The relative detector response is plotted on the y-axis and analyte concentration is plotted on the x-axis. In analytical measurements, the concentration of a given analyte is obtained by calculating peak area ratios, which is accomplished by dividing the absolute detector analyte peak area by the deuterated internal standard peak area. Deuterated internal standard is a molecule synthesized to have the same exact chemical structure as the analyte, with the exception of hydrogen atoms. In the deuterated compound, particular hydrogen atoms are replaced by deuterium atoms. This enables the internal standard to have the same chemical properties as the analyte, but allows separation by mass spectrometry due to the heavier mass of added deuterium atoms. This approach compensates for variation in sample preparation steps. The linearity model is ideally described by the following equation:

$$y=mx+c \qquad \textit{...Eq 1.3}$$

As the concentration of the analyte in the sample increases, so too must the response increase. To create the linearity curve, the ideal method is for blank sample matrix to be spiked with increasing analyte concentration. This, however, is not possible with wastewater analysis, since no blank matrix can be obtained. Collected wastewater influent will always have the chance of containing the analytes of interest. Thus it is difficult to obtain blank wastewater matrix containing no analytes of interest. If a wastewater pool is collected and the average concentration of each analyte determined the calibration curve can be adjusted accordingly to subtract the existing analyte pool concentration from the spiked calibrator standards. On the other hand samples which contain analytes of interest can be collected daily and the linearity as well as the concentration of analyte present in the collected sample can be assessed by using of the method of standard addition.

A large wastewater pool containing a representative sample of wastewater matrix can be used to perform the linearity assessment. The calibration graphs may not pass through the origin, since analyte may already be present in the matrix. Equal volumes of sample matrix are taken and separately spiked with increasing known quantities of analyte. The relative response for all signals are calculated and plotted. A least squares regression curve is calculated from the collected data and a curve is fitted through the data points. The fitted regression curve is extrapolated backwards to obtain an x intercept. This x intercept will represent the unknown concentration of analyte, with an uncertainty associated to its measurement. The r square value of the regression is a measure of goodness of fit, or linearity. A graphical representation of the method of standard addition can be seen in Figure 1.10. The method standard addition is also used when the sample matrix between days may vary significantly, as the case is with wastewater.

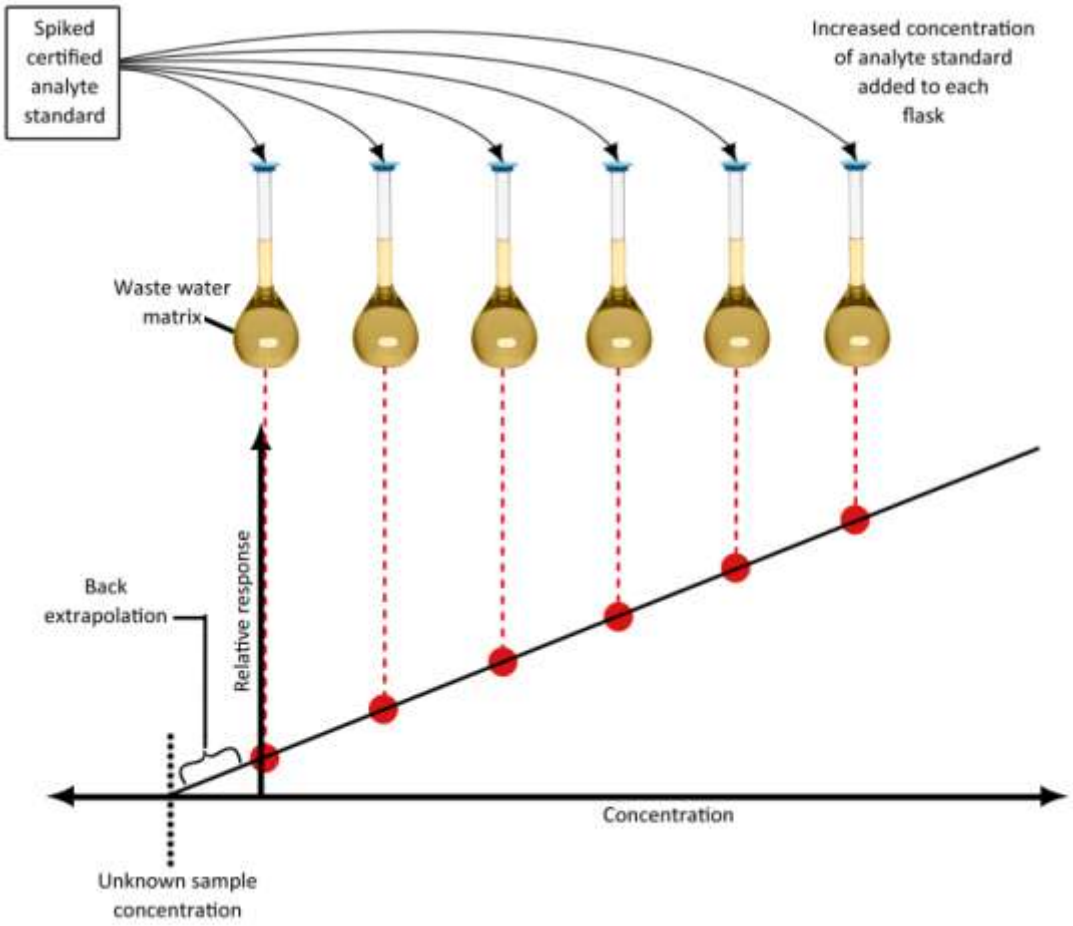

FIGURE 1.10 METHOD OF STANDARD ADDITION USED TO OBTAIN UNKNOWN CONCENTRATION OF ANALYTE BY BACK EXTRAPOLATING THE FITTED REGRESSION LINE.

1.3.3.3 PRECISION

Precision describes the amount of spread between individual measurements of the same homogenous sample (Figure 1.11). Precision is considered at three different levels: repeatability (within day), intermediate precision (between day) and reproducibility (intra laboratory). [15] Precision is usually expressed as percentage relative standard deviation (%RSD):

$$\%\text{RSD} = \left(\frac{\text{Standard deviation } (\sigma)}{\text{Mean concentration value } (\bar{x})}\right) \times 100 \qquad \textit{...Eq 1.4}$$

Since standardization of this method will not be developed between laboratories, intra laboratory measurements are not necessary. Only repeatability and intermediate precision will be considered. Precision needs to be measured at both extremes of the calibration model, thus high as well as low controls are incorporated in both within-day and between-day measurements.

1.3.3.4 ACCURACY

Accuracy can be described in terms of both systematic error (bias) as well as random error (precision). Accuracy is usually calculated as the percentage deviation of the measured value from the expected value (Figure 1.11). Internal quality control samples must be prepared from lot numbers other than those used for calibration and ideally by another analyst. [15] Acceptable criteria are ±15% from reference and ±20% at the lower limit of quantification. [37] Accuracy is calculated as follows:

$$\text{Accuracy}(\%) = \left(\frac{\text{Amount expected}}{\text{Amount measured}}\right) \times 100 \qquad \textit{...Eq 1.5}$$

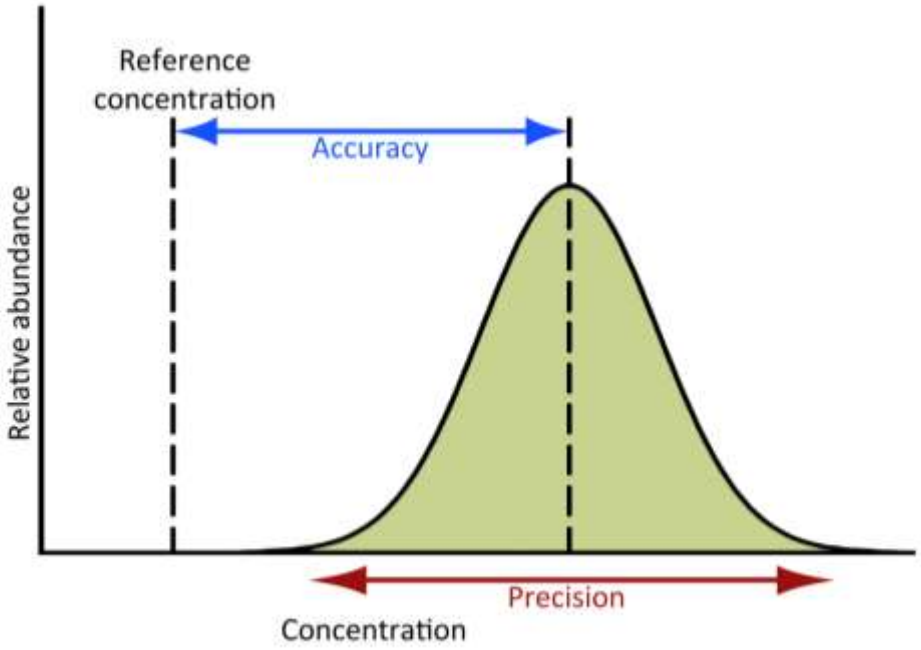

FIGURE 1.11 ACCURACY VERSUS PRECISION OF A CHROMATOGRAPHIC MEASUREMENT [25]

1.3.3.5 LOWER LIMIT OF QUANTIFICATION (LLOQ)

The lower limit of quantification can be defined as the minimum concentration of analyte that can be measured with a precision of ±20% RSD and an accuracy of ±20%. [15,37] It is recommended that the LLOQ value be chosen close to the lowest concentration used in the linearity tests and that it be performed separate from the linearity experiments. [15] If a value falls below the lowest concentration linearity standard used, as a result of the intrinsic analytical imprecision, this value will be deemed unquantifiable, since no value below the lowest linearity calibrator may be quantified. [15] The value of the lowest calibrator can be chosen as the LLOQ on the condition that acceptance criteria of precision (±20% RSD) and accuracy (±20%) are met. Replicates should be performed, with all adhering to a precision of ±20% RSD and an accuracy of ±20%. Any measurement value below the LLOQ is considered to be qualitative; thus, no quantitative data can be obtained from concentration measurements below the LLOQ.

The limit of detection (LOD) is defined as the lowest concentration of an analyte that an analytical method can reliably differentiate from background noise. [37] Detection of analyte concentrations below LLOQ can be considered as semi-quantitative. [38] The signal-to-noise ratio (S/N) is the criterion used to evaluate LOD. Typically a S/N of greater than three is

considered significantly dissimilar to the background signal and is characterized as the limit of detection. [38] Sample replicates are injected and diluted until a signal-to-noise ratio of 3 is obtained. The concentration at this particular dilution will be considered the limit of detection for its respective analyte.

1.3.3.6 Errors in quantitative analysis

Quantitative analysis plays a dominant role in any analytical laboratory, and as such, the errors associated with each measurement are exceedingly important. No quantitative result of any kind merits value when not accompanied by a measure of error or uncertainty. A measurement taken from any biological sample has a random spread around the mean. Thus, for a measurement to be significantly disparate, i.e. lie outside the Gaussian distribution, the error of the measurement needs to be known. There are three types of error: gross, random and systematic. Gross error can be described as errors relating to complete failure of the particular test, i.e. dropping a sample or instrument failure. [39] Random error is described as replicate results differing from one another so that individual measurements are a spread around a mean. Systematic error causes all of the results or the mean to be shifted towards a more negative or positive value, causing an emergent bias to be observed. [39] All of these errors play a central role in the reporting of an analytical measurement. If a result is reported, it should undoubtedly contain a measure of uncertainty, i.e. value ***X*** ± error ***Y***.

1.3.3.7 Statistical outliers

A quantitative measure is needed in order to classify whether a measurement result may be categorized as an outlier and rejected. According to Miller *et al.* [39], some statistical packages flag outliers by means of comparing the y residual of the suspected value to $S_{y/x}$ of the regression fit, and the suspected value is then excluded from analysis. The y residuals ($\hat{y}$) are the y-values calculated from the regression equation using the known x-value concentrations. The $S_{y/x}$ is given by Equation 1.6.

$$S_{\frac{y}{x}} = \sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n-2}} \qquad \textbf{...Eq 1.6}$$

In Equation 1.6, y_i denotes the experimentally obtained y-values and *n* denotes the sample size. As mentioned previously, Miller *et al.* [39] proposes a comparison of the $S_{y/x}$ and the $\hat{y}$ (y residual), suggesting that the $\hat{y}$ value should not be larger than two (or a certain multiple) of the $S_{y/x}$-value. If the aforementioned situation is true, a value may be deemed an outlier, although no definite criterion exists. Before the suspect value can be compared to the other measurements, a regression curve needs to be fitted through all the data except the suspect value. This regression curve is necessary because the $\hat{y}$ values are calculated from the curve's equation.

1.3.3.8 Regression

This section will describe the calculation of the best fit, which assumes a linear relationship between increased concentration on the x-axis and relative response on the y-axis. Before linearity can be assumed, the product moment correlation coefficient (r) needs to be calculated. The r is a measure of "goodness of fit" for the measured values and can range from r<1 to r>-1. [39] The product moment correlation coefficient is calculated by the following Equation 1.7.

$$r=\frac{\Sigma\left\{\left(x_i-\bar{x}\right)\left(y_i-\bar{y}\right)\right\}}{\left\{\left[\Sigma\left(x_i-\bar{x}\right)^2\right]\left[\Sigma\left(y_i-\bar{y}\right)^2\right]\right\}} \qquad \textit{...Eq 1.7}$$

As the r approaches 1 or -1, the goodness of fit for a linear regression increases. An r-value of +1 indicates a perfect positive slope correlation; conversely, -1 indicates a perfect negative slope correlation. After this measure of linearity is calculated and appropriate r-values are obtained for each plot, a regression curve may be fitted through the data. This curve will be calculated using the method of least squares. Since no error is assumed in the x direction, the regression line will be fitted to minimize error in the y direction. [39] This method endeavours to minimize the sum of the y residuals ($\hat{y}$), or in other words, the deviation in the y direction. [39] The following equations are used to determine the slope and the intercept of the linear regression line:

$$\text{slope } m=\frac{\Sigma\left\{\left(x_i-\bar{x}\right)\left(y_i-\bar{y}\right)\right\}}{\Sigma\left(x_i-\bar{x}\right)^2} \qquad \textit{...Eq 1.8}$$

$$\text{Intercept } c=\bar{y}-m\bar{x} \qquad \textit{...Eq 1.9}$$

In Equation 1.9, $\bar{y}$ denotes the mean of all y-values, and $\bar{x}$ denotes the mean of all x data points. These equations describe the regression line of y on x, representing how the y-values vary for a given set of x-values. It should also be noted that these equations describe homoscedastic data, meaning that equal variance in y is observed across the whole concentration range for the selected x-values. [39]

1.3.3.9 Weighted regression

In the situation of unequal variance across each x concentration range value, data is termed heteroscedastic. [39] Errors in the y direction may either increase as the x concentration decreases, or the inverse may be true. [39] The error may increase at both ends of a concentration range while having modest values at the centre of the range. When heteroscedastic data is suspected, the variance for each individual point is calculated and analysed. If the variance increases or decreases with concentration, heteroscedastic data is suspected and weighted regression lines should be used. [39] For example, it is more important for the regression curve to pass closely to the points at lower concentration that a lesser amount of variance than to points showing greater variance at high concentrations. [39] This is achieved by adding additional weight to the lower concentration y-values. Each concentration level is given a weight inversely proportional to the variance at that specific point. [39] These weights can be calculated by the following Equation 1.10.

$$w_i = \frac{s_i^{-2}}{\sum s_i^{-2} / n} \qquad \textbf{...Eq 1.10}$$

The individual x control level concentration weights are denoted w_i. Each concentration level has a variance denoted by s_i with the total amount of concentration levels denoted by n. [39] After each weight has been calculated, the slope and intercept of the weighted regression line can be calculated using the following equations [39]:

$$m_w = \frac{(\sum w_i x_i y_i) - (n \bar{x}_w \bar{y}_w)}{(\sum w_i x_i^2) - (n \bar{x}_w^2)} \qquad \textbf{...Eq 1.11}$$

$$c_w = \bar{y}_w - m \bar{x}_w \qquad \textbf{...Eq 1.12}$$

The terms $\bar{y}_w$ and $\bar{x}_w$ correspond to the coordinates of the weighted centroid, through which the regression curve must pass. [39] These coordinates are given by Equation 1.13 and Equation 1.14 [39]:

$$\bar{x}_w = \sum w_i x_i / n \qquad \textit{...Eq 1.13}$$

$$\bar{y}_w = \sum w_i y_i / n \qquad \textit{...Eq 1.14}$$

The goodness of fit of a regression line can be estimated using the product moment correlation coefficient. The aim of this section of method validation is to establish whether the analyte response plotted against increasing concentrations retains a linear relationship. All outlying data has been discarded and thus the product moment correlation coefficient can be calculated from the viable data points.

1.3.3.10 Method of standard addition statistics

When the sample itself is used to calculate the unknown concentration of the analytes in the sample, as well as to plot the calibration graph, the method of standard addition is utilized. Since a calibration graph is obtained for each analyte, the unknown analyte concentration obtained from the calibration graph is subject to error and this error value is calculated using extrapolation. For Equation 1.15 – 1.29, common numerators as well as denominators are listed. [40,39] The x-value coordinates are denoted by x, in this case, concentration. The y-axis values will be denoted by y (relative response). The term x_i and y_i denote each individual x- and y-value of the 6 respective measurements conducted. The term *n* denotes the sample size, in this case, 6. As mentioned earlier, $\hat{y}_i$ denotes the individual y-values calculated from the regression curve's equation. Finally, m denotes the slope of the regression curve. The equation for the regression slope is given by Equation 1.19. The slope as well as intercept of this regression line is calculated by the method of least squares regression and is formulated in both Equation 1.8 and Equation 1.9. Using these equations, the slope, intercept, and product moment correlation coefficient are calculated.

$$\bar{x} = \frac{\sum x_i}{n} \qquad \textit{...Eq 1.15}$$

$$\bar{y} = \frac{\sum y_i}{n} \qquad \textit{...Eq 1.16}$$

$$Sy/_x = \sqrt{\frac{\sum (y_i - \hat{y})^2}{n-2}} \qquad \textit{...Eq 1.17}$$

$$S_{X_E} = \frac{Sy/_x}{m} \sqrt{\frac{1}{n} + \frac{\bar{y}^2}{m^2 \sum (x_i - \bar{x})^2}} \qquad \textit{...Eq 1.18}$$

$$y = mx + c \qquad \textit{...Eq 1.19}$$

The method for calculating the error in measurement involves extrapolation of the regression line through the scatter plot, and obtaining an uncertainty in the x direction. [40,39] The equation for obtaining the standard deviation in the x direction is given by Equation 1.18. With the use of this equation the standard deviation of the unknown concentration or "x", value is calculated and subsequently the confidence interval using the *t-test* can be determined. The equation for calculating the confidence limits for x is as follows:

$$x \pm t_{(n-2)} S_c \qquad \textit{...Eq 1.20}$$

The corresponding value for *t* can be obtained in any statistical *t* test table with the corresponding degrees of freedom (n-2). A percentage confidence limit can then be read from the table and the correct *t* value obtained. It should be noted that in using the method of standard addition, the x-intercept and the slope are calculated from the same data set and thus they are not independent of one another. This results in a covariance term that is significant, and may influence the error associated with the measurement.

The use of the standard addition method to obtain the desired weekly wastewater profile will be tabulated and discussed in detail later in the paper.

1.3.3.11 STATISTICAL SIGNIFICANCE OF RESULTS

The simplest way of deciding significance between two sample means, would be to organize the means in increasing order and compare the means of adjacent values with a quantity called the least significant difference (LSD). [33] In order to establish whether the difference in calculated doses between days of the week differ significantly, the LSD is calculated using the Equation 1.21.

$$LSD = s^2\sqrt{\frac{2}{n}} \times t_{df(n-1)} \qquad \textbf{...Eq 1.21}$$

Where s is the within sample estimate of standard deviation (σ), and $t_{df(n-1)}$ is the tabulated t-test value with associated degrees of freedom.

1.3.4 COMPARISON OF GC VERSUS LC COUPLED WITH MASS SPECTROMETRY FOR WASTEWATER ANALYSIS OF DIFFERENT ANALYTES

Different instrument configurations can be employed to measure analytes in wastewater matrix. Although it would be difficult to compare these methods on equal terms, a comparison will be attempted. Sample pre-treatment steps will also influence the performance of an analytical instrument to a great extend. For example if a SPE pre-treatment step is employed which concentrates the analyte as it is extracted, the amount of analyte injected onto the instrument increases significantly, depending on the extraction. Since the LOD value reported is a measure of analyte concentration in sample before any sample preparation is employed, SPE extraction will greatly affect the concentration limit at which the analyte can be detected by the specific method. The configuration of an analytical instrument will also have an effect on what types of analytes can be measured and how well these analytes are separated and detected. A comparison of sample preparation employed as well as instrumentation configuration used will be listed in Table 1.3. Four different studies will be compared namely: Study 1 [41], Study 2 [42], Study 3 [1] and Study 4 [6]. It is clear from Table 1.3 that enhanced sensitivity is obtained by using LC-MS/MS compared to GC-MS.

TABLE 1.3 A COMPARISON OF DETECTION LIMITS, SAMPLE PRE-TREATMENT, ANALYTES MEASURED AND INSTRUMENTAL CONFIGURATION FOR MEASURING ANALYTES IN WASTEWATER

	Sample pre-treatment	Instrumentation	Analytes	LOD
Study 1 [41]	Liquid –liquid and solid –liquid extractions (magnetic stirrer)	GC-MS	Organic phosphates, musks, and plasticizers	2.3 ng/L
Study 2 [42]	Sample evaporation 1 L to 1 ml	GC-MS	Toxaphene	76 µg/L
Study 3 [1]	Solid phase extraction	LC-MS/MS	Amphetamines, opiates, cocaine, cannibinoids	<1 ng/L
Study 4 [6]	Solid phase extraction	LC-MS/MS	Amphetamines, opiates, cocaine, cannibinoids	<1 ng/L

1.3.5 CONCLUSION

As seen from this section, a variety of factors need to be taken into account before an analytical result can be reported. The method used for analysis should adhere to certain constraints concerning selectivity, accuracy, linearity etc. The unknown concentration of the analyte should be reported with its uncertainty or error. Only then will the reported quantitative concentration be of any significance, allowing a conclusion to be drawn from the result.

1.4 SAMPLE PREPARATION TECHNIQUES AND WORK-UP

1.4.1 INTRODUCTION

The highly sensitive and complex instrumentation involved in measurement of trace analytes would not be possible if a chemist were unable to separate the analyte of interest from the sample matrix and deliver the separated analyte into a detection system. This process of preparing the sample for analysis begins with the sampling of wastewater, various factors must be considered, like sampling time, volume, frequency and location. The separation or extraction of analytes from the sample matrix entails a consecutive process of sample pre-treatment steps, to enable the detection of the analyte. Sample preparation is the very first step in the analysis of compounds. Care must be taken with sample preparation in order for the analytical results to fall within the acceptable constraints of the validated method. If errors are made during the sample preparation stage, they contribute to the total error of measurement. Sample preparation techniques such as solid-phase extraction and derivatization will be discussed in this chapter. The eventual goal of sample preparation is to separate the analytes from sample matrix, and present them in a suitable form for a specific detection system.

1.4.2 ANALYTE EXTRACTION

Liquid-liquid extraction (LLE) can be credited as one of the most basic types of extractions. LLE demands the use of two immiscible liquid phases. The analyte in question possesses varying degrees of solubility in each of the liquid phases; hence, one of the phases will contain a more concentrated quantity of the analyte. Agitation of the two phases creates a dispersion of one phase into the other in the form of droplets and is termed the dispersed phase. [15] The phase that is not dispersed into droplets will be termed the continuous phase. Movement from one phase to the other is a consequence of a difference in affinity or chemical potential for neutral or ionic species, creating a thermodynamic driving force. [15] When phase distribution equilibrium is reached, the movement of species between the two phases is arrested. After equilibrium is reached the drops of the agitated dispersed phase coalesce to form a definite separation line between itself and the continuous phase.

Each ionisable functional group of a molecule has a certain pK_a value that determines at which specific pH the group will be ionized or in the free form. It is common practice to alter the pH to above or below two units of the pK_a in order to obtain a more that ninety percent free or ionized form. This will be discussed in more detail in Section 1.4.2. The K_a of a solution is termed the acid dissociation constant and is a measure of the concentration of ionized vs. free form states of an acid in solution. [43] Similarly the dissociation constant for a basic functional group is termed K_b, and the same math can be applied. K_a is described by both Equation 1.21 and Equation 1.22. [43] The pK_a of a molecule in solution is described by Equation 1.23. [43]

$$[\text{R-OH}] \leftrightarrow [\text{R-O}^-] + [\text{H}^+] \qquad \textit{...Eq 1.21}$$

$$K_a = \frac{[\text{H}^+][\text{R-O}^-]}{[\text{R-OH}]} \qquad \textit{...Eq 1.22}$$

$$pK_a = -\log_{10} K_a \qquad \textit{...Eq 1.23}$$

$$pH = pK_a + \log \frac{[\text{R-O}^-]}{[\text{R-OH}]} \qquad \textit{...Eq 1.24}$$

Another type of analyte extraction is solid-phase extraction (SPE) is a broad term that is used to describe the separation that occurs when analytes contained in a solution come into contact with a modified solid surface and adsorb to these solid particles. [44] In a subsequent step, analytes are released from the solid surface by means of an elution solvent. [44] SPE sample preparation techniques are employed for purifying, as well as concentrating complex samples before analysis by gas chromatography. [45] SPE can remove unwanted interfering compounds from the sample matrix. [45] In some instances, SPE has been used as a substitute for LLE. [45] LLE has the disadvantage of requiring profuse amounts of organic solvent, as well as being a technique that is difficult to automate. [45]

In SPE, analytes are partitioned between a porous polymer solid phase and a mobile liquid phase. [45] The polymeric stationary phase usually contains functional groups that have a greater affinity for the analyte than the sample matrix. [45] The affinity for a species to distribute, or in this case interact or bind, to a solid phase also determines the compound's concentration on the solid phase. A major advantage of SPE is that the column cartridge

actually concentrates the sample on the sorbent bed as it passes through the column. This is especially important in this study, where the samples contain extremely low concentrations. The cartridge type used in this study was a mixed mode cation exchange.

1.4.2.1 SOLID-PHASE EXTRACTION STEPS

SPE is the adsorption of the analyte of interest onto a solid bonded phase. This reversible bond can be utilized in such a way that analytes can be removed from a complex matrix and concentrated into an elution solvent of choice. The solid phase extraction method used in this study consisted of the following steps, in order:

- Conditioning of the SPE cartridge
- Loading of the sample
- Wash
- Drying of cartridge
- Elution

The basic components of an SPE cartridge are shown in Figure 1.19.

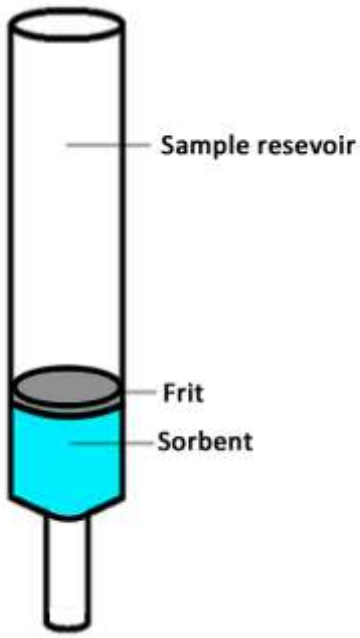

FIGURE 1.19 SPE CARTRIDGE ILLUSTRATION

Conditioning of the SPE cartridge will be discussed in the following paragraph. A solvent capable of solvating the sorbent is brought into contact with the solid phase. This type of

solvation, or wetting, is necessary to maximize interaction of the sorbent, or bonded phase, with the analytes. [46] Figure 1.20 shows the mechanism of wetting the bonded phase. The sorbent is usually wet first using the main constituent of the elution solvent, so as to maximize the interaction of the elution solvent with the bonded phase.

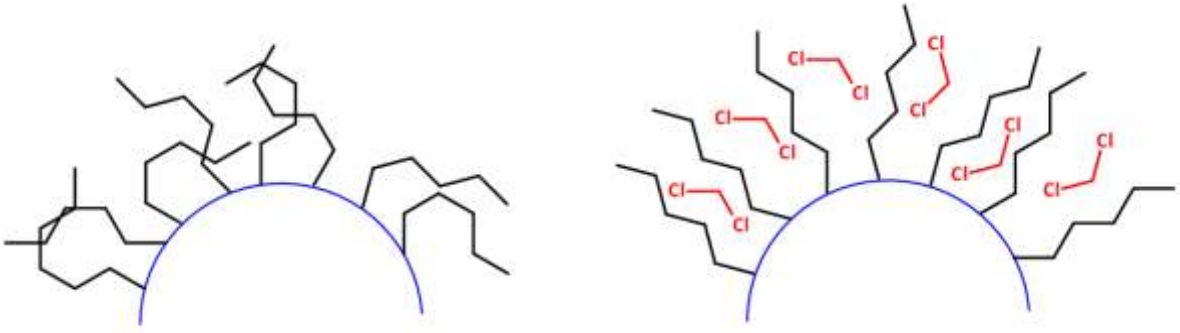

FIGURE 1.20 SOLVATION/WETTING OF A SPE CARTRIDGE BONDED PHASE

The loading procedure for the SPE cartridge involves introducing pH-adjusted wastewater matrix into the SPE cartridge. The cartridge reservoir holds the sample volume to be loaded onto the sorbent. A detailed explanation regarding the loading steps employed in this study will be provided in the experimental section of this dissertation (Section 2.1.2.3.2, Loading wastewater sample).

After sample loading has taken place, the analytes are now bound to the solid phase by the various types of interactions explained above. These interactions are relatively strong, and thus a comparatively strong solvent interaction is needed to break these interactions and elute the analytes of interest. The purpose of the wash, or clean up, is to remove interfering matrix compounds while still retaining analytes of interest. Thus, the wash solvents employed should not have a pH range or non-polarity capable of breaking the bonds between the analytes and bonded phase. They should, however, have the ability to wash off other matrix compounds retained by the bonded phase.

A very important step in the SPE work-up process is drying the cartridge before sample elution is performed. The drying step ensures that no aqueous medium is present on the cartridge packed bed before elution with an organic solvent is completed. This is of the utmost significance, seeing as that the silylation reagents required in subsequent steps are sensitive to moisture.

The analyte components that are bound to the solid sorbent phase after washing and drying need to be removed from the sorbent and transferred into a liquid phase. This liquid phase is termed the elution solvent. The elution solvent contains certain properties in order to disrupt or break the interactions between the analyte and the SPE sorbent. The SPE sorbent backbone can consist of different chemistries, and two of these chemistries will be discussed.

1.4.2.2 Silica based sorbents

Silica-based sorbents have been primarily used in solid-phase extraction applications. [45,47] A wide variety of bonded phases are available for a multitude of applications. SPE silica sorbents have notable disadvantages, however—the same as those seen with columns used in HPLC systems. [45] These columns have a limited pH range (pH 2-pH 7), as well as the presence of surplus silanol groups. [45] If a certain set of pH conditions are met (pH values around 4.5–4.7), the bonded phase silanol groups become ionized and are negatively charged. Positively charged analytes in turn interact with the negatively charged silanol functional groups. [45] These strong ionic interactions can rarely be broken by an organic solvent elution, and thus influence basic analyte concentration. [45] For silica-based sorbents, it is critical to ensure that the bonded phase does not dry out in between experimental steps. [45] This deactivation or “dewetting” can adversely affect the properties and recovery of the bonded phase as well as the analytes. [45] Silica base sorbents were not employed in this study because the presence of negatively charged silanol groups would adversely affect the recovery of positively charged amine functional groups. Furthermore if the bonded phase dries out in the experimental procedure, recovery of the analytes of interest would be adversely affected.

1.4.2.3 Polymer based sorbents

Polymer. [45] Harsh solvents and buffers are tolerated exceedingly well by polymeric sorbents sorbents have been a great advancement in SPE sample preparation, providing a number of advantages compared to their silica-based counterparts. Firstly, polymer sorbents can be used at a markedly wider pH range compared to silica-based sorbents. [45]

Silica-based sorbents also have an irregular shape compared to the spherical polymeric-based sorbent. [45,44]

The spherical particles of the polymeric-based sorbents make room for a much more homogenous bed packing. [45] As result of the tighter bed packing, less Eddy diffusion is observed compared to silica sorbents. [45,25] Factors such as particle size and shape influences Eddy diffusion. [45,25] From a practical stand point, a lower amount of Eddy diffusion is desired, which results in lower back pressure and more consistent flow rates of solvents through the cartridge. The polymeric sorbents are usually constructed from a polystyrene divinylbenzene polymer backbone. [45] A polymer-based sorbent can be seen in Figure 1.12A [47] and a silica-based sorbent in Figure 1.12B [48].

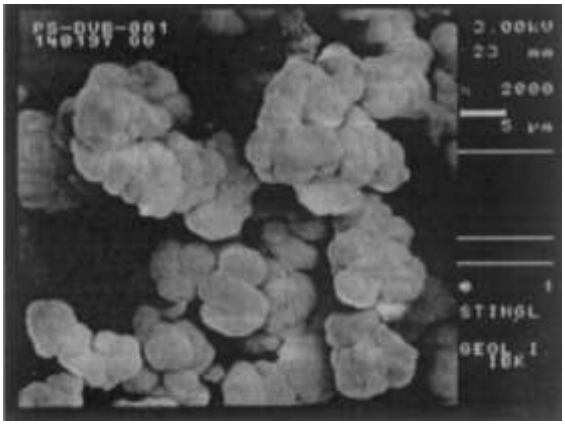

FIGURE 1.12A POLYSTYRENE DIVINYLBENZENE MICROSCOPIC STRUCTURE. (47)

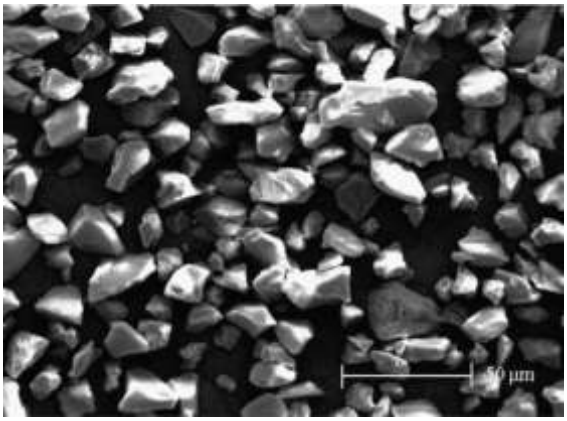

FIGURE 1.12B SILICA BASED SORBENT MICROSCOPIC STRUCTURE. (48)

A big advantage of polymeric-based sorbents is that they are not sensitive to de-wetting. The polymeric particle surface wets easily, and even after drying, the surface around the particle remains wetted, leaving analyte recovery unaffected. [45] There are numerous manufacturers that produce polymeric based cartridges, because their products were employed in this study, two of these manufacturers will be discussed.

1.4.2.4 OASIS MCX™ CARTRIDGE

The Oasis MCX™ cartridge was developed by Waters™, and works on the principles of mixed mode cation exchange. [49] The Oasis line of SPE extraction products are all constructed from the same molecular backbone, a proprietary polymer consisting of two different functional groups, divinylbenzene and N-vinylpyrolidone. The divinylbenzene groups interact with

analytes that are of lipophilic nature, and the N-vinylpyrolidone group interacts with analytes that are hydrophilic of nature. [50]

1.4.2.5 PHENOMENEX™ STRATA C-X™ CARTRIDGE

The Phenomenex™ Strata C-X™ cartridge is constructed from a polymer base divinylbenzene but contains no N-vinyl pyrrilidone functional groups. Along with providing increased proficiency and stability, the Phenomenex™ Strata C-X™ was the cartridge used in the study because of an interference caused by the Waters™ Oasis cartridges. This interference will be discussed in detail in the experimental section of this document. The technical aspects of the Phenomenex™ cartridge will be discussed in the following paragraphs.

The Phenomenex™ cartridge consists of a polymer-based sorbent which contains a benzenesulfonic acid group. This functional group is characterized as a strong cation exchanger. According to Guthrie *et al.* [51], the pK_a of toluenesulfonic acid is -2.6, which means the sulfonic acid group is a strong cation exchanger. The sulfonic acid group will be negatively charged at a low pH (in ionized form). When a sample (pH 5) is loaded onto the SPE cartridge, the positively charged, basic analytes (in ionized from) will coordinate with the negatively charged sulfonic acid groups. The inverse of this is true as the pH is increased past the pK_a of the analyte's basic functional group. The sulfonic acid groups of the resin remain negatively charged (in ionized form), while the basic analyte molecules are deprotonated and carry no charge (in free form). The Phenomenex™ and Waters™ cartridges are both polymer-based, using a divinylbenzene backbone rather than the older octadecylsiloxane-bonded silica sorbent. The Phenomenex™ cartridge uses three modes of interaction or bonding to adsorb analytes. These bonds include ionic, van der Waals, and covalent pi bonds. The sulfonic acid group is responsible for ionic bonds, the benzene rings in the backbone are responsible for pi bonding, and the divinylbenzene polymer is responsible for van der Waals hydrophobic interaction. These interactions are shown in Figure 1.13A. [52] As previously mentioned in the mechanisms describing LLE, the dispersed phase of the partitioning will be facilitated by the divinylbenzene backbone. As result of the divinylbenzene backbone being organic as well as hydrophobic, it is termed the reverse phase of the mixed mode cation exchange cartridge. Thus the name 'mixed mode' indicates

that the Phenomenex™ cartridge has a dual mode of action, including both ion exchange and reverse phase extraction mechanisms.

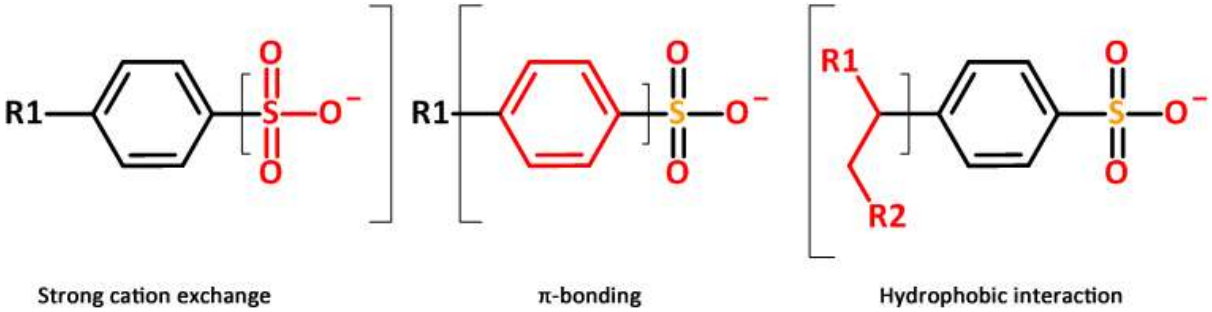

FIGURE 1.3A PHENOMENEX™ANALYTE MECHANISMS OF INTERACTION WITH THE SPE SORBENT [52]

The Phenomenex™ cartridge's polymer-based sorbent extracts analytes in three ways:

1. First, via the sulfonic acid group (ion exchange)
2. Second, via non-polar van der Waals interactions with the divinylbenzene groups (reverse phase)
3. Last, via π-bonding of benzene groups.

In this study, the reverse phase divinylbenzene groups interacted with the non-polar molecules; namely, 11-nor-9-carboxy-Δ^9-THC and benzoylecgonine. These analytes both have an acidic functional group that remains uncharged (in free from) at a pH below their pK_a. The free forms of these two analytes are less water soluble and will interact with the divinylbenzene sorbent via reverse phase hydrophobic interactions. The basic analytes of interest in this study (codeine, morphine and 6MAM) are primarily positively charged at a pH below their pK_a.

Codeine, morphine and 6MAM, interacted with the sorbent via cation exchange principles. The ionized analyte functional groups have a high affinity for the negatively charged sulfonic acid groups. There is still, however, a slight amount of reverse phase contribution. As the pH is increased, fewer basic functional groups are charged, depending on their pK_a values. If more molecules are in free form, a larger portion of total retention is attributed to the reverse phase mechanism [50]. This mechanism can be visualized in Figure 1.13B. [50]

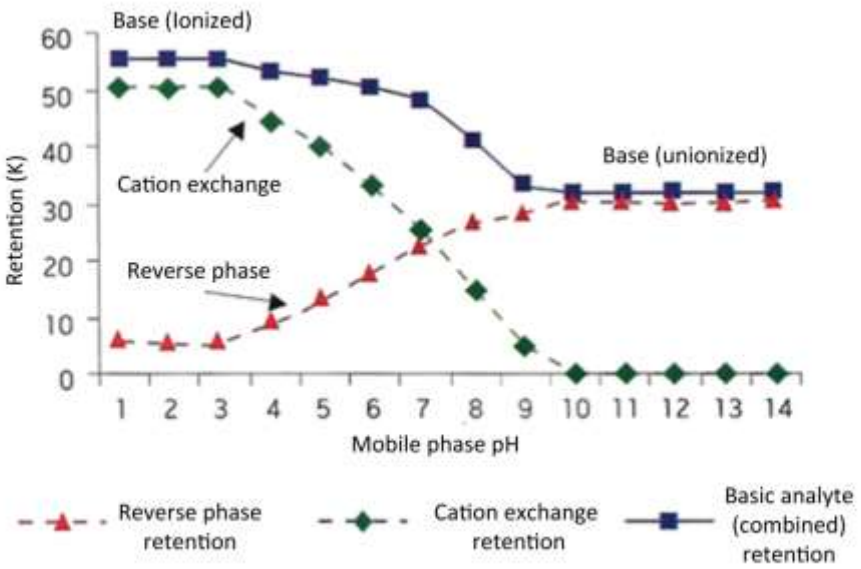

FIGURE 1.13B CATION EXCHANGE VERSUS REVERSE PHASE RETENTION INCLUDING A COMBINED RETENTION PLOT [50]

To give a better understanding of how each analyte is ionized a graphical representation of each analyte and its respective ionisable functional groups will be depicted in the section to follow. A single analyte may contain more than one ionisable functional group, like with morphine and the other opioids measured. The ion exchange characteristics of the polymer sorbent will be discussed next.

1.4.2.6 ION EXCHANGE IN ANALYTES

Ionization data for analytes are easy to come by and are some of the most valuable parameters used during ion exchange extraction. Most of the drug analytes that were analysed are of basic nature, with the exception of benzoylecgonine and 11-nor-9-carboxy-Δ^9-THC. An analyte's dissociation constant K_a is a measure of the amount of molecules that will dissociate into their respective ionic products when in solution. The pK_a value is a logarithmic measure of the acid dissociation constant as a result of the K_a, spanning many orders of magnitude. It should be noted that some molecules have more than one pK_a, this means that the molecule has two or more ionisable functional groups. For basic functional groups an anlyte will be in free form (non-ionised) at $pH \geq pK_a+2$ and for acidic functional groups at $pH \leq pK_a-2$. For basic analytes ionisation sets in at $pH < pK_a+2$ and at $pH > pK_a-2$ for

acidic functional groups. Figure 1.14 illustrates the equilibrium shifts for both acidic and basic molecules at set pK_a values.

FIGURE 1.14 RELATIONSHIP OF PH TO THE PK_A OF AN ACIDIC OR BASIC FUNCTIONAL GROUP

Examples of the ion exchange occurring in two analytes, morphine and 11-nor-9-carboxy-Δ^9-THC, will be given in the following paragraphs. In addition, the pK_a values for the rest of the analytes measured in this study will be specified. Morphine was chosen as an example because this molecule contains two functional groups, each with its own pK_a value. 11-nor-9-carboxy-Δ^9-THC was chosen as an example because it has only one ionisable group. Figure 1.15 is a representation of morphine and the respective pK_a states that accompany its functional groups.

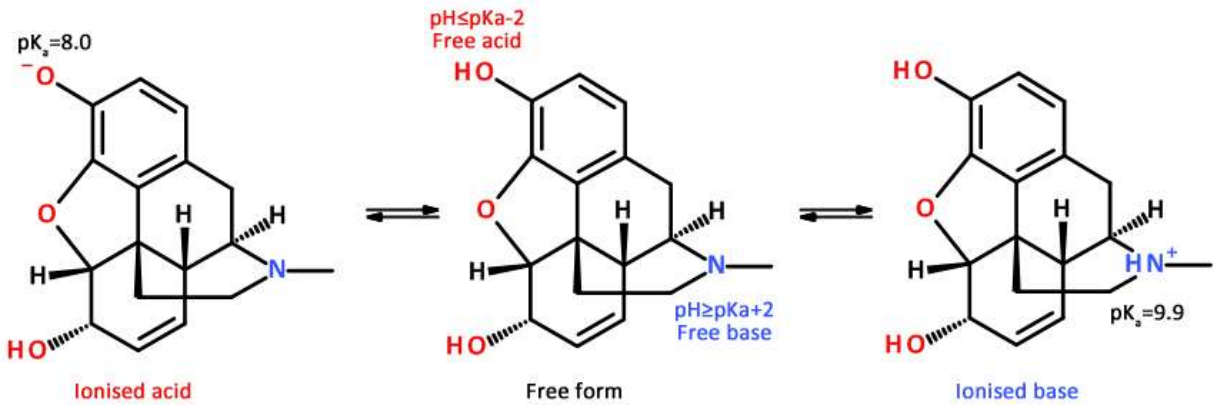

FIGURE 1.15 FREE FORM AND IONISED STATES OF MORPHINE

As can be seen in Figure 1.15, morphine has two types of functional groups, a tertiary amine as well as a phenolic hydroxyl group. The pK_a values for the amine and phenolic hydroxyl groups are 9.9 and 8.0, respectively. [53] When the pH of a solution increases, the proportion

of free amine groups will also increase. At 2 pH units above the amine functional group's pK_a, approximately 100% of all amine groups will be in free base form. The same principle can be applied to the phenolic hydroxyl group of morphine, which has a pK_a of 8.0. At 2 pH units below the phenolic hydroxyl pK_a, approximately 100% of all phenolic hydroxyl groups will be free acid (in non-ionised) form. According to Zhang *et. al.* [54], tertiary amine groups can readily be protonated to give rise to a positively ionised nitrogen group. Figure 1.16 is an author drawn illustration of the morphine ion species present at any given pH.

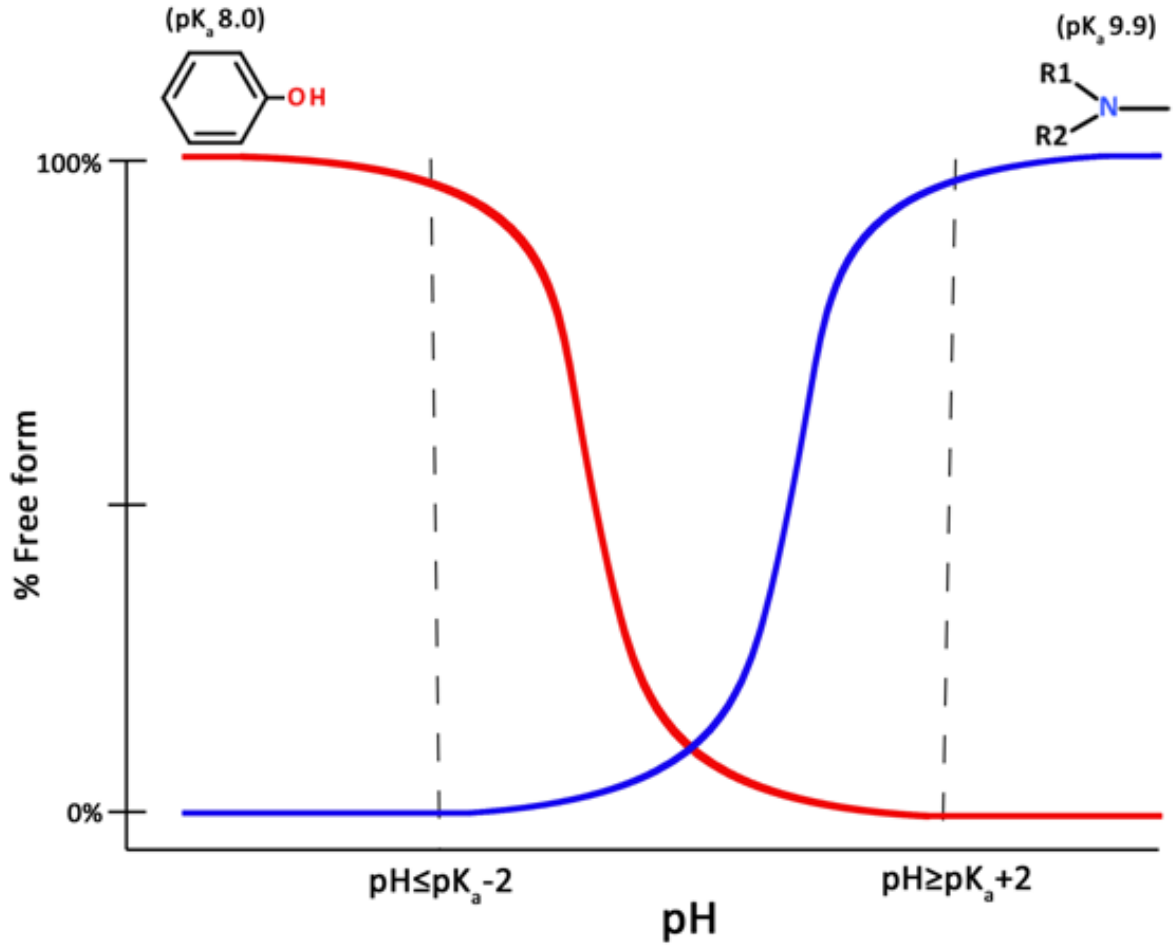

FIGURE 1.16 PERCENTAGE FREE FORM OF MORHPINE FUNCTIONAL GROUPS RELATIVE TO AN INCREASE IN PH

The above figure is a graphical representation of the degree of ionization for morphine's functional groups as a result of changing pH. At low pH, the amine groups are ionized, and as the pH increases, more amine groups become available in free base form. As the pH increases further, the free acidic phenolic hydroxyls become ionized.

The next example that will be discussed is 11-nor-9-carboxy-Δ^9-THC. As the name suggests, this compound contains a carboxyl group, which has a pK_a of 4.5 and can be ionised. [55] This group will be in free acidic form (non-ionised), below a pH of 2.5. This is important as the free form of the metabolite is of interest. The mixed mode SPE cartridges used contain both ion exchange and reverse phase sorbent sites. If 11-nor-9-carboxy-Δ^9-THC is ionised, it carries a negative charge, which will not interact with the negative sulfonic acid groups present on the sorbent of the cartridge. If, on the contrary, 11-nor-9-carboxy-Δ^9-THC is protonated and in free form (non-ionized) at a pH of 2.5, no interaction will occur between 11-nor-9-carboxy-Δ^9-THC and the negative sulfonic acid groups. The free form of 11-nor-9-carboxy-Δ^9-THC is also less water soluble and more lipophilic. The lipophilic divinylbenzene (reverse phase sorbent) will be the functional group that facilitates binding through van der Waals interactions occurring between the free form 11-nor-9-carboxy-Δ^9-THC and the divinylbenzene polymer. In Figure 1.17, a depiction of both the free and ionized forms of 11-nor-9-carboxy-Δ^9-THC is shown.

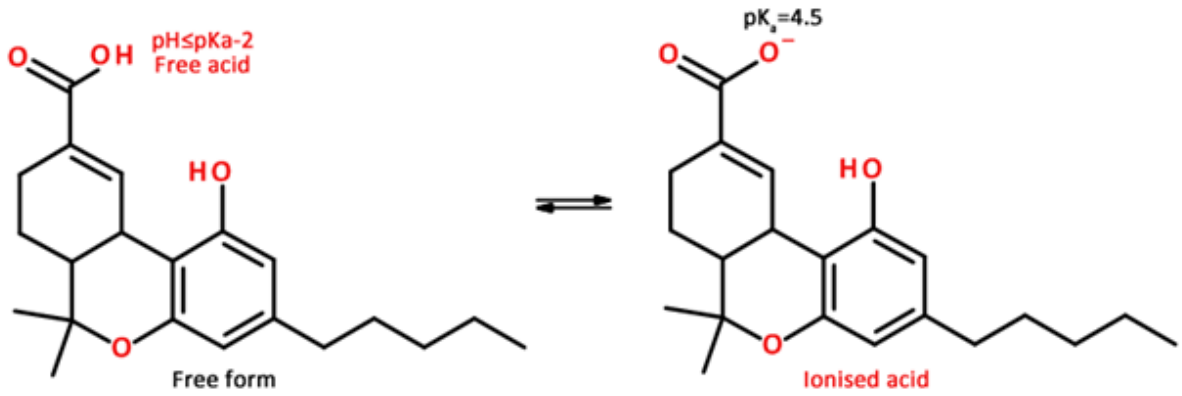

FIGURE 1.17 FREE FORM AND IONIZED STATES OF 11-NOR-9-CARBOXY-Δ^9-THC

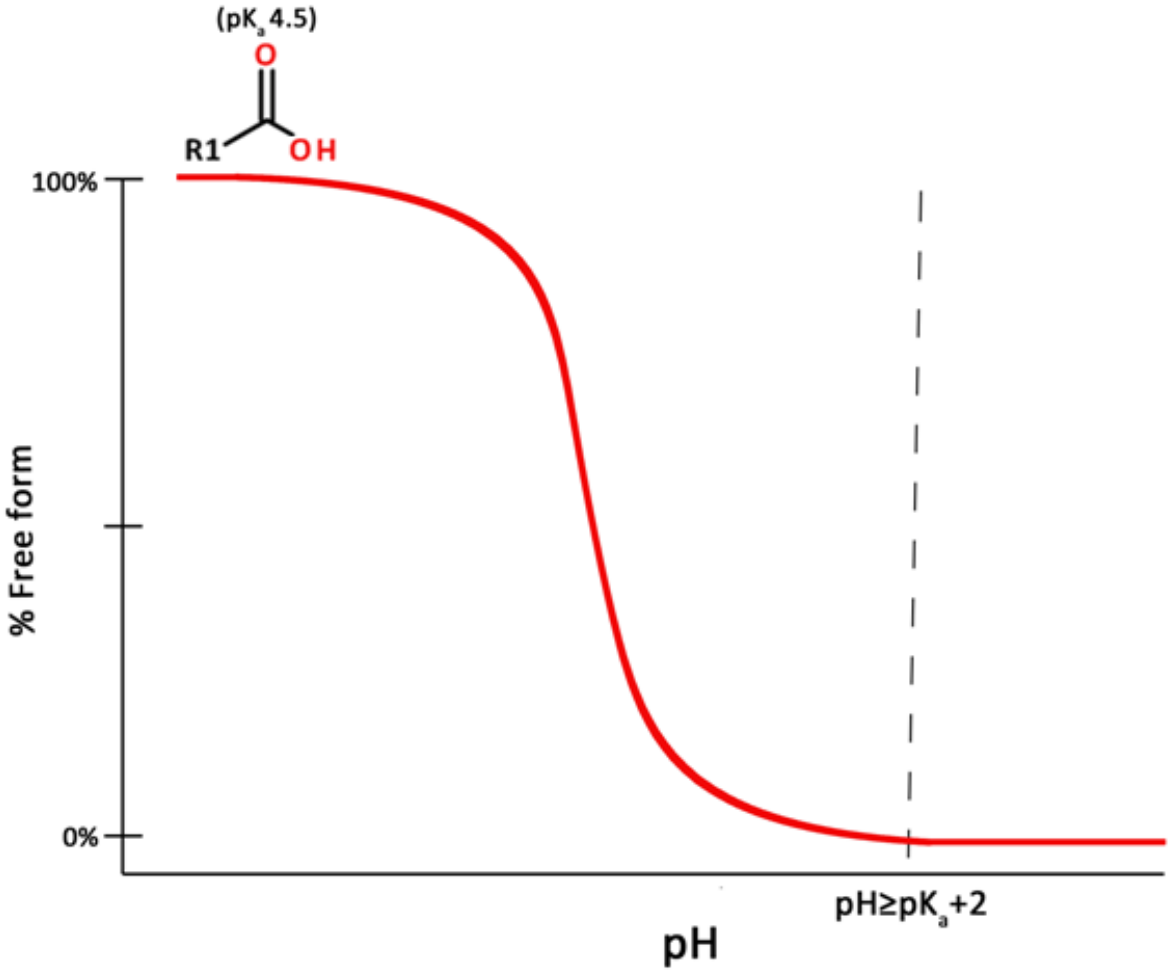

FIGURE 1.18 PERCENTAGE FREE FORM OF 11-NOR-9-CARBOXY-Δ9-THC FUNCTIONAL GROUP RELATIVE TO AN INCREASE IN PH

As seen in the pK_a curve, complete ionisation of the carboxyl group will occur at $pH \geq pK_a+2$. A $pH \leq pK_a-2$ value will ensure that approximately 100% of the metabolite is in non-ionised

form. With regard to all of the analytes studied, each respective pK_a value is provided in Table 1.4.

TABLE 1.4 pK_A VALUES OF THE MEASURED ANALYTE METABOLITES

Analyte	pK_a
benzoylecgonine	-
morphine	8.0, 9.9
codeine	8.2
6-monoacetyl morphine	-
11-nor-9-carboxy-Δ^9-THC	4.5

1.4.3 DERIVATIZATION

As mentioned in Section 1.2.3, all compounds entering a biological system are transformed via enzymatic Phase I and Phase II reactions to diminish the compounds' lipophilicity and ease excretion. When compounds are in their metabolized hydrophilic forms, they are rarely suitable for direct injection into a gas chromatographic system. The polar groups present frequently decrease the volatility as well as thermal stability of a compound, both important factors for GC analysis. Certain types of hydrogen atoms complicate GC analysis due to the fact that they are able to form hydrogen bonds with active sites on the column as well as on the inlet liner. [15] Derivatization is the process of substituting certain functional groups of an analyte molecule, with the aim of improving its chromatographic and/or detection properties. This is accomplished by increasing an analyte's molecular weight, thermal stability, and volatility, as well as decreasing its polarity. [56] One of the most advantageous characteristics of employing derivatization reactions is the increased selectivity of the analytes. The derivatization reaction mechanism introduces selectivity through the addition of molecular fragments to certain analyte functional groups. These derivatives also produce improved fragmentation patterns and better ionisation of analytes, which in turn results in improved mass spectrometric detection. [15] As a result of the derivatized analyte having a larger structure than the original underivatized counterpart, the daughter ions that are produced by the electron impact have a larger mass-to-charge ratio. The increased m/z in turn helps to separate the analyte from the low m/z interfering matrix compounds. Analytes suffering from stability issues may also be derivatized to increase their stability.

Disadvantages of derivatization include increased analysis and sample preparation time, increased cost due to additional reagents used, and finally, low derivative yields. If more than one functional group is present on the analyte, mixed derivatives may be formed. In some cases, by derivatizing a molecule, another analyte is manufactured. This is the case when morphine is derivatized using acetic anhydride. The acylation reaction produces heroin, also known as diacetylmorphine. In light of these drawbacks, the criteria for successful derivatization include the following:

- Selective derivative formation
- High reaction yields
- Stable derivatives

Different types of reaction mechanisms are employed for different types of derivatization reagents. The four main types of derivatization reactions are silylation, acylation, alkylation and esterification. [56] The three types of derivatization reactions employed in this study are silylation using Methyl-N-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA), acylation using propionic anhydride (PA), and esterification using pentafluoropropanol (PFPOH). A variety of different derivatization reagents were tested in this study; propionic anhydride and pentafluoropropanol yielded the best results with the minimum amount of interferences.

1.4.3.1 SILYLATION

Functional groups that have relatively acidic hydrogens are termed protic. Nearly all functional groups exhibiting a protic nature are able to react with a silylating agent to form either silyl esters or ethers. [15] This type of derivatization reaction is thus the most widely used. Silylation occurs by proton displacement in the following functional groups (COOH, OH, NH, SH) by an alkylsilyl. The silylation usually forms either a trimethylsilyl (TMS) or a tert-butyldimethylsilyl (TBDMS) derivative. The abilities of various hydrogen-containing functional groups to form a silyl derivative are as follows:

alcohols > phenols > carboxylic acids > amines > amides.

Trimethylsilyl derivatives are commonly used because of their chemical stability, thermal robustness, and volatility—all of which are important for gas chromatographic analysis. [57]

Derivatization reagents may include the following: *N,O*-bis- trimethylsilyl-trifluoroacetamide (BSTFA), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), and Methyl-N-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA). The former two are the most widely used derivatization agents, and the latter, MTBSTFA, was developed to increase the hydrolytic stability due to an apparent disadvantage that silylating agents possess. All sylilating reagents are sensitive to moisture and as a consequence, procedures are followed to ensure anhydrous reactions as well as storage conditions. The main drawback of MTBSTFA is the difficulty it exhibits in derivatizing sterically-hindered functional groups. As mentioned previously, a TBDMS group is donated in the reaction of MTBSTFA, and trifluoroacetamide is produced as by-product. MTBSTFA is used to derivatize hydroxyl, carboxyl, and thiols, as well as primary and secondary amines. These derivatives usually have high *m/z* ratios, thus adding the desired selectivity. TBDMS derivatives are usually characterized by a $[M-57]^+$, molecular mass minus a *tert*-butyl group. [57] The fragmentation of ions occur in the mass spectrometer ion source and the $[M-57]^+$ ion is usually an indication of a TBDMS derivative. The metabolite derivatized by means silylation in this study is 11-nor-9-carboxy-Δ^9-THC, specifically at the hydroxyl and carboxyl functional groups. Both of these functional groups react with MTBSTFA, and a TBDMS group is added while a hydrogen atom is displaced. The reaction mechanism is illustrated in Figure 1.21.

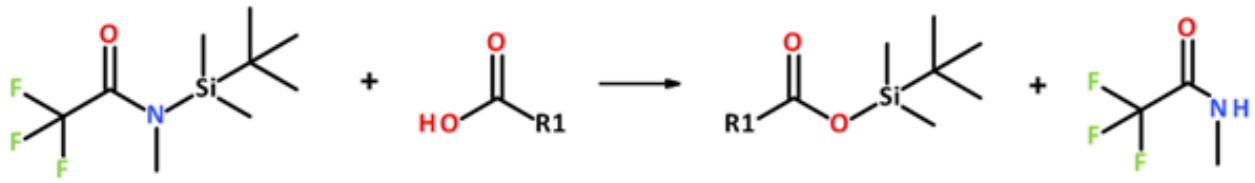

FIGURE 1.21 REACTION MECHANISM OF MTBSTFA WITH A CARBOXYL FUNCTIONAL GROUP.

As seen in Figure 1.21 above, a *tert*-butyldimethylsilyl (TBDMS) group is added to the carboxyl functional group of the metabolite, and trifluoroacetamide is produced by the reaction. The trifluoroacetamide by-product is both non-ionized and volatile. [57] In the derivatization of 11-nor-9-carboxy-Δ^9-THC, both the carboxyl as well as the hydroxyl groups are derivatized. A non-polar derivative is formed that exhibits improved chromatographic properties. This derivative is illustrated in Figure 1.22. It should be noted that this is the

only derivative in this study formed by means of silylation. A m/z of 572 is achieved through derivatization with MTBSTFA.

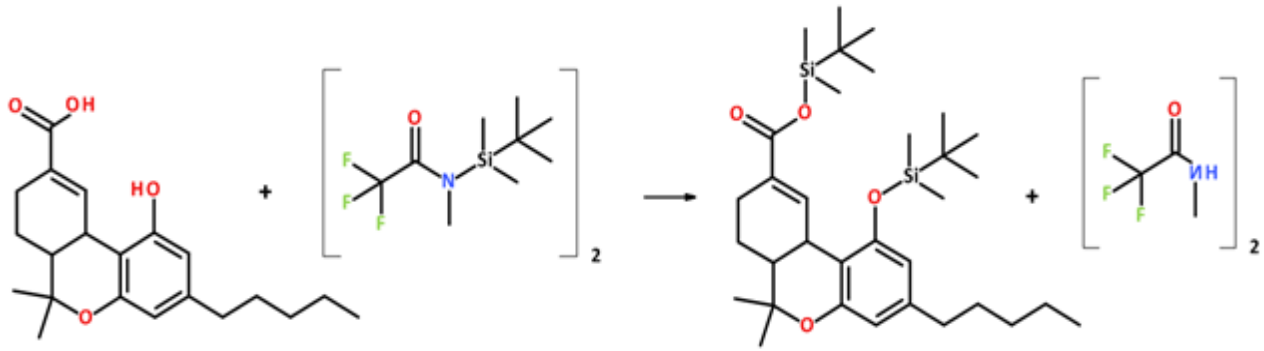

FIGURE 1.22 11-NOR-9-CARBOXY-Δ^9-THC TBDMS DERIVATIZATION REACTION AND BY-PRODUCTS

1.4.3.2 ACYLATION

The reagent propionic anhydride employs an acylation reaction mechanism to attach a propyl group to any alcohol, phenol, or amide analyte functional groups. Acylation can be carried out using three types of reagents: acyl halides, acylated imadazoles, and acid anhydrides. [15] As a result of the strong acidic nature and by-products of acid anhydrides and acyl halides, in this study they are accompanied by a proton acceptor such as pyridine or dimethylaminopyridine (DMAP). Acid anhydrides are preferred to acyl halide because of the ease of removing the acidic by-products, which may interfere and diminish GC column lifetime. The acidic by-products are removed by evaporation, facilitated by their high volatility. The reaction mechanism involving propionic anhydride and an alcohol or phenol group is shown in Figure 1.23.

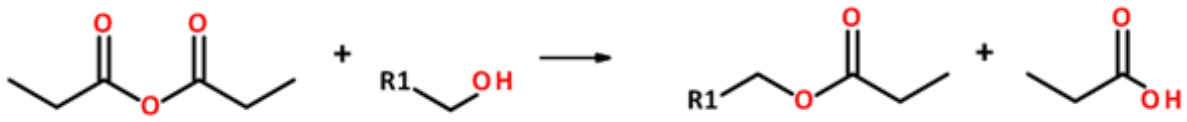

FIGURE 1.23 REACTION MECHANISM INVOLVING ACID ANHYDRIDES AND ALCOHOLS

Propionic acid is one of the by-products formed from this derivatization reaction. This excess propionic acid is volatile and should be removed prior GC analysis since the compound will degrade the GC column. The surplus hydrogen protons formed by the reaction are usually neutralized by a proton acceptor such as pyridine or DMAP. The

chemical structures of the opioids measured in this study, as well as their respective acylated derivatives, are shown in Figure 1.24. Propionic anhydride was used as the derivatization agent in addition to DMAP. The phenolic hydroxyl functional group is where derivatization takes place. 6MAM as well as codeine can only form one type of derivative; conversely, morphine is able to form three different types. With morphine, a propionic group can be attached to either the number 3 phenolic or the number 6 hydroxyl group. If a propionic group is attached to both phenolic groups, a complete or full derivative is formed. A derivative formation where a propionic group is attached to both the phenolic and hydroxyl functional groups is favoured.

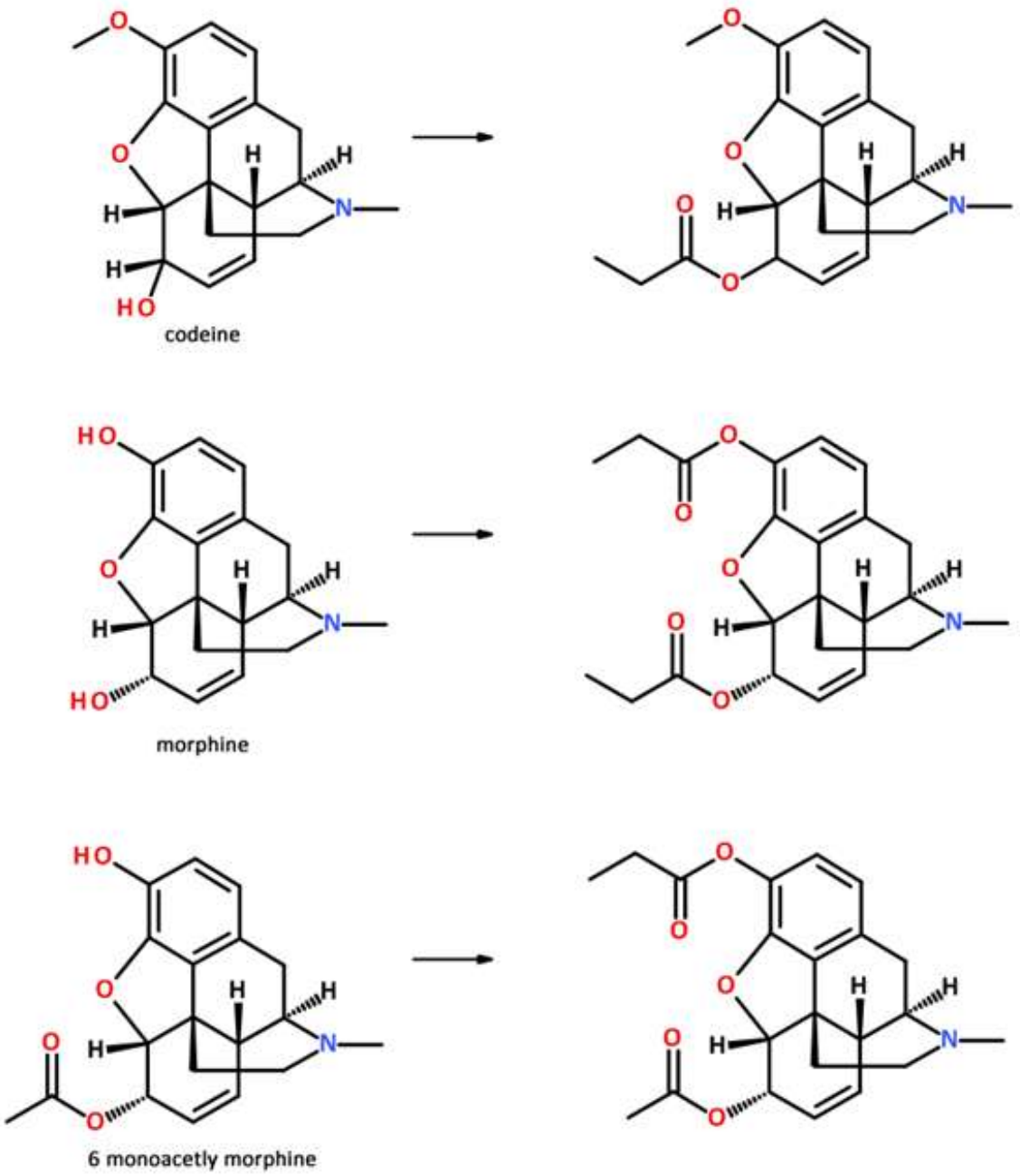

FIGURE 1.24 CHEMICAL STRUCTURES OF OPIOIDS ANALYTES AND THEIR ACYL DERIVATIVES.

1.4.3.3 ALKYLATION

Alkylation involves the exchange of an active hydrogen atom with an alkyl group, or in some cases, an aryl group. [57] The replacement of the hydrogen atom can either form an ester or an ether. [15] The functional groups that can form alkyl derivatives are the following: carboxylic acids, alcohols, thiols, phenols, primary and secondary amines, amides and sulfonamides. Methylation is of interest when employing gas chromatography as result of these derivatives being low in molecular mass and being volatile. Alkyl halides are some of

the most popular alkylation reagents employed to form derivatives. These include aliphatic bromides and iodides, as well as benzyl and substituted benzyl bromides. [57] Esterification by means of alcohols was employed in this study to derivatize the benzoylecgonine carboxyl group.

High molecular mass alcohols as well as halide-containing alcohols are utilized to obtain a derivative's molecular mass. Most of these alkylation reactions need to take place in the presence of a catalyst such as potassium carbonate or pyridine. [15] Carboxylic acid esterification is accomplished by means of an acid catalyst (hydrochloric acid or sulfuric acid) that is added to the alcohol reagent. PFPOH is the alcohol that is used for the derivatization of benzoylecgonine. In the study, since propionicanhydride was used to derivatize the opioid metabolites, the propionic acid by-product served as the acid catalyst for the esterification of benzoylecgonine. Both propionicanhydride as well as PFPOH were added to the metabolite mixture to form derivatives of both opioids as well as benzoylecgonine.

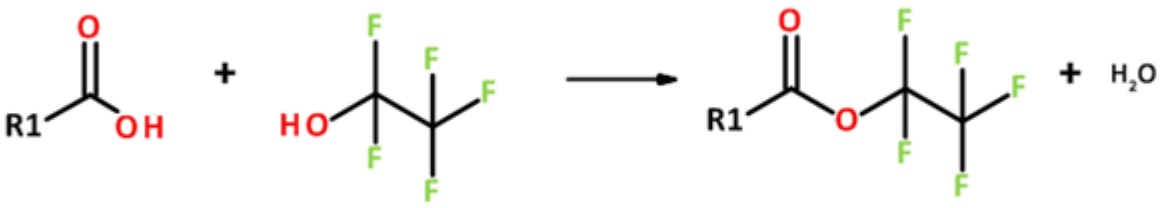

FIGURE 1.25 REACTION MECHANISM OF ALCOHOLS WITH CARBOXYLIC ACIDS

As seen in Figure 1.25 above, alcohols react with carboxylic acids to form alkyl esters, and water is produced as a by-product. This same reaction is used to derivatize benzoylecgonine to form a pentafluoro ester complex. Although benzoylecgonine has two ionisable functional groups, only one has the potential to be derivatized via alkylation. The esterification reaction can be seen in Figure 1.26. Benzoylecgonine was the derivative with the lowest m/z in this study and consequently eluted first with the shortest retention time.

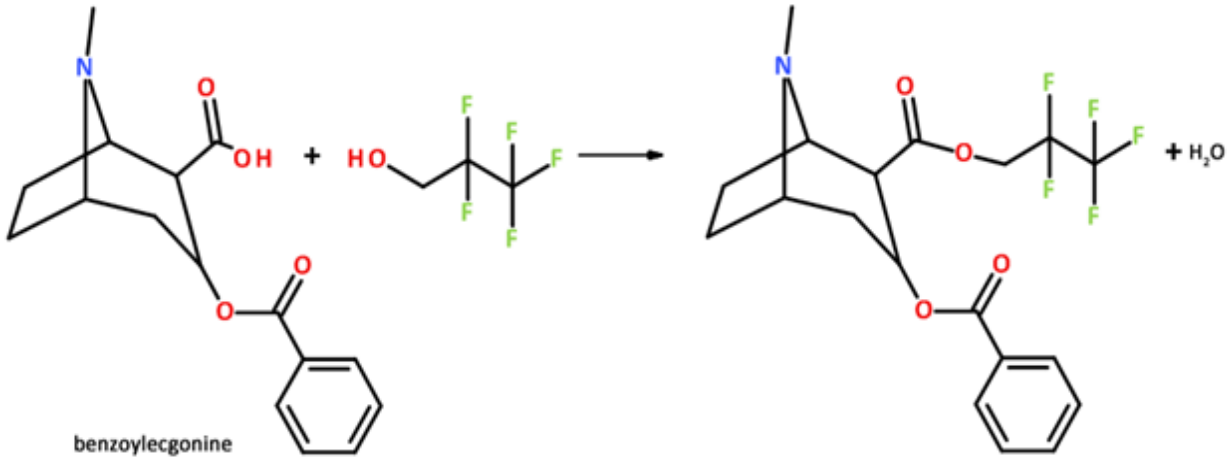

FIGURE 1.26 BENZOYLECGONINE PENTAFLUORO DERIVATIVITAZION REACTION AND BY-PRODUCTS.

In summary, SPE is used to extract analytes from the wastewater matrix by means of ion exchange and reverse phase interactions. The procedure also concentrates the analytes in the elution solvent. After the extraction, analytes of interest are derivatized to increase their selectivity and chromatographic performance. Derivatization reaction by-products need to be removed before injecting the sample into the instrument.

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2. EXPERIMENTAL

2.1 METHOD DEVELOPMENT AND MATERIALS USED

2.1.1 INTRODUCTION

Even though an abundance of literature is available on the analysis of drugs of abuse in wastewater, gas chromatography (GC) as a separation technique is not commonly used. The favoured technique of separation for this type of analysis is liquid chromatography due to the ease of sample preparation and the ability to separate analytes without derivatization. The sensitivity offered by liquid chromatography coupled with dual mass spectrometry is also unparalleled. Each molecule has a unique fragmentation pattern, and when two mass spectrometers are coupled in series, the transition from one mass fragment to another can be examined. Furthermore, the transition from each first dimension mass fragment to second dimension fragment is also unique. The mass spectral transitions present in tandem mass spectrometry offer the ability to measure very low concentrations (high sensitivity) while separating analytes from the matrix with high efficiency (selectivity). Selectivity is also provided by the separation technique employed before measured on the mass separation detector. Both Liquid as well as gas chromatography provide additional selectivity to analytical instrumentation. Nonetheless, if such instrumentation is not available, as was in the case of this study, alternative instrumentation must be employed. In this study, a Leco (LECO Africa (Pty) Ltd 3 Vuurslag Ln, Spartan extension 7) two-dimensional gas chromatographic system coupled with time-of-flight mass spectrometry (GCxGC-TOF/MS) was employed to attain the selectivity and sensitivity required for wastewater analysis. Although a variety of literature is available on the subject of wastewater analysis, very limited literature was found to have employed gas chromatography as separation technique for the analysis of drugs of abuse specifically. Method development for the use of gas chromatography (GC) for separation required a different approach to sample work-up. Using GC, samples needed to be derivatized. Different sample clean-up and extraction procedures also needed to be followed. Ultimately, the epidemiological results from this study matched those obtained using liquid chromatography. However, since the means of

obtaining those results differed considerably, the developed method can be considered as novel in every regard.

2.1.2 METHOD DEVELOPMENT

The complete analytical method for obtaining adequate separation, selectivity and sensitivity was developed in this study. The method's development, from sampling to analysis, will be discussed in this Section.

2.1.2.1 WASTEWATER SAMPLING

Sampling wastewater involves an assortment of factors that need to be considered in order to obtain a sample that is representative of the population. The Daspoort wastewater plant receives about 50 million litres of influent per day, a figure roughly estimated from the flow data obtained. This influent flow data can be found in the results Section of the dissertation. The average human consumption of water in an average developed area is about 130 L per person. [(1)] Taking the total influent divided by average daily water use, gives us a figure of approximately 385 000 residents. This influent is processed at the treatment plant by first removing solid and synthetic aggregates such as toilet paper, after which the influent is divided equally between a number of sediment pools where solid organic waste particles are removed. After solid organic waste is removed the wastewater is transferred to biofilters. Each sediment pool runs on a 20 minute cycle where the influent gets pooled until the batch is transferred to the biofilter. Sampling took place after sedimentation and prior to biofiltering (Figure 2.1).

FIGURE 2.1 WASTEWATER SEDIMENTATION POOL WHERE SAMPLING TOOK PLACE

Sampling was executed using an ISCO auto-sampler (Teledyne Isco, Lincoln, NE 68504 U.S.A.) with 24 sampling bottles. The sampler collects the wastewater and a robotic arm rotates in a 360° arc to dispense the wastewater samples into the bottles. The sampler is equipped with a liquid detector that is used to measure the volume of sample being dispensed at a given time. It also has a control unit from which the sample volume and frequency can be set. In order to collect the water, a plastic tube is connected to the sampler at one end and submerged in the wastewater at the other end. This tube is used to transfer the sample from the wastewater pool to the sampling bottles. The dimensions, including the internal diameter and length of the tube, are programmed into the sampler control unit. The passage of wastewater sample through the tube is controlled by a peristaltic pump, which is also able to reverse direction of flow. When a cylinder is placed into a body of water, the air in the cylinder initially displaces some of the water until the pressure is equalized, upon which time the cylinder is filled with water until it is level with the surrounding body of water. Capillary force also facilitates the movement of water into the sampling tube when submerged. When a sample is collected by the ISCO sampler, the three-meter plastic collection tube is filled with wastewater. If successive samples are to be taken at this time,

the water present in the sampling tube is collected rather than a “fresh” wastewater sample from the sedimentation pool. However, the ISCO auto sampler follows a simple yet extremely effective procedure for producing a fresh sample, rather than collecting an old sample from the sampling tube. Since the peristaltic pump is able to reverse itself, a reverse pump cycle is implemented to discharge any residual wastewater from the sampling tube before sampling commences. The peristaltic pump is then immediately reversed and a forward pump cycle is initiated. Wastewater is pumped up the collection tube until the liquid detector is reached, at which time a given volume of wastewater is collected. After collection, a second reverse pump cycle is implemented to expel the remaining wastewater from the sampling tube. Figure 2.2 shows the Isco auto sampler setup.

FIGURE 2.2 ISCO AUTO SAMPLER USED FOR ACQUIRING WASTEWATER SAMPLES

One litre samples were collected every hour during a 24-hour cycle starting at 6am on a Monday morning, over a period of 7-days. The 24-hour samples were pooled together to form a 24-hour representative sample for one day.

2.1.2.2 WASTEWATER MATRIX PRE-TREATMENT.

The full wastewater matrix pre-treatment step will be discussed later in the chapter, because of interferences encountered during method validation.

The pH of the pooled wastewater samples was adjusted to 2.5-3 using 6 M HCl and the pool stored at 1-2 °C. It should be noted that sodium phosphate was initially employed for pH adjustment. However, after the eluate from SPE was dried, sodium phosphate crystals were observed. Changing the acid used for pH adjustment solved this problem, as HCl evaporated readily and no residue was observed. The wastewater matrix was then filtered using 1.6 µm pore size glass fibre filter paper (Whatmann GFA, Merck (Pty) Ltd, Modderfontein, South Africa). If filtration was not implemented, the SPE cartridges would have become obstructed by fine particulate and adequate flow rates could not be maintained. Nylon and cellulose acetate filters showed decreased recovery characteristics, as the drugs of abuse analytes interacted with these filters and bound irreversibly. The same irreversible binding is described by Lindenberg, M *et. al.* [(2)] The glass fibre filters, on the other hand, can be classified as inert, not interacting with the analytes of interest. Following wastewater filtration, 600.00 ml sample volumes were aliquoted into volumetric flasks.

The deuterated internal standard was initially added to the 600.00 ml sample after the filtration process. A variation of this step will be discussed later in the chapter after method validation results are discussed.

The 600.00 ml sample volume was chosen for subsequent SPE because tests revealed that breakthrough of the 11-nor-9-carboxy-Δ^9-THC occurs at volumes greater than 600.00 ml. To test this, different volumes of wastewater matrix (250.00 ml, 500.00 ml, 600.00 ml and 700.00 ml) were aliquoted into separate volumetric flasks. Sample preparation was performed for each of the volumes and the concentrations of the four samples were plotted against volume. The results indicated that after 600.00 ml of wastewater is passed through the SPE cartridge, no further adsorption of the THC metabolite takes place, i.e., a plateau is reached.

A 100.00 µg aliquot of deuterated internal standard for each measured analyte was added to 600.00 ml of wastewater matrix, giving a standard concentration of 166.00 µg/L. The samples were then agitated using a magnetic stir bar for 10 minutes to ensure proper homogenization of the wastewater and the added deuterated standard.

2.1.2.3 SOLID-PHASE EXTRACTION

The following SPE method was developed and used to extract drugs of abuse metabolites from the wastewater matrix. Phenomenex™ Strata c-x™ SPE cartridges were used. These cartridges were syringe type with a reservoir volume of 6ml and a sorbent bed volume of 500 µg. Dichloromethane was used as the first conditioning solvent, after which an intermediary solvent miscible in both dichloromethane and water—methanol—was used as the second conditioning solvent. 6 ml of dichloromethane followed by 6 ml methanol followed by 6 ml 0.1 M HCl (pH 3.5-4) was used to condition the SPE cartridge. This transition step was needed for the aqueous matrix to be able to interact with the bonded phase and not be repelled by dichloromethane. After conditioning, the cartridge bonded phase was subjected to a buffered solution of deionized water containing HCl. This solution was first adjusted to pH of 3.5-4 (0.1 M HCl) to ensure that the cartridge was at approximately the same pH as the adjusted sample matrix.

2.1.2.3.1 LOADING WASTEWATER SAMPLE

The sample loading procedure was executed using a E2M1.5 two stage rotary vane pump (Edwards Crawley, West Sussex, UK) connected to a SPE manifold (Waters Corporation, Milford, MA, USA). Sample loading commenced by means of bottle feeding. Specially constructed teflon tubing as well as syringe plungers were used to fashion air tight seals around the bottoms of the syringe SPE cartridges. The teflon tubes extended from the syringes to the bottom of the sample volumetric flasks. The sub-ambient pressure maintained by the pump ensured continuous suction and flow of sample from the volumetric flasks through the SPE packed beds (Figure 2.3).

FIGURE 2.3 SPE MANIFOLD SETUP, WITH VOLUMETRIC FLASKS FILLED WITH WASTEWATER SAMPLES.

A solvent trap was positioned between the SPE manifold and the vacuum pump to avoid the passage of liquid through the vacuum pump. Samples were loaded onto the SPE cartridges overnight at a pressure of less than 2 inches of mercury. As sample loading progressed the SPE cartridge frit saturated with sub 1.6 µm particles, this in turn caused variation in loading flow rates for each individual SPE cartridge. Because the flow rate for each individual SPE cartridge cannot be measured, although not ideal, it was decided that constant sub-ambient pressure will be maintained. Slow sample loading ensured adequate interaction of the sample analytes with the resin of the SPE cartridges. In addition to breakthrough of cannabinoids occurring at sample volumes higher than 600.00 ml, these higher sample volumes caused obstruction of the syringe frits because they contained small solid particles (<1.6 µm). This blockage was more evident after large sample volumes had been loaded. With the sample volume of 600.00 ml, the obstruction was not as pronounced and workable flow rates could be achieved more easily. It should also be noted that if analyte loss did occur during filtration, through interaction with teflon lines, or during the loading phase, the loss of deuterated internal standard will be proportional to the loss of analyte. When the

ratio of analyte to deuterated internal standard is calculated, the deuterated internal standard compensates for any variation caused by loss of analyte.

2.1.2.3.2 WASHING THE SPE CARTRIDGE

Each cartridge was washed with 10.00 ml of 0.1 M HCl buffer, after which a 10.00 ml 30:70 mixture of acetonitrile (Acn) and 0.1 M HCl was loaded. The Acn:HCl mixture ensured that all of the buffer solution had been expelled from the packed bed, in addition to removing interfering matrix compounds. The wash solution exhibited a yellow to light brown colour. The wash solution was analyzed and found to contain no analytes of interest. The low pH accompanied by the relatively low concentration of acetonitrile ensured that no analytes of interest were eluted during this step. Weakly retained matrix compound did however elute during the washing step, as observed by the colour of the solution.

2.1.2.3.3 DRYING OF THE SPE CARTRIDGE

The Acn:HCl wash step was necessary to obtain reasonable drying time of the cartridges, since acetonitrile exhibits accelerated evaporation and volatility compared to other aqueous solvents. Each cartridge was dried for 30 minutes at a pressure greater than 15 inches of mercury. As explained in section 2.1.2.3.2, variable flow rates are produced for each individual cartridge, because of the saturation of the SPE frits. Instead of reporting the flow rate although not ideal only constant sub-ambient pressure is reported. A condensation line was observed on the outer surface of the plastic cartridges. This condensation line moved down as suction of air continued to flow from top to bottom of the packed bed; furthermore, the line was a good measure of how the drying process was advancing. No analyte losses were expected from the Acn:HCl wash step because of the enhanced affinity the THC analyte showed for the divinylbenzne sorbent. The HCl decreases the pH of the wash solution which in turn promotes the occurrence of the free form THC analyte. The free form of the THC analyte in turn will show increased lipophilicity. This is explained in more detail in the next section.

2.1.2.3.4 ELUTION OF THC METABOLITES

It was thought that the elution of 11-nor-9-carboxy-Δ^9-THC would be increased by a non-polar solvent mix. The first solvents implemented for elution were hexane and ethylacetate

at a ratio of 7:3. Common practice in the literature has shown that the hexane:ethylacetate mixture (Hex:EtAc) should yield the best results. The predicted outcome was not achieved, however, and after a low recovery yield for 11-nor-9-carboxy-Δ^9-THC, a pH-adjusted approach was taken. It was discovered that the THC analyte showed enhanced affinity for the divinylbenzne sorbent functional groups, and thus elution could not be performed using the Hex:EtAc (7:3) mixture. Instead, an increase in pH was used to transform 11-nor-9-carboxy-Δ^9-THC to its ionised form in order to decrease its lipophillicity and reverse binding to the sorbent polymer. It should be noted that with an increase in pH, 11-nor-9-carboxy-Δ^9-THC, benzoylecgonine and the opioids show a decreased affinity for the sorbent. With the adjustment, the acidic analyte functional groups obtain a negative charge and amines revert back to free non-ionised from as the sample pH approaches their pK_a values. This negative charge on the acidic functional groups increases the polarity of 11-nor-9-carboxy-Δ^9-THC, in turn, decreasing the strength of the van der Waals interaction with the DVB backbone. Furthermore, the ionic bonds formed between the positive functional groups of the basic analytes and the negative sulfonic acid groups are broken as basic analyte groups become non-ionised. Thus, it was anticipated that delicate balance existed between obtaining increased recovery yields for 11-nor-9-carboxy-Δ^9-THC and decreased recovery yields in the second opioid elution step. The new solvent mixture consisted of Hexane:Isopropanol (Hex:Isoprop), and the buffers used to adjust pH were 6 M HCl for a pH below 7 and NH_4OH for a pH above 7. All samples were spiked with equal amounts of deuterated internal standard and analyte standard. pH adjusted (4,6,8,10,12 and 14) elution solvents were prepared. An organic elution solvent which was not pH adjusted was also experimented with to compare to the pH adjusted samples. The relative analyte response was then plotted according to the pH of the elution solvent employed for that specific sample. 6MAM was expected to have the lowest concentration in the wastewater samples. A loss in recovery of 6MAM would influence the detection limit of the specific analyte negatively. Thus the relative response of both 6MAM and 11-nor-9-carboxy-Δ^9-THC was calculated and plotted on a cartesian plane, in order to visualize the 11-nor-9-carboxy-Δ^9-THC relative response increasing as 6MAM relative response declines. This experiment was undertaken to study the anticipated increase in THC yields and diminishing opioid recovery.

A mixture of Hex:Isoprop (70:30) with HCl (17.00 µl, 6 M) per 100.00 ml was prepared. 6 M HCl was added to adjust the solvent pH to 5. 10.00 ml of the Hex:Isoprop mixture was aspirated onto the packed bed and no vacuum was applied. Due to the volatile nature of the solvents, acceptable flow rates were maintained by gravity only. After 10.00 ml of eluate had been collected in amber sampling vials, the residual eluate was expelled into the vials by means of a sub-ambient pressure greater than 20 inches of mercury. The first elution step only facilitated the elution of analytes bound to the sorbent by means of van der Waals interactions, i.e., reverse phase interactions.

2.1.2.3.5 Drying of the SPE cartridge

No second wash step was employed prior to elution of the basic ionic analytes. The drying step was merely performed to remove any residual hexane or isopropanol still present on the packed bed. A 10-minute drying time at a sub-ambient pressure pressure greater than 20 inches of mercury was used for the SPE cartridges.

2.1.2.3.6 Elution of basic analytes

The elution of the basic analytes was done using a non-polar solvent. The eluent needed to have its pH adjusted in order to be able to break the ionic bonds between the analytes and the solid phase sorbent. The solvent elution mixture used was dichloromethane:isopopanol:ammonium hydroxide (DCM:Isoprop:NH_4OH) at a ratio of 77:20:3. It was evident that aqueous ammonium hydroxide alone would not be miscible with dichloromethane. Thus, an intermediary solvent, isopropanol, which is miscible with both ammonium hydroxide and dichloromethane, was utilized. The elution solvent was made by dissolving 3.00 ml of ammonium hydroxide in 20.00 ml of isopropanol. These two solvents were mixed thoroughly before 77.00 ml of dichloromethane was added. If ammonium hydroxide and isopropanol were not mixed before addition of dichloromethane, a liquid phase separation occurred. The same liquid phase separation also occurred when larger volumes of ammonium hydroxide were used. A 10.00 ml volume of this elution solvent was aspirated into each cartridge and was allowed to flow through by gravity alone. After all of the eluate had been collected in amber sampling vials, a final sub-ambient pressure greater than 20 inches of mercury was applied to remove all residual solvent from the packed bed.

The mechanism by which the ionic bonds of the analytes were broken was achieved through a pH shift caused by the ammonium hydroxide. The elution solvent had a pH of 12. In the reaction, analyte amine groups are transformed to their free, non-ionised form by donating their protons. Conversely, the hydroxyl and carboxyl functional groups are deprotonated to form their charged ionised form. The transition of amine groups to their free form breaks the ionic bond interactions with the negatively charged sulfonic acid sorbent. A brown residue present on the SPE cartridges eluted during step. This appeared to be matrix compounds which has affinity for the ion exchange mechanism of the cartridge and eluted as a result of the pH adjusted solvent aspirated onto the SPE cartridge.

In this method, analytes are eluted by means of the DCM:Isoprop:NH_4OH solution. The reason for using a volatile organic solvent such as DCM is because this solvent exhibits very fast drying times. 10.00 ml of methanol must dry for about 2-3 hours before a volume of approximately 1 ml is reached. For an equivalent volume, the DCM:Isoprop:NH_4OH solution exhibits a drying time of 30 minutes.

After all of the solid-phase extraction steps were completed, 11-nor-9-carboxy-Δ9-THC was present in its own sampling vial while morphine, codeine, 6MAM and benzoylecgonine were present in their own sampling vial. These sample sets were then processed individually to prepare them for GC-MS analysis.

2.1.2.4 Derivatization

The metabolites of interest in this study cannot be detected or quantitated reliably using gas chromatography without first being derivatized. Thus, this step of the sample work-up procedure can be deemed as having the utmost importance. The following list of derivatization reagents were used in this study (Sigma-Aldrich Corporation, St. Louis, MO, United States of America).

- N-Methyl-N-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA)
- Pentafluoropropanol (PFPOH)
- Pentafluoropropionic anhydride (PFPA)
- Propionic anhydride

The aim was to find a suitable derivatization reagent with the least amount of interference. During all of the tests of derivatization reagents, at least one analyte reacted in a satisfactory manner, while others co-eluted or specific ions were clouded by abundant interfering ions of the same mass. The answer to these limitations was to separate the analytes into two classes; namely, cannabinoids and opioids + cocaine. This separation allowed for silylation of cannabinoids and alkylation plus esterification for the opioids + cocaine group. For the first-eluted cannabinoid groups, MTBSTFA was used as the derivatization agent, adding *tert*-butyl-trimethylsilyl groups to the cannabinoid functional groups. The opioids + cocaine class was derivatized using a combination of propionic anhydride and pentafluoropropanol.

The derivatization procedure consisted of first drying the eluate (elution solvent + analyte mixture). This procedure was completed by placing the amber sampling collection vials under a stream of filtered compressed air at a temperature of 50 °C. Due to the high molecular mass of both the cannabinoid and opioids + cocaine group, an increased drying temperature was chosen to facilitate quicker drying times. The eluates of both extractions were dried under a stream of filtered compressed air until a volume of about 1 ml remained present in the amber sampling vials. The eluate was then transferred to a GC vial and further dried until complete dryness was achieved. This drying step was necessary due to the moisture sensitivity of the derivatization reagent. A dry residue was present at the bottom of the vial for both extractions. After drying, the derivatization procedure differed for the two different class extractions.

The cannabinoid group of analytes was derivatized using MTBSTFA. A volume of 40.00 µl of MTBSTFA was added with 20.00 µl of acetonitrile. This derivatization mixture was then vortexed for 30 seconds to ensure that the dry residue was properly dissolved. The GC vial was then capped and derivatized using microwave assisted derivatization (MAD), which entailed the use of microwave energy instead of the conventionally-used thermal energy.

2.1.2.5 Instrumental analysis parameters

When the sample work-up, including extraction and derivatization, was completed, the samples present in the GC vials were ready to be injected onto the instrument. At first, experiments were carried out using one-dimensional GC coupled with mass spectrometry. However, separation of the analytes was a tedious process and no clear separation of

analytes could be visualized using one-dimensional GC. The instrumental parameters were adjusted to yield maximum performance.

The temperature program was started at 225 °C because of the high boiling point attributed to the analytes of interest. The oven program proceeded to 320 °C at a rate of 3 °C/min and was isothermally maintained at this temperature for 1 minute to ensure that all analytes as well as matrix components eluted off the column. Due to the high boiling point and heavy molecular nature of the derivatized compounds, a high oven temperature was needed in order to ensure complete elution of the analytes of interest. When propionic anhydride was used, all of the analytes of interest eluted off the column at an oven temperature of 310 °C. Based on this elution pattern, it is clear that a GC column capable of high temperature separation must be used. Polar wax columns thus have a clear disadvantage, as they are only able to reach an average temperature of 220 °C.

The glass inlet liner is an important facet that also needs to be considered for the injection of a sample. Most importantly, this liner needs to exhibit inert character; no interaction of sample analytes with the glass silanol groups should occur. The glass inlet liner, and specifically the silanol groups were deactivated by submerging the inlet in a deactivation mixture for 30 minutes and then rinsing the inlet in methanol. The deactivation mixture consisted of 10 % Dimethylchlorosilane in toluene. This ensured proper deactivation of all active sites that could interact with the injected analytes and cause peak splitting, tailing or unsymmetric peaks etc. The importance of deactivating inlet liners is explained by RESTEK®. [3] Also, due to the fact that wastewater samples contain an abundant amount of matrix components and can be classified as “dirty,” it is important to ensure that newly cleaned inlet liners are installed at regular intervals. As the result of the non-volatile nature of some of the matrix compounds, residue deposits are formed on the inside of the inlet line. These adversely affect signal strength, as they are not inert and thus may interact with the analytes of interest. Therefore, in this study, a clean deactivated inlet liner was installed after each 50 injections. A double taper RESTEK® Blue sky split/splitless deactivated glass liner without glass wool was employed. Pulsed splitless injection was used and maintained at 30 psi for 2 minutes after which the carrier gas flow rate was returned to 1 ml per minute. The modulator period was 5 seconds with a hot pulse time of 1.2 seconds.

Column selection determines the separation of analytes and needed to be carefully considered for analysing the samples. A variety of different columns were tested for suitability, including the following:

- Zebron ZB-5 (95% Dimethylpolysiloxane, 5% phenyl)
- Zebron ZB-1701 (86% Dimethylpolysiloxane, 14% cyanopropylphenyl)
- Zebron ZB50 (50% Dimethylpolysiloxane, 50% phenyl)
- Restek Rxi-17sil MS (50% Dimethylpolysiloxane, 50% phenyl)

Different combinations of first as well as second dimension columns were also tested. Due to the popularity and established track record of the ZB-5 column, the ZB-5 (30 meters) with an Rxi-17sil MS (2 meters) in the second dimension was tested first. The separation of the analytes was unsatisfactory and co-elution of analytes with matrix compounds occurred. Co-elution of certain opioid metabolites also occurred. Due to the short length of the Rxi-17sil MS in second dimension, the increased polar nature of the column did not have adequate theoretical plates to facilitate separation of the polar analytes. The separation profile for the aforementioned column configuration yielded a very flat spread of compounds in the y-axis and inadequate separation in the x-axis. It is evident from this type of profile that a more polar first dimension column needed to be implemented to ensure proper separation of both matrix and analyte compounds. The compounds contained in the sample exhibit hydrophilicity; thus, a GC column that would allow greater interaction with the column rather than pure boiling point separation needed to be investigated. The following first dimension columns with increased polarity were tested: the ZB-50 column, which has 50 % more phenyl groups than ZB-5, the Rxi-17sil MS, which has a similar phase as the ZB-50, and finally the ZB-1701. The column that yielded the most promising results was the Restek Rxi-17sil MS. This GC column facilitated more than adequate separation of all analytes, including the cannabinoids, opioids and benzoylecgonine. Thus, in the first dimension a (Rxi-17sil MS, 30 meters, i.d. = 0.25 mm, df = 0.25 µm) and in the second dimension a (Zebron ZB5, 2 m, i.d.=0.25 mm, df = 0.25 µm) were employed. It was expected to find immense overlap of analyte compounds with wastewater matrix compounds and two dimensional gas chromatography coupled with mass spectrometry was employed.

The following figures, Figures 2.4A-C, Figures 2.5A-C, Figures 2.6A-C, Figures 2.7A-C and Figures 2.8A-C are representative chromatograms of all analytes measured. A 3D chromatogram, a colour plot, and a reconstructed 1st dimension chromatogram are provided for each analyte. The quantifier ion for each analyte was extracted and plotted in order to visualize the peaks more clearly. The x-axis is the retention time in the 1st dimension, while the z-axis is the 2nd dimension retention time. The y-axis is the relative abundance of the selected ion. A total ion chromatogram will be shown in Figure 2.11A-C to show massive matrix component overlap.

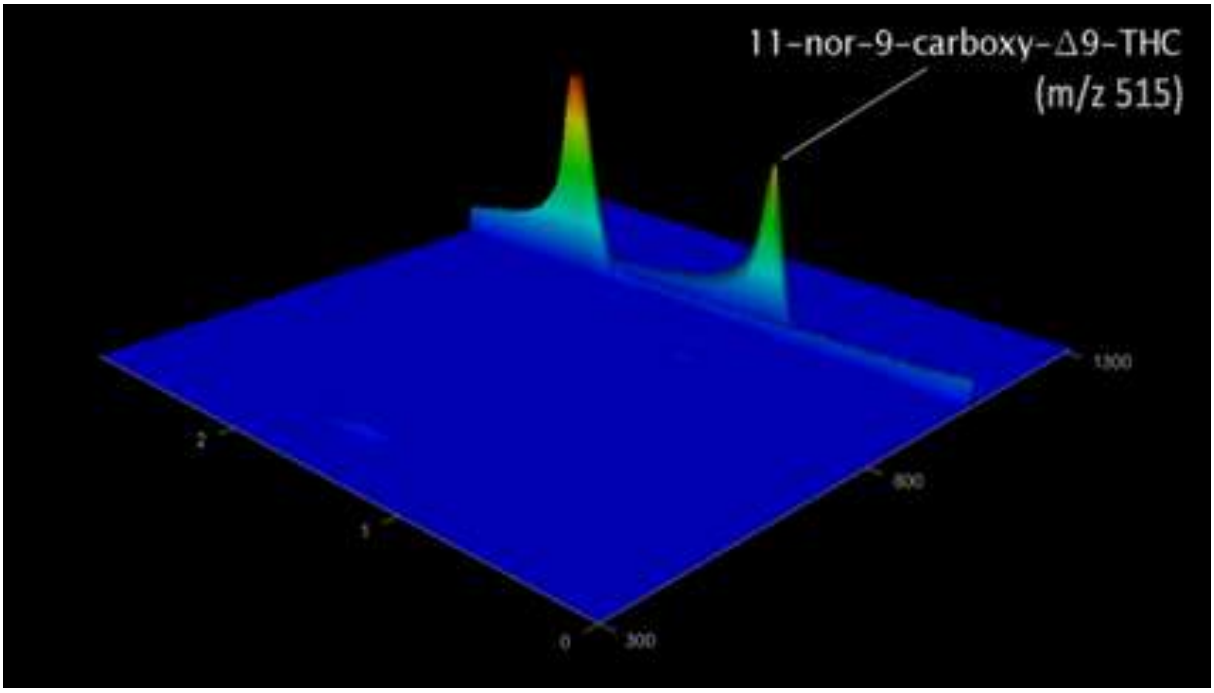

FIGURE 2.4A THREE DIMENSIONAL SELECT ION GCxGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 11-NOR-9-CARBOXY-Δ9-THC USING (M/Z 515). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

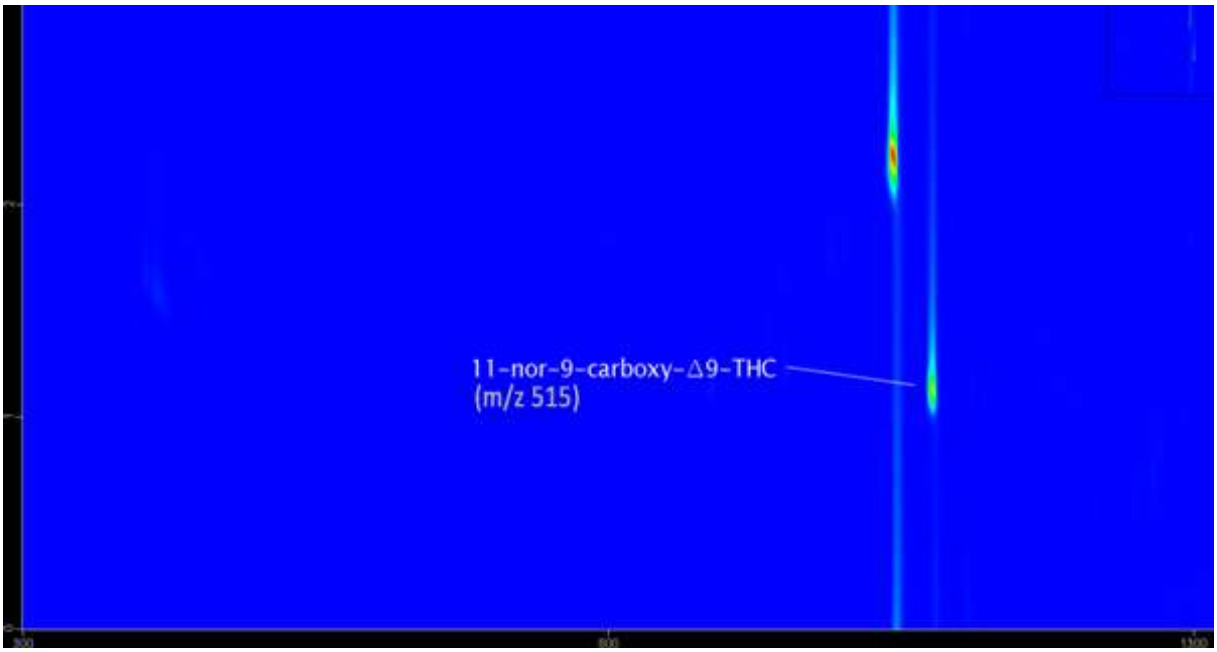

FIGURE 2.4B TWO DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 11-NOR-9-CARBOXY-Δ9-THC USING (M/Z 515). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

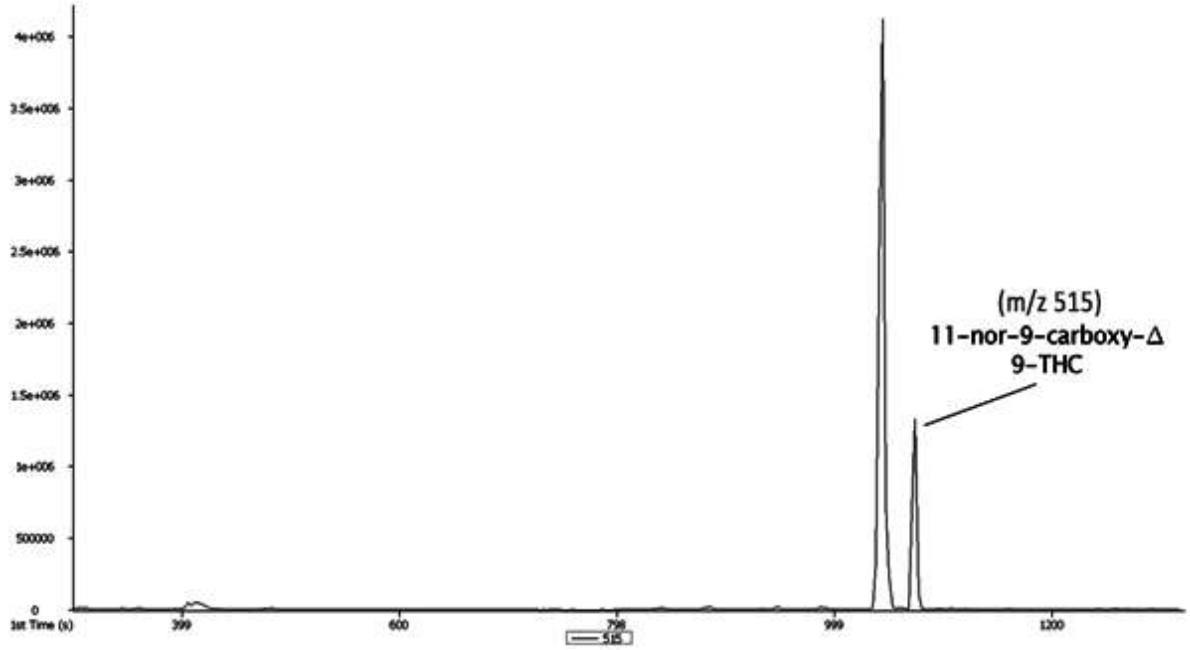

FIGURE 2.4C A RECONSTRUCTED 1ST DIMENSION SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 11-NOR-9-CARBOXY-Δ9-THC USING (M/Z 515). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

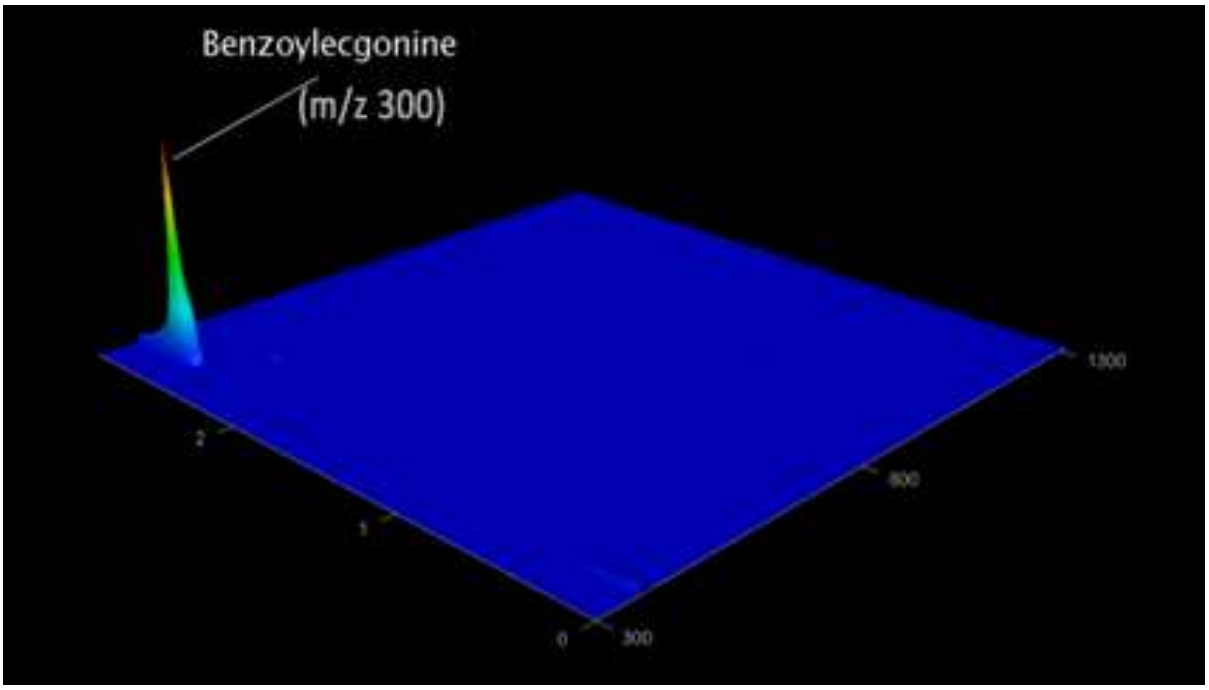

FIGURE 2.5A THREE DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED BENZOYLECGONINE USING (M/Z 300). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

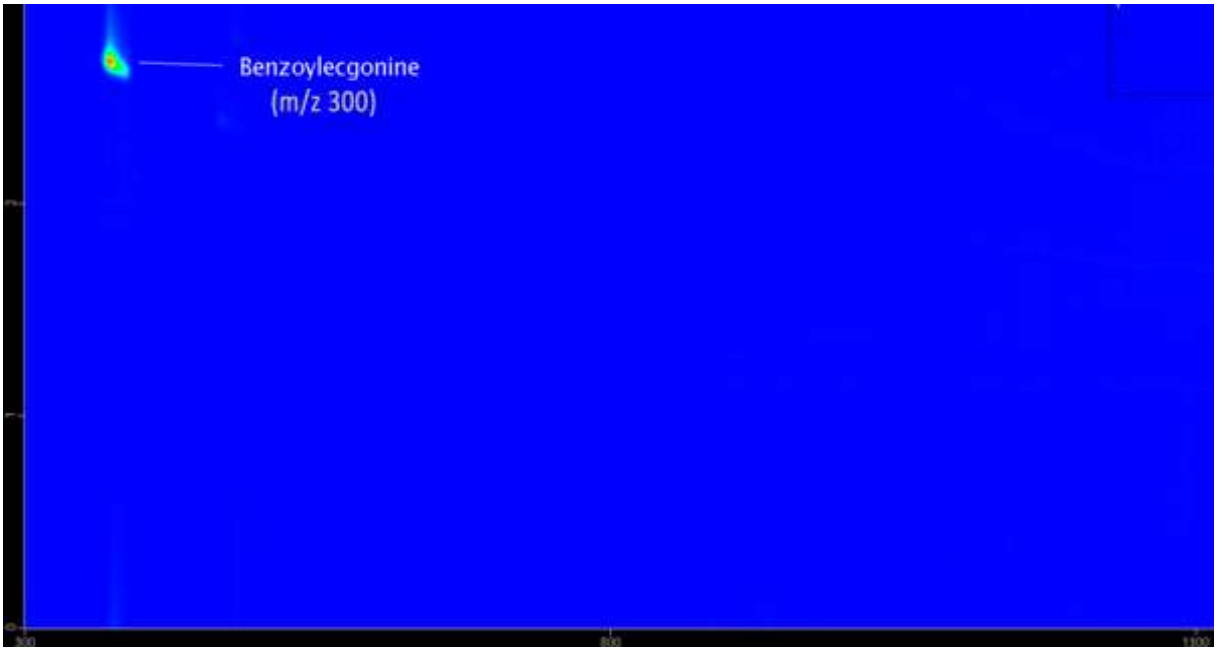

FIGURE 2.5B TWO DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED BENZOYLECGONINE USING (M/Z 300). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

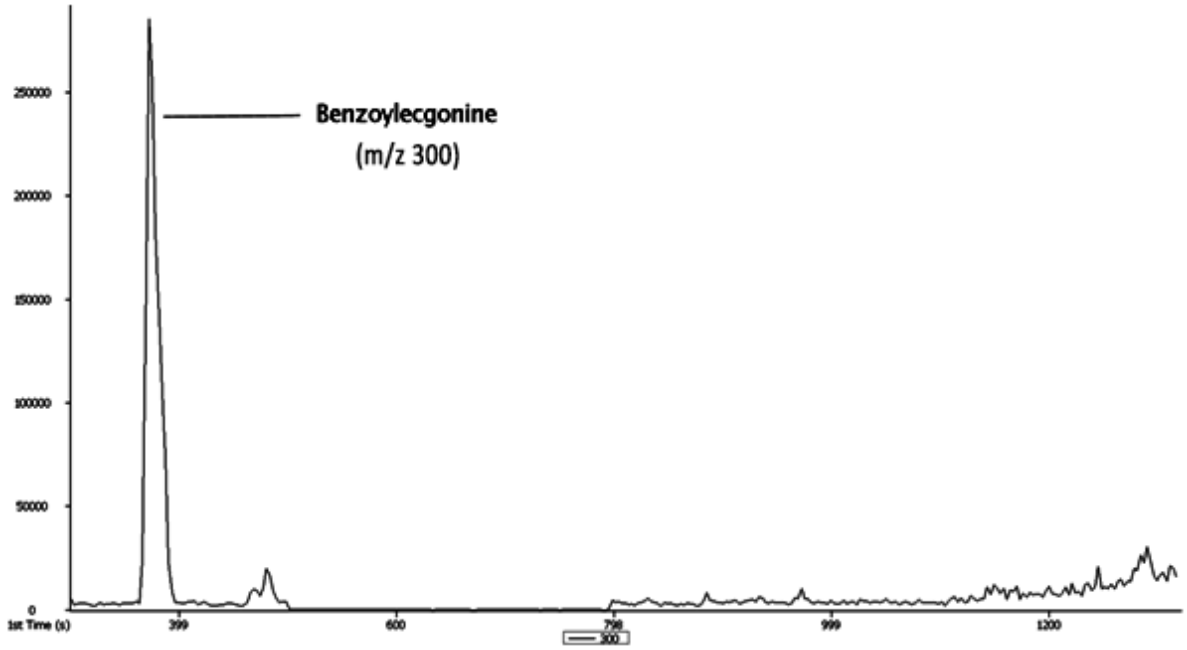

FIGURE 2.5C A RECONSTRUCTED 1ST DIMENSION SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED BENZOYLECGONINE USING (M/Z 300). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

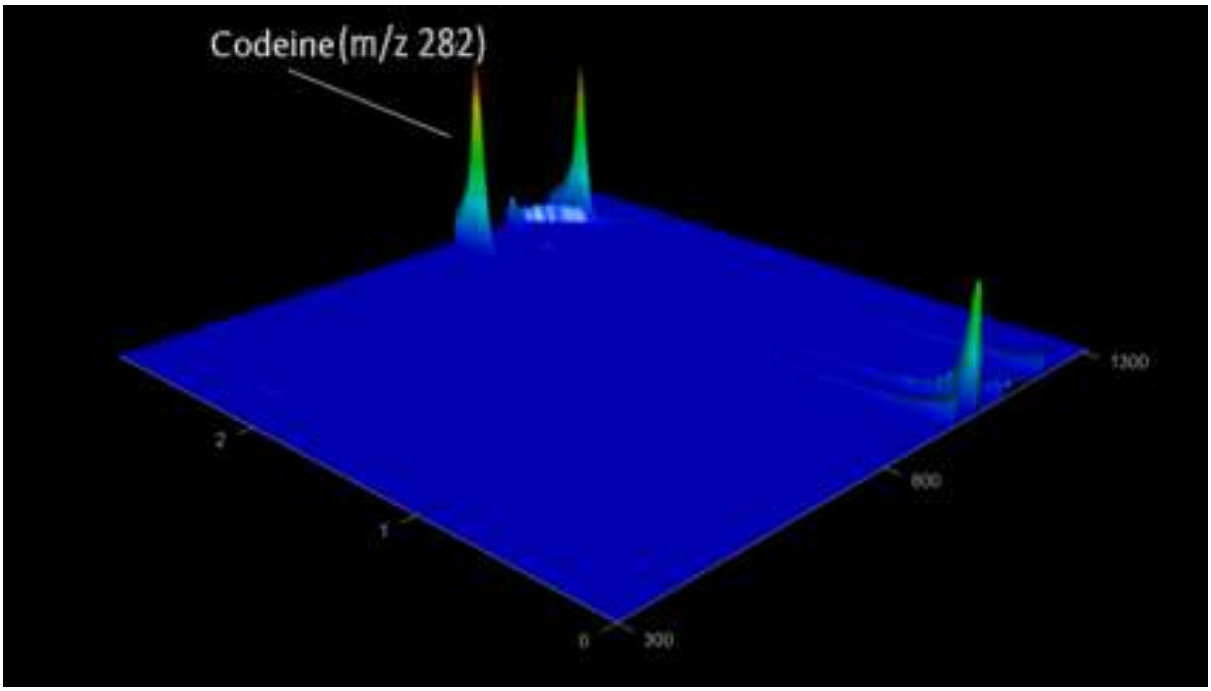

FIGURE 2.6A THREE DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED CODEINE USING (M/Z 282). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

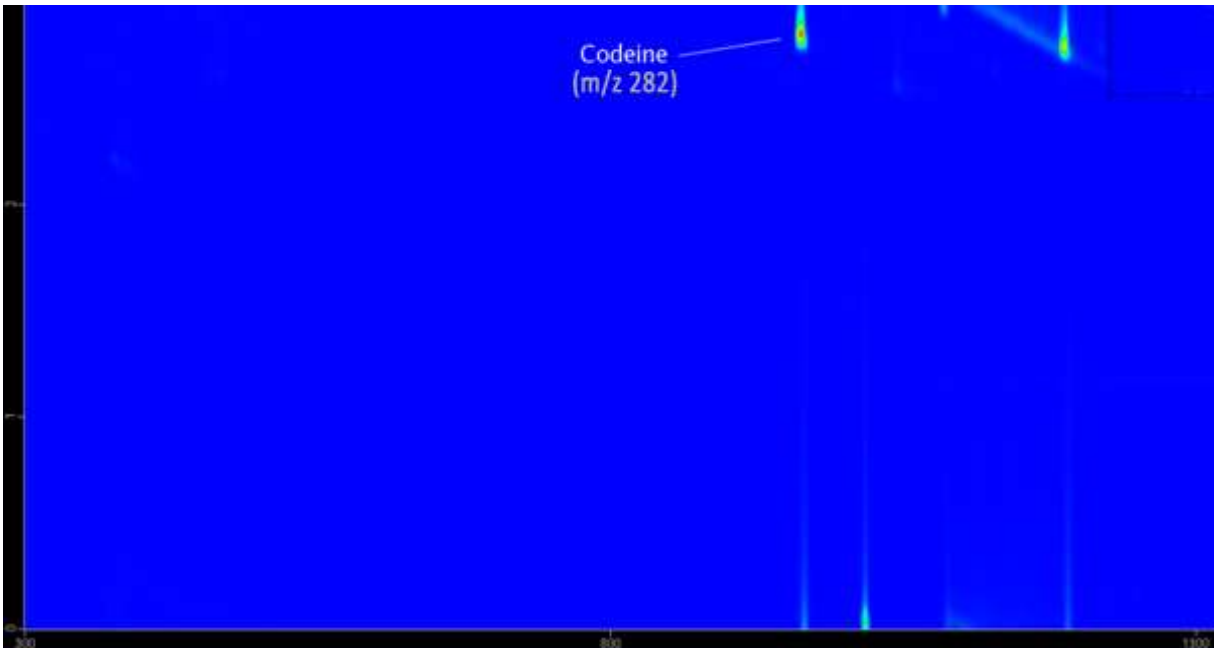

FIGURE 2.6B TWO DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED CODEINE USING (M/Z 282). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

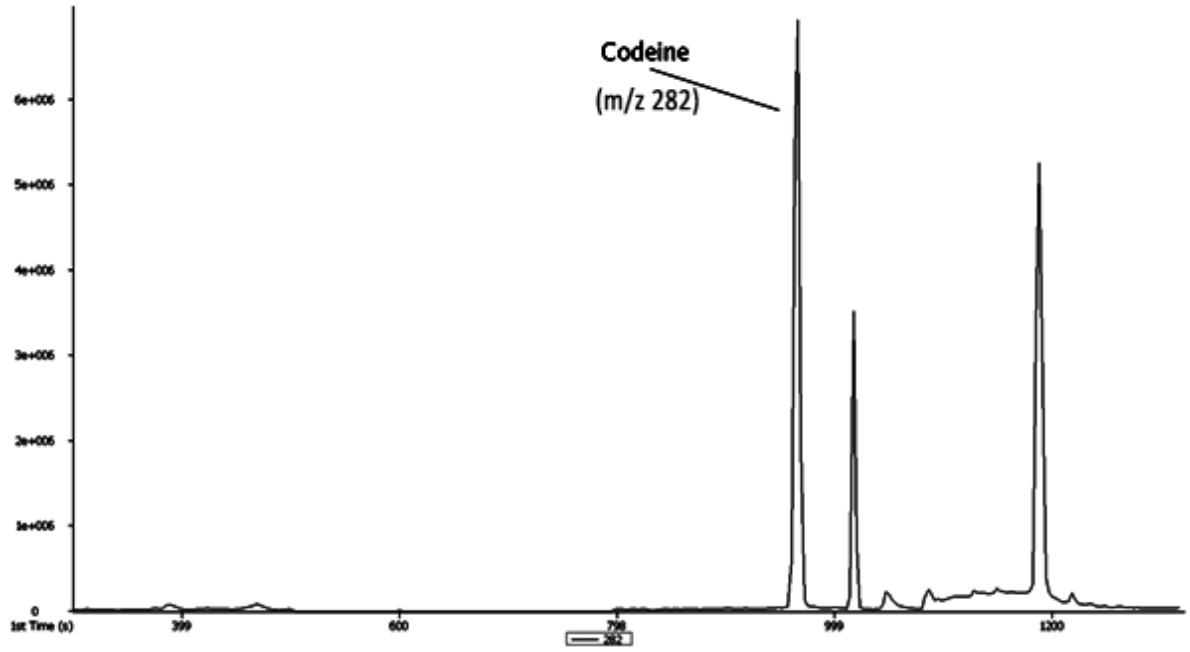

FIGURE 2.6C A RECONSTRUCTED 1ST DIMENSION SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED CODEINE USING (M/Z 282). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

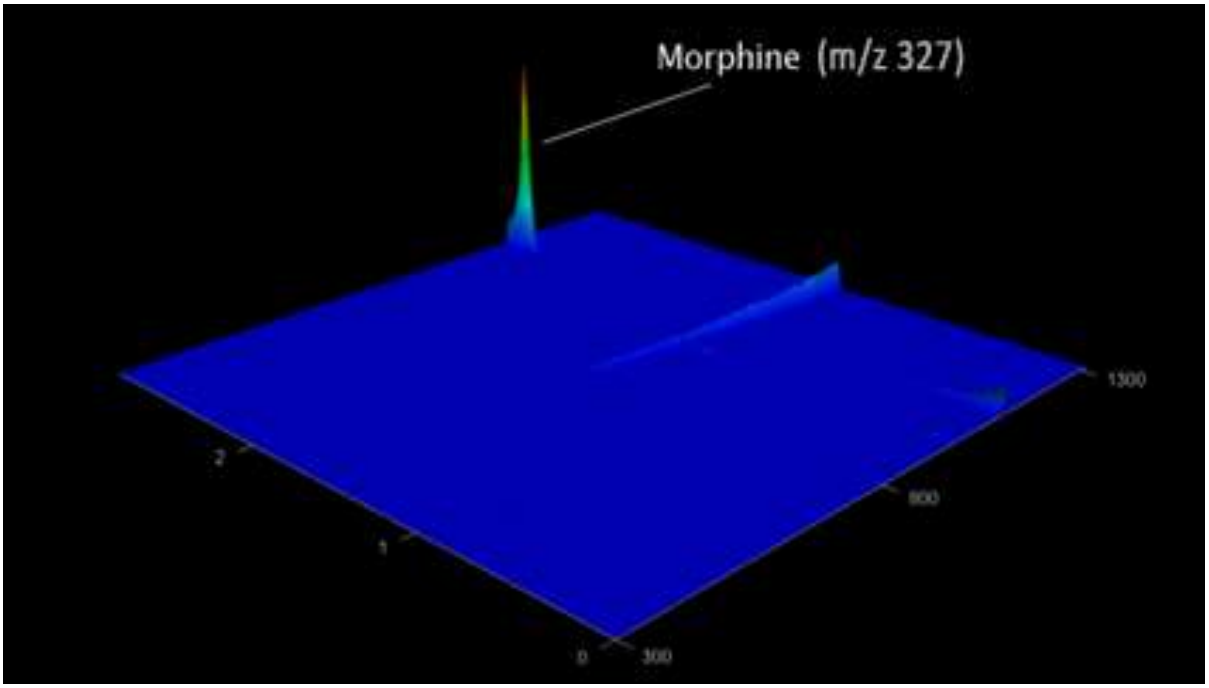

FIGURE 2.7A THREE DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED MORPHINE USING (M/Z 327). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

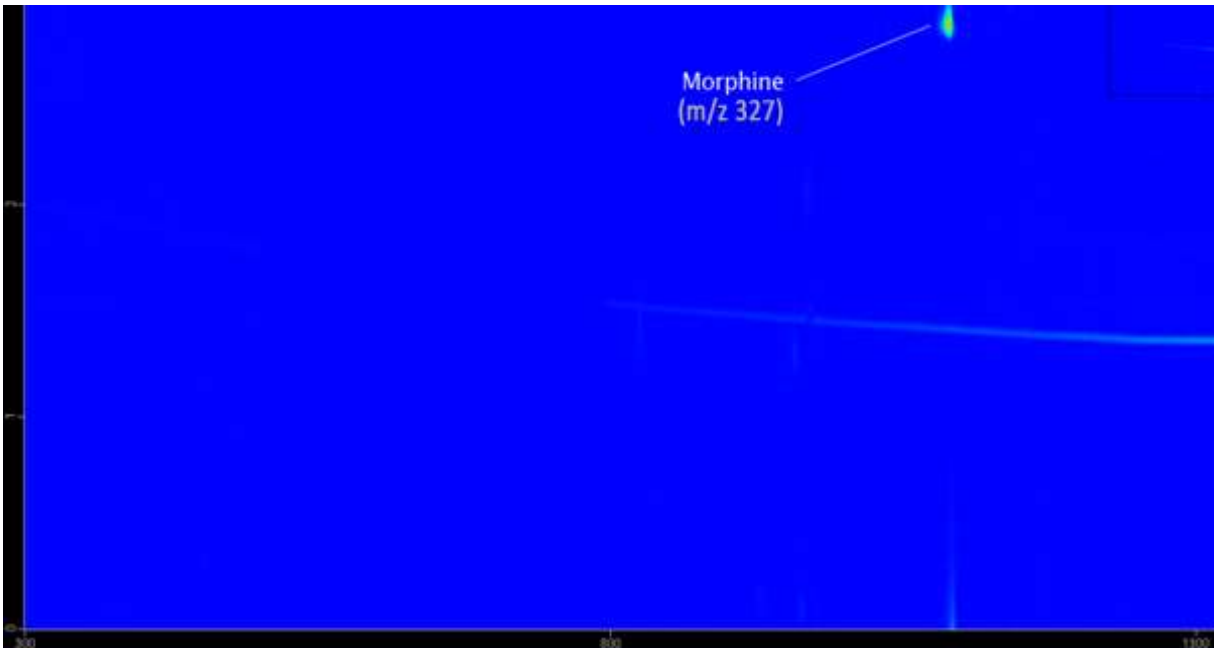

FIGURE 2.7B TWO DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED MORPHINE USING (M/Z 327). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

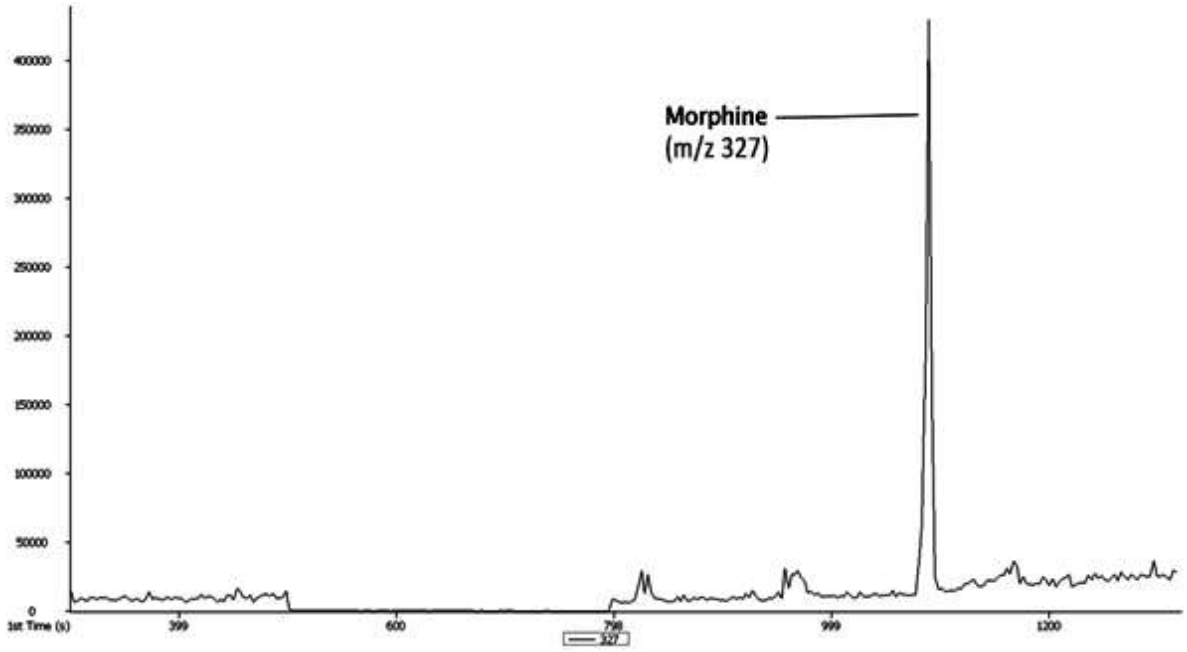

FIGURE 2.7C A RECONSTRUCTED 1ST DIMENSION SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED MORPHINE USING (M/Z 327). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

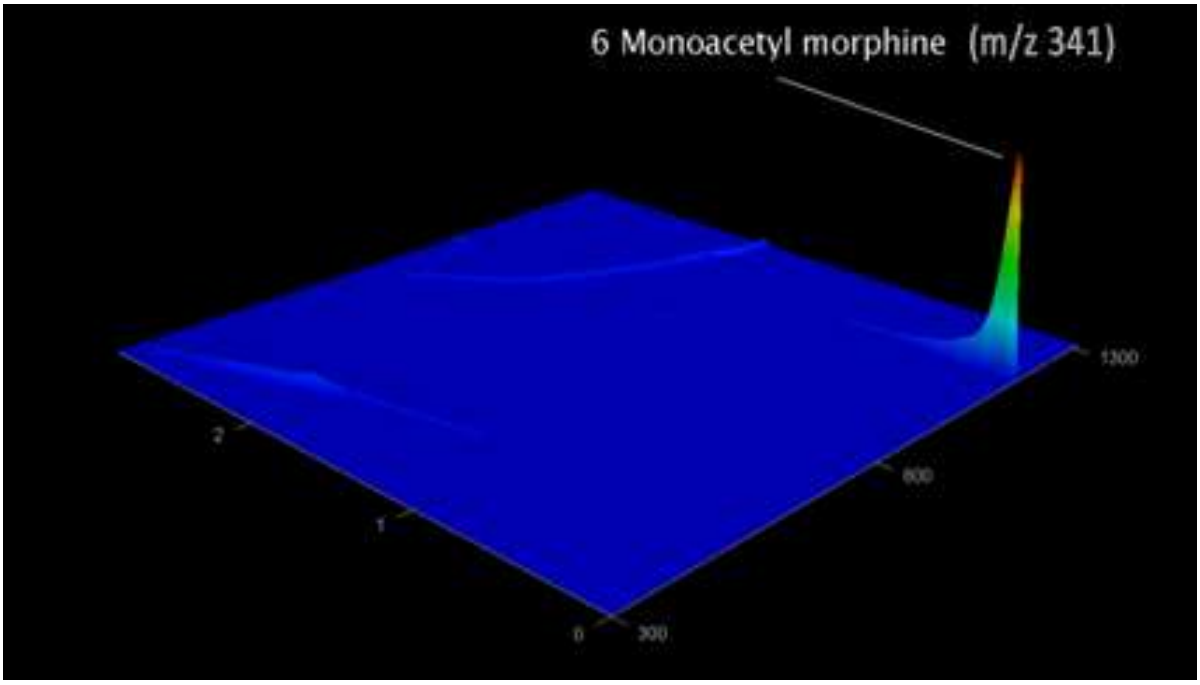

FIGURE 2.8A THREE DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 6 MONOACETYL MORPHINE USING (M/Z 341). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

FIGURE 2.8B TWO DIMENSIONAL SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 6 MONOACETYL MORPHINE USING (M/Z 341). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

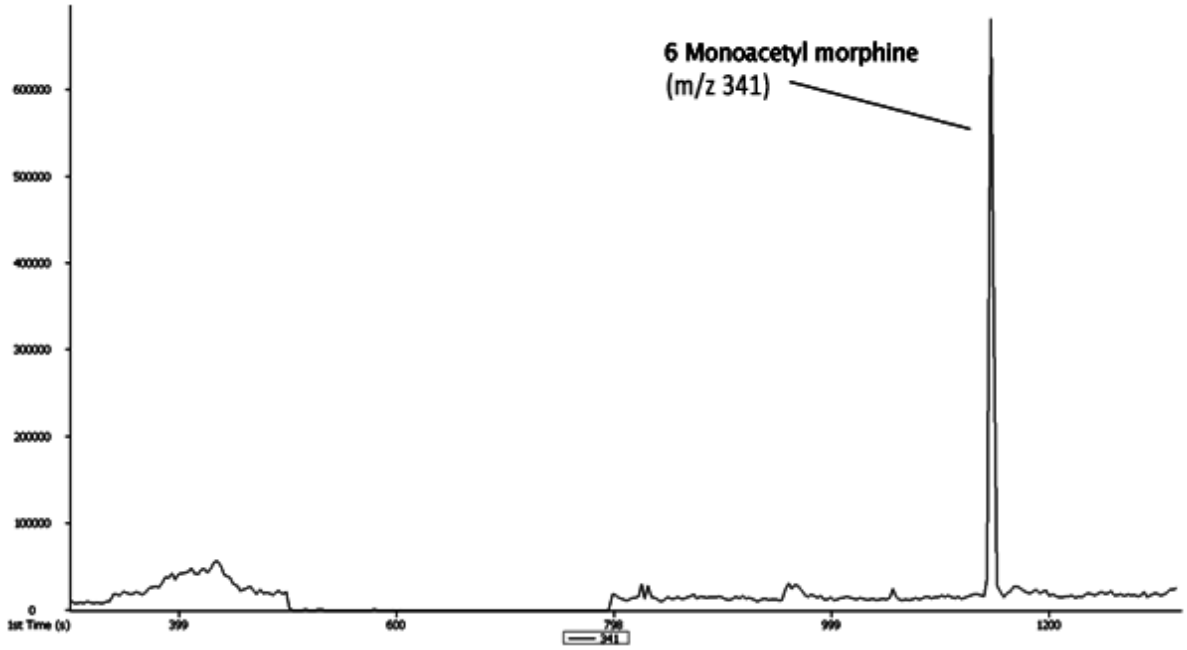

FIGURE 2.8C A RECONSTRUCTED 1ST DIMENSION SELECT ION GCXGC/TOF-MS CHROMATOGRAM OF DERIVATIZED 6 MONOACETYL MORPHINE USING (M/Z 341). MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

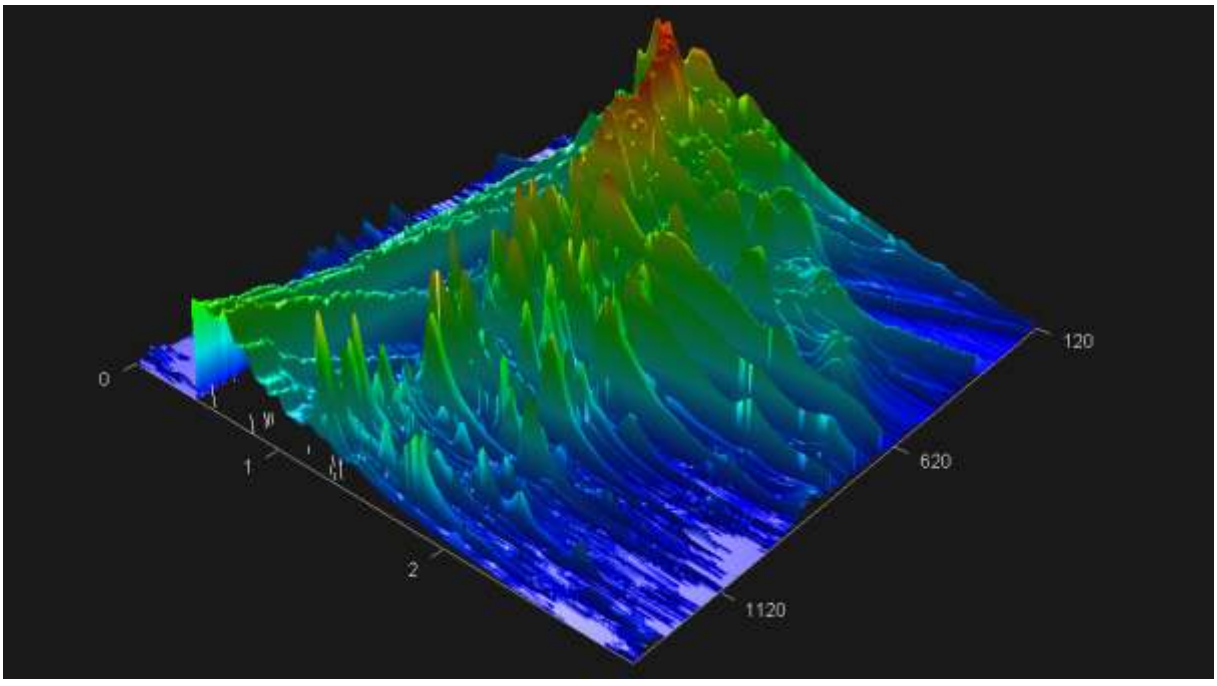

FIGURE 2.9A THREE DIMENSIONAL TOTAL ION GCXGC/TOF-MS CHROMATOGRAM OF A DERIVATIZED WASTEWATER SAMPLE CONTAINING ALL ANALYTES. MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

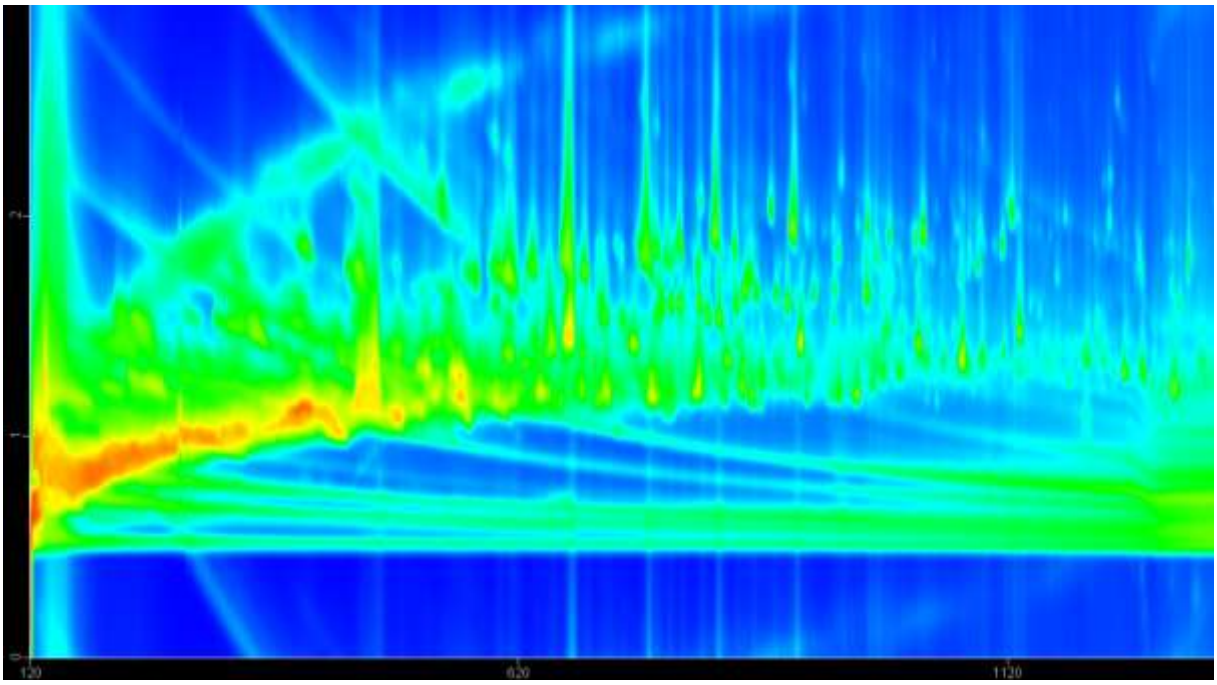

FIGURE 2.9B TWO DIMENSIONAL TOTAL ION GCXGC/TOF-MS CHROMATOGRAM OF A DERIVATIZED WASTEWATER SAMPLE CONTAINING ALL ANALYTES. MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

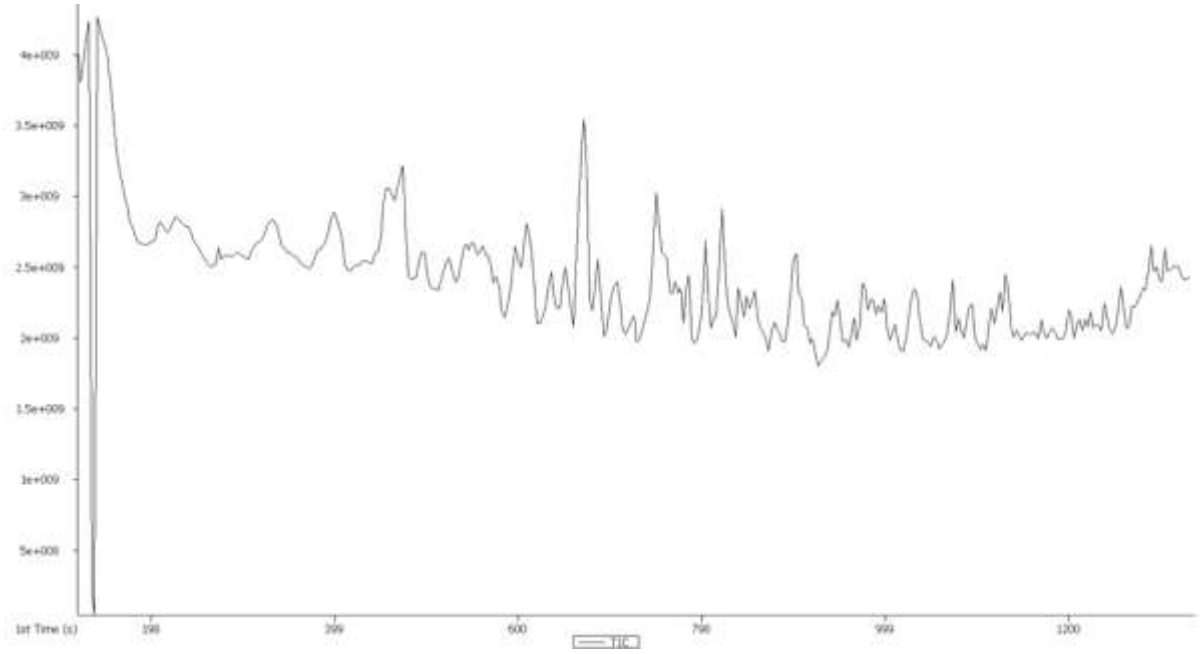

FIGURE 2.9C A RECONSTRUCTED 1ST DIMENSION TOTAL ION GCXGC/TOF-MS CHROMATOGRAM OF A DERIVATIZED WASTEWATER SAMPLE CONTAINING ALL ANALYTES. MS CONDITIONS REPORTED IN SECTION 2.1.2.5.4 MASS SPECTROMETRY.

The mass spectrometer employed was a Pegasus® 4D GCxGC-TOFMS (Leco, St. Joseph, Michigan.). A mass spectrometer with high acquisition rates is required for separations performed using two-dimensional gas chromatography. The acquisition rate of this particular mass spectrometer was 500 spectra per second. The mass spectra collected by this instrument ranged from 100 to 650 m/z using the current method. The transfer line temperature from GC to MS was set at 300 °C. MS ion source voltage was set at 70eV. For the analytes of interest, three qualifier ions as well as one quantifier ion was selected for identification of each analyte. These qualifier ion ratios were matched against certified synthesized analyte standards ratios and were required to stay constant throughout the study. A certificate of analysis is supplied with each certified standard indicating the molecules molecular structure confirmed by NMR (Nuclear magnetic resonance), as well as purity (>99 %) of the standard. If the same method and values for ionization are employed, these qualifier ion ratios should not fluctuate and can therefore be used to identify the analyte of interest. As result of the high matrix interference and background noise in the samples, it would be impossible to visualize an analyte peak in a total ion chromatogram. Thus, an extracted ion plot was chosen as the only means of visualizing the analytes of interest. Deuterated internal standard was also added. All certified standards as well as deuterated standards were purchased from (Cerilliant Corporation, Round Rock, Texas, USA)

The guidelines given by TIAFT [4] were followed, and all repeated sample runs adhered to the specified tolerances. The qualifier ions selected for each analyte and their ion fragments can be visualized in Figure 2.10.

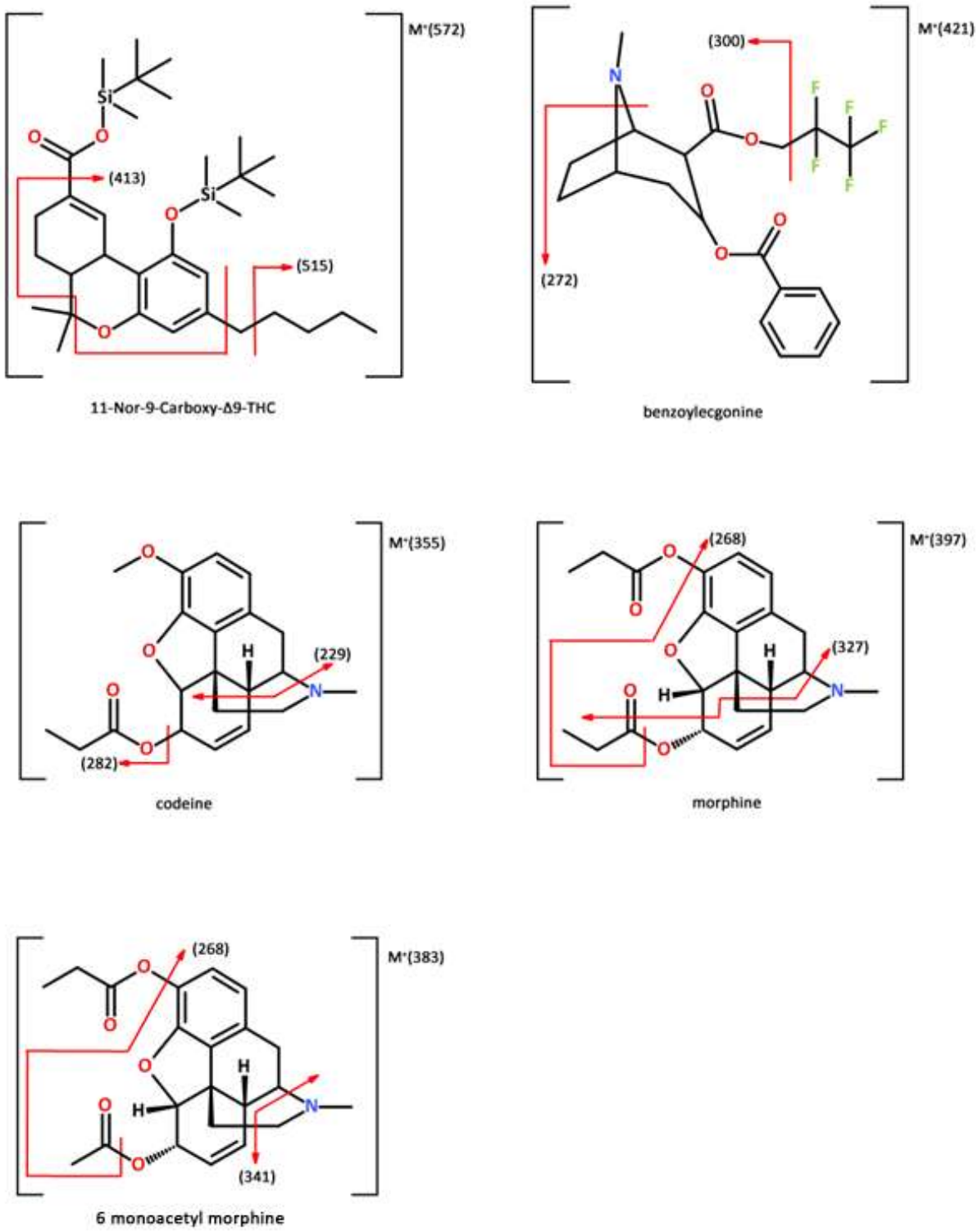

FIGURE 2.10 ANALYTE EI SPECTRUM ION FRAGMENTS

As seen in Figure 2.10, a quantity of 3 ions was used to identify each analyte and consequently, these ions were designated as qualifier ions. The ions used to quantify each of the analytes, including the certified analytical standard, are as follows: 11-nor-9-carboxy-

Δ^9-THC (515), benzoylecgonine (300), codeine (282), morphine (327) and 6 monoacetyl morphine (341). The quantifier ion selected for the deuterated internal standards were as follows: 11-nor-9-carboxy-Δ9-THC-D9 (524), benzoylecgonine-D8 (303), codeine-D6 (288), morphine-D6 (333), 6-monoacetylmorphine-D6 (347). The mass spectrum for each analyte are shown in Figures 2.11A-E. Each quantifier ion was manually integrated at each peak base, from baseline to baseline, to obtain the instrument peak area.

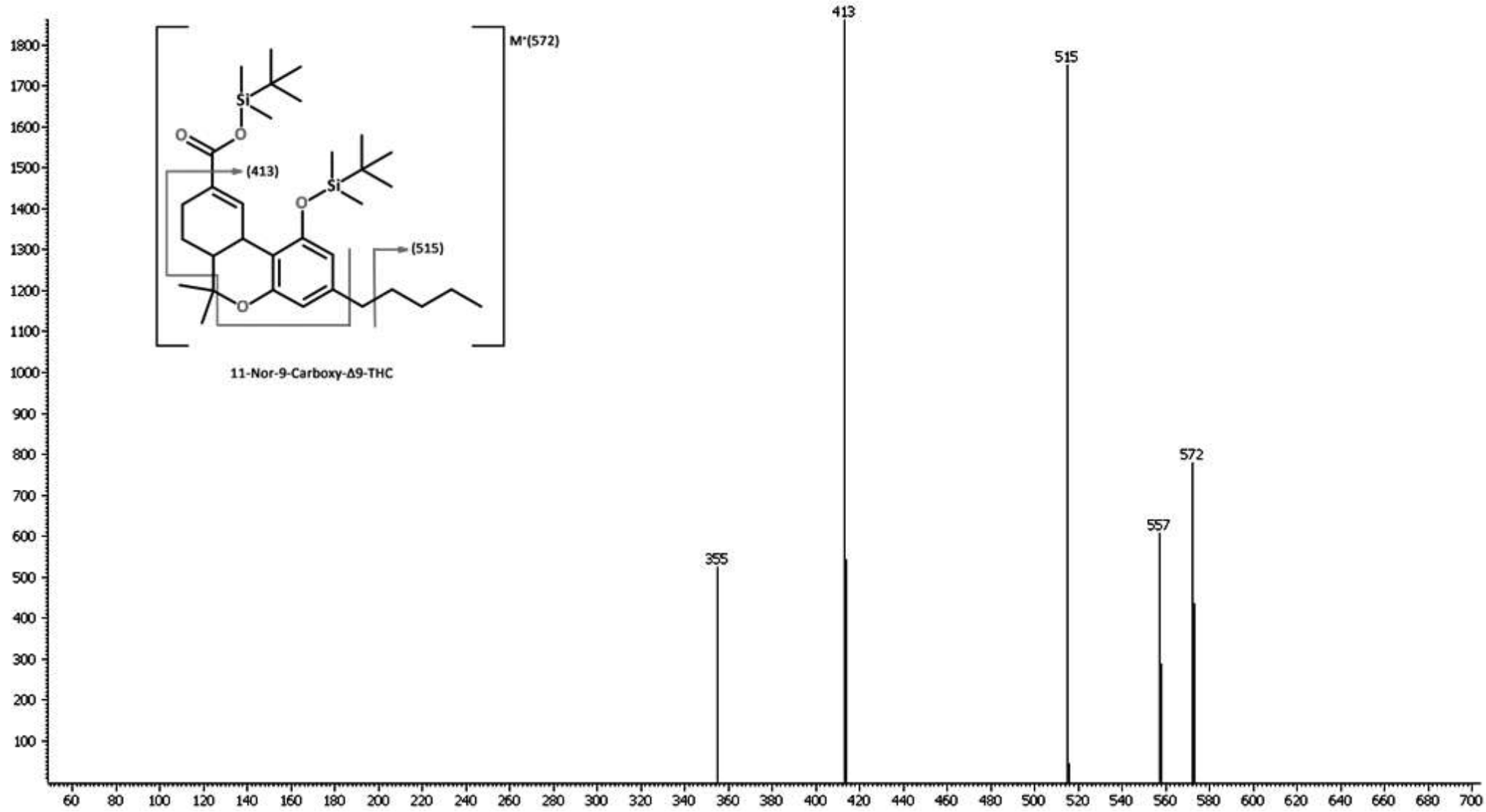

FIGURE 2.11A MASS SPECTRUM OF DERIVATIZED 11-NOR-9-CARBOXY-Δ^9-THC

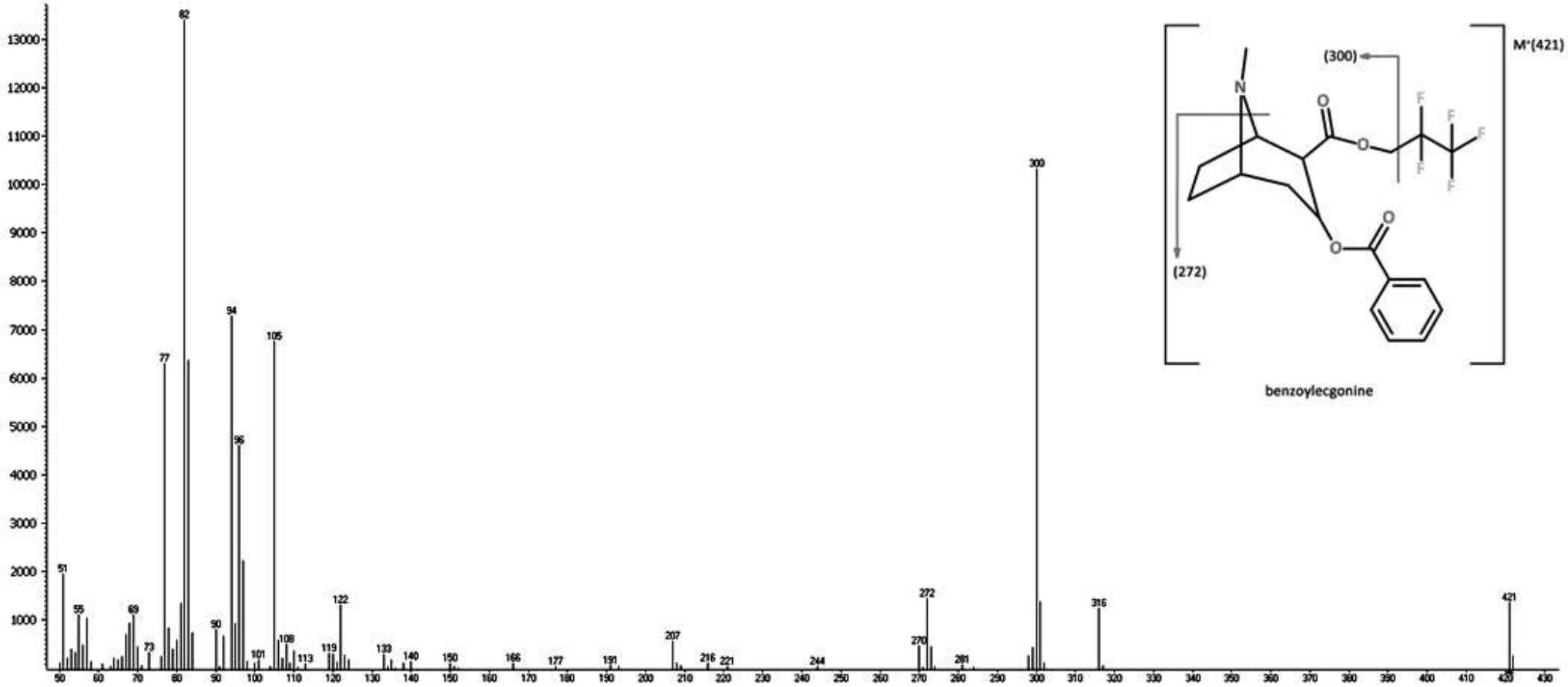

FIGURE 2.11B MASS SPECTRUM OF DERIVATIZED BENZOYLECGONINE

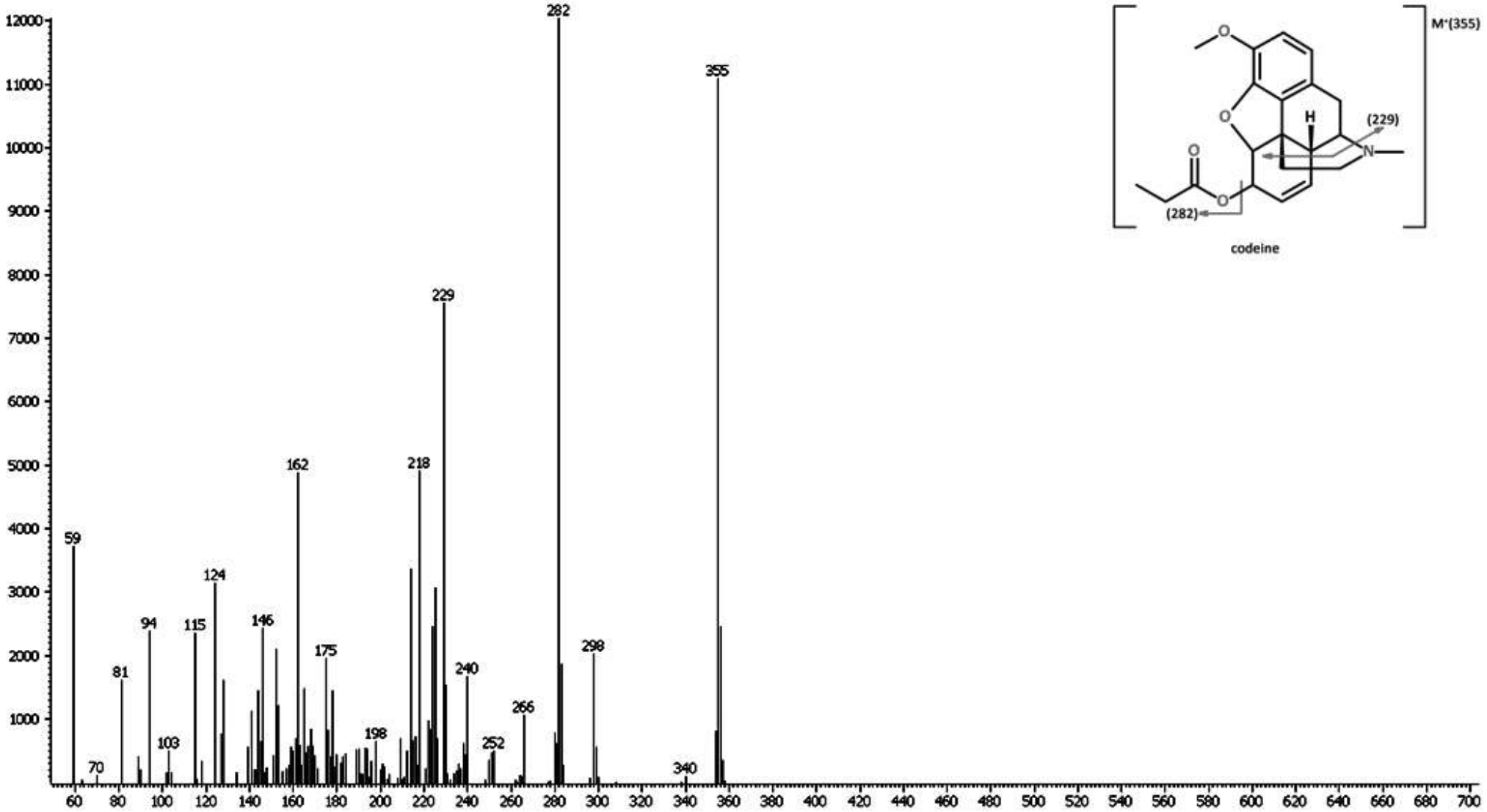

FIGURE 2.11C MASS SPECTRUM OF DERIVATIZED CODEINE

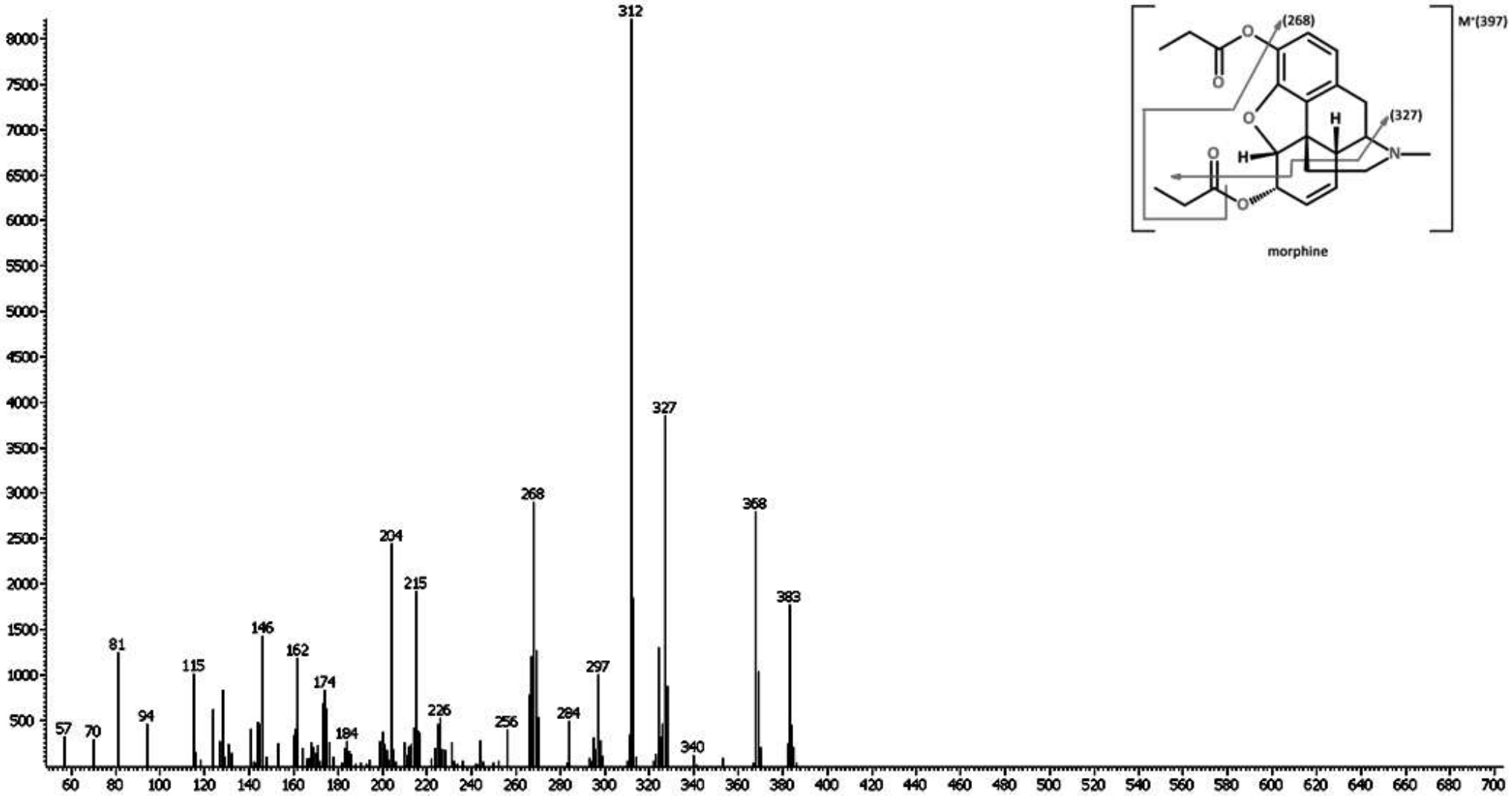

FIGURE 2.11D MASS SPECTRUM OF DERIVATIZED MORPHINE

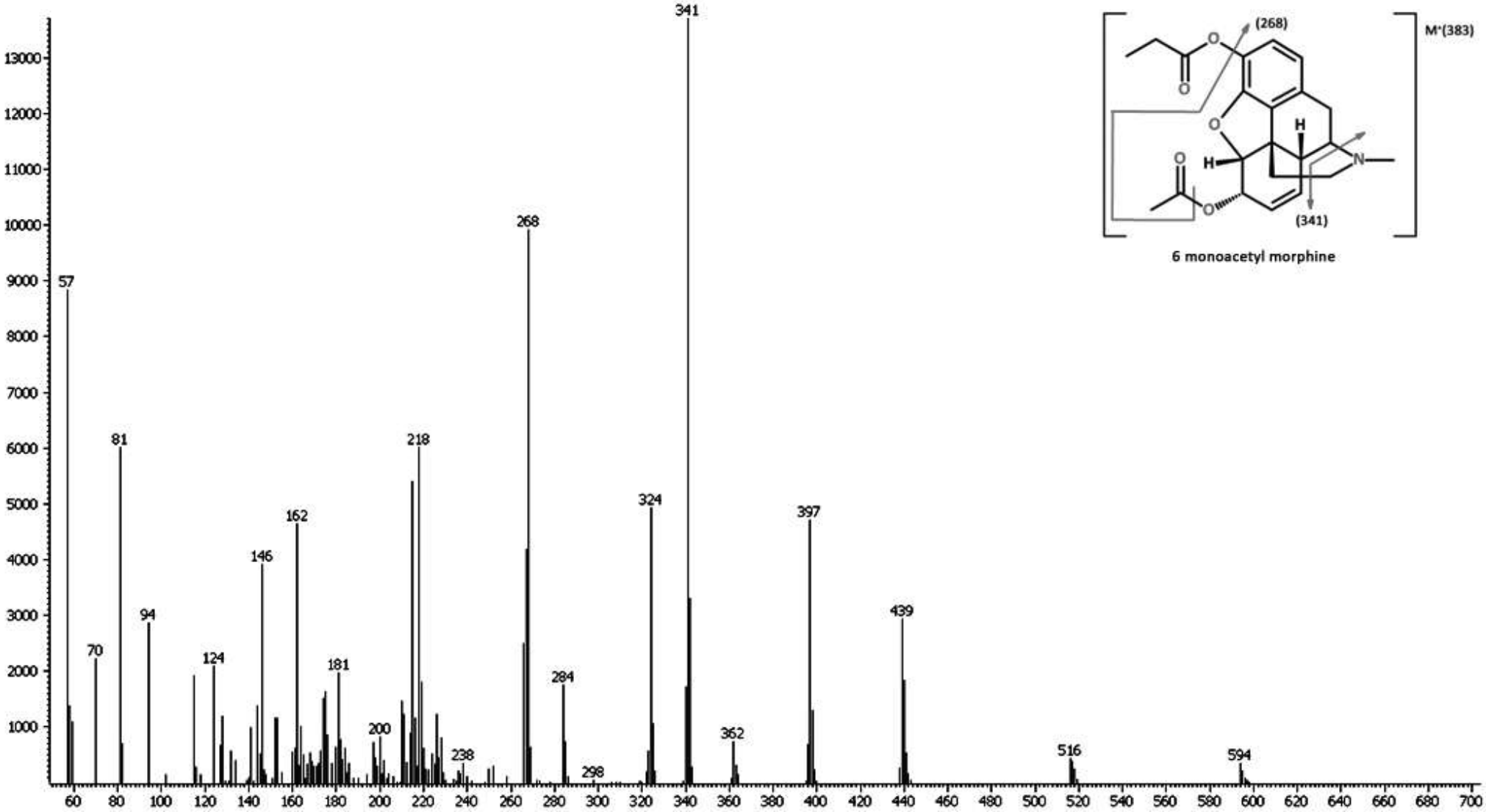

FIGURE 2.11E MASS SPECTRUM OF DERIVATIZED 6 MONOACETYL MORPHINE

2.1.3 METHOD VALIDATION

Any analytical method needs to adhere to certain criteria to be classified as an accepted repeatable method. As described in Section 1.3.3, these criteria include: selectivity, linearity, precision, accuracy, LLOQ and limit of detection (LOD).

In order the confirm that these criteria were met, a full method validation was attempted, as described by Boleda *et al.* [5] and Zucatto *et al.* [6] A large representative wastewater matrix pool was collected to ensure diminutive variation in the sample matrix composition. A 60-litre wastewater pool was collected over a period of 5 weekend days to ensure an adequate representation of the ever-changing wastewater matrix.

2.1.3.1 SELECTIVITY

Since no blank wastewater can be collected to test for the absence of response, selectivity was tested by comparing GC retention times and the mass spectra of both the analyte and the added deuterated internal standards. Since it can be assumed with confidence that no deuterated standard is present in unaltered wastewater samples, analysis of the added internal standard is a sufficient measure of selectivity. Seven samples of unaltered wastewater were analysed in addition to 7 samples with added deuterated standard. In these, no interferences were present for the ions of interest and the method was classified as adequately selective.

2.1.3.2 LINEARITY

Linearity requires that the detector response to increasing concentrations of the analyte be linear across the entire concentration range. In analytical measurements, the relative response is calculated by dividing the peak area of the analyte by the peak area of the deuterated internal standard. Since blank wastewater is usually spiked with increasing amounts of analyte to determine linearity, an external linearity calibration was attempted using the sample matrix itself. A blank and 5 calibrator controls were prepared. Different concentration ranges were prepared for each analyte. The blank and the calibrator aliquots were used to adjust the y-axis relative response of the fitted curve, since the statistical model assumes negligible error in the x direction. The blank aliquots were used as a measure of the concentration of analyte already present in the sample matrix before the addition of control concentrations.

A total of 6 aliquots of the wastewater sample pool were prepared each day for a period of 6 days. Each batch consisted of a blank and 5 aliquots spiked with increasing concentrations of analyte. The concentration ranges of spiked analyte for 11-nor-9-carboxy-Δ9-THC, morphine, 6 mono acetylmorphine and benzoylegconine were 10.00, 20.00, 60.00, 180.00 and 360.00 ng/600.00 ml. For the analyte codeine, the concentration range was increased due to the high amount of analyte present in the wastewater. This adjustment was necessary in order quantify the analyte concentration without the unknown sample concentration falling outside of the calibrated range. The range for codeine was 150.00, 300.00, 500.00, 800.00 and 1100.00 ng/600.00 ml. The previously mentioned ranges were all converted to ng/L in order to generate unknown concentration results in ng/L instead of ng/600.00 ml.

2.1.3.3 Precision

Five replicate aliquots for both high and low control concentrations were run each day for a period of 4 days. Within-day precision was determined by the 5 replicate aliquots that were measured in a day. Between-day precision was calculated by repeating the experiment for 4 days and comparing the results using a one way analysis of variance (ANOVA). The high and low control concentrations for all analytes except codeine were as follows: Low control 17.00 ng/L and high control 600.00 ng/L. The low control for codeine was 250.00 ng/L and high control was 1833.00 ng/L. Due to the result obtained, which is described in Section 3.2.3, a sample homogenization step was included in the method to counteract the variation between samples. The Statistical method of standard addition compared to external calibration was employed from this point onward.

2.1.3.3.1 Sample homogenization

Three different approaches can be utilized to achieve minimal variation between samples.

1. The first approach would be to spike deuterated internal standard into the large pooled wastewater sample at a certain concentration. This pooled wastewater sample will then need to be homogenized properly so that equal distribution of the internal standard is achieved throughout the solution pool. If proper distribution of internal standard molecules is attained, the particulate matter can then be allowed to settle at the bottom of the container. Although analyte interaction will occur with the particulate, it will consequently also occur with the deuterated internal standard, thus compensating for

variation between samples since relative response is measured. It should be noted that filtration of the wastewater sample is improved when there is less particulate matter present. The particulate matter impedes the glass fibre filter's ability to allow the sample solution through. A significantly decreased quantity of filter paper is demanded when particulate is allowed to settle. Another point of note would be the large quantity (10.00 mg of each analyte) of internal standard that would need to be spiked into the 60 litre container in a single dose in order to attain the desired concentration level.

2. The second approach would be to keep the samples properly homogenized throughout the experimental work-up procedure. This can be achieved by using inert magnetic stir bars. Deuterated internal standard is spiked during the first step of sample work-up for each individual sample. The sample should be homogenized for a period of 15 minutes at a high revolutions per minute (rpm) setting. It should be noted that with this approach, the use of a large amount of glass fibre filters will be needed to counteract the saturation effect produced by the homogenized particulate present in the sample.
3. The third and final approach would be to employ a different form of sample work-up, where variation between days can completely be disregarded. This approach is called the method of standard addition, where a calibration graph is obtained for each individual sample. When the determination of the unknown sample concentration and the calibration graph must be performed using the sample itself, the method of standard addition is applied. This approach effectively calibrates for each individual sample. Between day variance can therefore not be calculated since each respective sample has its own calibration graph. Each sample is divided into a predetermined amount of aliquots and each individual aliquot is spiked with increasing amounts of certified analyte standard. All aliquots are also spiked with an equal amount of deuterated internal standard. The relative response is calculated and plotted against concentration. After a graph is obtained, the regression line can be extrapolated to obtain the unknown concentration of analyte present in the sample. It should be noted that when routine application of sample measurements is demanded, a full method validation is recommended since a minimum of 6 calibration points are needed per sample. When routine sample preparation is employed, the costs associated with preparing a six-fold data set for each sample outweigh the benefits. This approach can produce high resource expenditure very quickly. The main advantage of this approach is that between-day and sample variation is completely abolished, and matrix variation has no effect on the repeatability since each sample is

individually calibrated. This is of immense importance since the problem of ever-changing wastewater matrix can be circumvented.

The first approach was attempted and between day sample concentration variations varied significantly. The observations recorder as well as results and discussion will be conducted in Chapter 3 of this dissertation. Because of the results obtained, another approach was attempted which would negate the need for between day sample variation.

A combination of two of the aforementioned methods was employed in order to circumvent the between day variation of samples. The between day sample variation was addressed primarily through the use of standard addition methodology. The within-day between-sample variation was dealt with by the homogenization of samples using deactivated glass magnetic stir bars. The combination of these two approaches was the solution to decreasing sample variation and error. The method of standard addition will be discussed in detail in Section 2.1.3.5.

2.1.3.4 Recovery of 11-nor-9-carboxy-Δ^9-THC

The method described for assuring minimum error and proper homogenization of the matrix solution used deactivated glass magnetic stirring and standards addition as a statistical tool. The homogenization of the sample and its particulate matter, however, presented a problem with the ability of 11-nor-9-carboxy-Δ^9-THC to stay in solution. The recovery of 11-nor-9-carboxy-Δ^9-THC completely diminished to the point where the analyte was no longer detected, even when certified analyte standard was spiked in solution.

Upon investigation, the particulate matter present seemed to display a non-polar nature. When the wastewater matrix was filtered using glass fibre filters and a sintered glass bed, a residue consistent with the visual appearance of lipids adhered to the sides of the filter. Due to the extremely non-polar nature of 11-nor-9-carboxy-Δ^9-THC, interaction between this analyte and the lipid-like particulate matter was to be expected. It is also a likely conjecture that, in contrast to urine samples, wastewater samples will contain escalated amounts of lipids due to the contribution of excrement. As result of this interaction, the recovery of 11-nor-9-carboxy-Δ^9-THC was negatively influenced. Most of the 11-nor-9-carboxy-Δ^9-THC analyte bound to the lipid-like particulate matter by means of Van der Waals interactions. The Van der Waals interactions are used in the same manner to retain 11-nor-9-carboxy-Δ^9-THC on the solid phase extraction sorbent

bed. Thus, it is expected that all analytes that bind to the reverse phase divinylbenzene functional part of the sorbent bed will be negatively influenced by the presence of lipid in the matrix.

The next logical proposition was to either remove all lipids completely from the solution and extract the analytes of interest using liquid-liquid extraction, or to increase the ability of 11-nor-9-carboxy-Δ^9-THC to stay in solution, in contrast to being bound to the lipid particulate matter.

An analyte may distribute itself between two partially miscible solvent phases in near contact with one another, resulting in an equilibrium partitioning of the analyte between the two phases. The equilibrium ratio of the analyte partitioned between the two phases is known as the distribution or partitioning coefficient, as mentioned by Sangster *et al.* [7] Well-known solvent, 1-octanol has been chosen to represent the non-polar phase in the equilibrium ratio, [7] and one of the criteria to describe a molecule's polarity is its octanol-water partition coefficient. Usually the value is transcribed on a log scale due to the value spanning several orders of magnitude (LogP=*"value")*. In the case of Δ^9-THC, the octanol-water partition coefficient is given by LogP=6.97. [8] This LogP value is quite high, which indicates favourable interaction with non-polar solvents and also indicates the non-polar nature Δ^9-THC. Since no octanol-water partition coefficient could be attained for 11-nor-9-carboxy-Δ^9-THC, and because of the similarity between this metabolite and Δ^9-THC, the LogP value of Δ^9-THC was regarded as a reference for the polarity of 11-nor-9-carboxy-Δ^9-THC.

2.1.3.4.1 Decreasing the polarity of the wastewater matrix solution

As mentioned above, two approaches can be followed to increase the recovery of lipophilic analytes. However, since it would be impractical to employ a liquid extraction after each successive filtration, the second approach—increasing 11-nor-9-carboxy-Δ^9-THCs' ability to stay in solution—was followed. To enable 11-nor-9-carboxy-Δ^9-THC to have a higher solubility in the aqueous wastewater matrix, an intermediary solvent both miscible with water and of increased non-polar nature needed to be employed. The first attempt involved the use of methanol as the intermediary solvent. Three 600.00 ml wastewater samples were diluted with methanol to contain 0.00 %, 10.00 % and 20.00 % methanol, respectively. The 0.00 % or blank sample was prepared to compare the previous sample composition (no methanol) to the methanol-containing wastewater matrix.

The sample pre-treatment included collecting the 24-hour wastewater pool. From this wastewater pool, 3.72 L of matrix was aliquoted into a large container. 310.00 µl of 2.00 ng/L deuterated internal standard, 17.00 ml of 6 M HCl, and 1.00 L of methanol was added to this wastewater pool. The concentration amounted to a 21.11 % methanol solution. Because it would add to the ease of sample preparation, it was decided that 1 litre of methanol would be added, thus circumventing the measurement and addition of fractional quantities of methanol. The prepared wastewater pool was mixed using magnetic stirring for a period of 2 hours on a high rpm setting. This preparation technique of magnetic stirring and early addition of deuterated internal standard ensured low variation and increased recovery. After stirring, the 6 individual solutions were aliquoted in such a manner that the quantity of wastewater present in the samples, not including the methanol added, still amounted to 600.00 ml. This was done by taking into account the 21.11 % methanol dilution. Thus, the total volume of the mixture aliquoted was 760.00 ml per sample.

The results obtained from the experiment will be discussed in Section 3.2.1, Recovery of 11-nor-9-carboxy-Δ^9-THC.

2.1.3.5 The method of standard addition

The method of standard addition was employed to test for linearity since the sample itself can be used as the matrix calibrator. A set of 6 calibration levels, including the sample blank, was prepared and injected over a period of 7 days. A total of 7 calibration curves were obtained for each of the analytes present. The statistical least squares method was employed to estimate the goodness of fit for each calibration curve. 600.00 ml of each unknown sample was aliquoted into 6 separate volumetric flasks. The flasks were spiked with increasing amounts of certified analyte standard and equal amounts of deuterated internal standard. A mass of 100.00 µg deuterated internal standard was spiked into each flask. Two different concentration ranges were employed for the certified analyte standards. As a result of the benzoylecgonine having interactions with the sediment particulate present in the water, when proper homogenization of the sample occurred the measured concentration of this analyte increased significantly. As a consequence of this significant increase a different scale needed to be used to measure benzoylecgonine. From the linearity experiments, a concentration range for each analyte could be estimated. Thus benzoylecgonine, together with codeine, required increased concentration levels as a result of their high matrix concentration, as compared with the other analytes. The concentration range of

certified analyte standard used for benzoylecgonine and codeine was as follows: 250.00 ng/L, 500.00 ng/L, 833.33 ng/L, 1333.33 ng/L, and 1833.33 ng/L. For all other analytes (11-nor-9-carboxy-Δ9-THC, morphine and 6 monoacetyl morphine) the range was as follows: 16.67 ng/L, 33.33 ng/L, 100.00 ng/L, 300.00 ng/L, and 600.00 ng/L. Only 5 concentrations levels are reported because the first aliquot was a blank containing no added certified analyte standard. As mentioned above, an equal amount deuterated internal standard was added to all aliquots.

2.1.3.6 Precision using method of standard addition

Precision describes both within as well as between day precision. With the use of standard addition, between day precision cannot be calculated. A precision value can be obtained for each individual measurement for each analyte by using Equation 1.4. The percentage relative standard deviation (%RSD) will be calculated by dividing standard deviation by the unknown concentration obtained through back extrapolation of the standard addition regression line. The value is multiplied 100 to obtain the %RSD.

2.1.3.7 Accuracy using method of standard addition

Accuracy is defined in Equation 1.5. The expected concentration value is divided by the measured concentration value. Since no external calibration model was implemented in this study no expected analyte concentration value can be obtained, since each sample is calibrated individually through standard addition. A calculated error can be obtained but accuracy percentage cannot with the method of standard addition.

2.1.3.8 Limit of quantification (LLOQ) and detection (LOD) using method of standard addition

When using the method of standard addition a calibration plot is obtained for each individual sample. An error is associated with each extrapolated concentration value. Because the limit of quantification is dynamic/changes for each individual measurement when using standard addition, each of the measured analytes will in turn have their own limit of quantification. If the individual measurement adheres to a 20% RSD at the lowest calibrator value, in this case unspiked sample, the measured concentration value can be classified as the limit of quantification for that specific analyte.

A calculated limit of detection will be employed since no replicate samples were analyzed. The method of standard addition fits a regression line through increasing concentration measurements of analyte. No one calibration point is analyzed in replicate. Thus a value below the quantificaiotn limit will be regarded as the detection limit (LOD < LLOQ).

2.1.4 Conclusion

Pooled daily wastewater samples were collected for a period of one week. Sample pre-treatment included addition of methanol to the wastewater pool as well as the addition of deuterated internal standard. Samples aliquots were prepared in accordance with the method of standard addition and spiked with increasing amounts of analyte standard. A solid phase extraction method was employed to concentrate the analytes of interest, and two separate elution steps were employed. First 11-nor-9-carboxy-Δ^9-THC was eluted and secondly all other analytes bound to the sorbent by means of ion exchange mechanisms. After elution derivatization was employed using microwave assisted derivatization together with MTBSTFA, PFPOH and propionic anhydride. Samples were dried and reconstituted with toluene. The samples were injected onto a GCxGC/TOF-MS, with Rxi-17sil MS, 30 meters, i.d. = 0.25 mm, df = 0.25 µm column in the first dimension and a (Zebron ZB5, 2 m, i.d.=0.25 mm, df = 0.25 µm) in the second dimension. Quantitative analytical data was obtained by single ion monitoring of the following ions: 11-nor-9-carboxy-Δ^9-THC (515), benzoylecgonine (300), codeine (282), morphine (327) and 6 monoacetyl morphine (341). The quantifier ion selected for the deuterated internal standards were as follows: 11-nor-9-carboxy-Δ9-THC-D9 (524), benzoylecgonine-D8 (303), codeine-D6 (288), morphine-D6 (333), 6-monoacetylmorphine-D6 (347). Method of standard addition was used to calibrate and calculate the unknown concentration of each analyte for each respective day. The method was able to measure low nanogram per litre analyte quantities in wastewater matrix. Despite the intricate nature of the method, once established, it can be relied upon to produce precise data. This reliability and precision is needed in order to draw any conclusions about population drug use.

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3. RESULTS AND DISCUSSION

3.1 INTRODUCTION

The contents of this chapter will present the results obtained using the developed method. The week wastewater profile results obtained by the method will be presented and discussed in detail. The data is of constructive nature since it has up to this point never been released in South Africa. A much improved estimation of the area's drug use can be obtained. It should be stated that these figures are not free from error, especially when the number of doses are back-calculated in order to obtain a population figure. There are certain dilution factors present in the wastewater from industry and commercial buildings, which skew the results obtained to produce underestimated drug use figures. Although underestimation may take place, this is the first data of its kind in South Africa to produce an acceptable data set for estimating total population drug use. It is important, however, to be able to provide the best result with the given equipment and resources available.

3.2 METHOD VALIDATION RESULTS

The results obtained during method development and validation will be discussed below. First the method development results will be discussed. These include increased recovery of 11-nor-9-carboxy-Δ^9-THC due to both addition of methanol during sample homogenization as well as a pH adjusted elution step. Derivatization interferences will also be discussed. As mentioned in Chapter 2, a full external calibration method validation was attempted, but due to enormous between day variations, the statistical model of standard addition was implemented. Results obtained during external calibration validation were responsible for changing the protocol used to validate the method. Some of these external calibration results will be discussed, followed by the newly adopted method of standard addition. The results presented as follows:

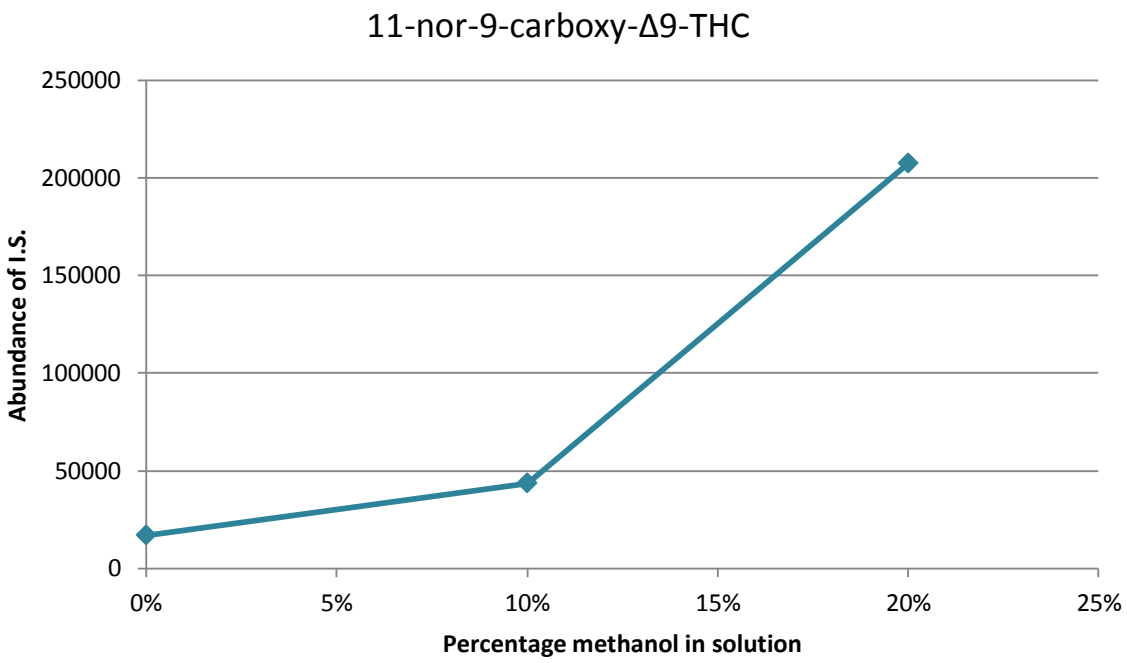
3.2.1 Recovery of 11-nor-9-carboxy-Δ9-THC

If analytes interact with the particulate matter present in wastewater, sources of variation between samples needed to be diminished. The between day variations were addressed primarily by using the method of standard addition. The within-day between-sample variation was dealt with by magnetic stir bar homogenization of the samples. The combination of these two approaches was the solution for decreasing sample variation and error, as described in Section 2.1.3.3.1 (Sample homogenization).

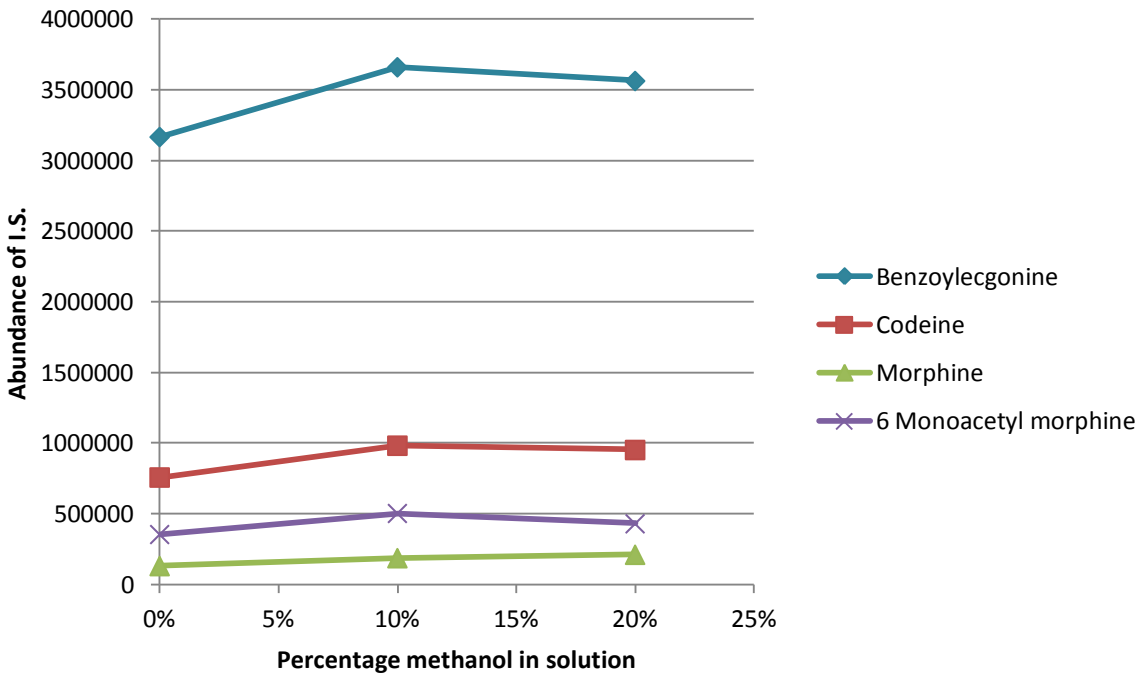
3.2.1.1 Using methanol to increase 11-nor-9-carboxy-Δ^9-THC recovery

Graph 3.1A-B shows the plotted results obtained from the experiment described in Section 2.1.3.4.1 (Decreasing the polarity of the wastewater matrix solution).

Graph 3.1A Relationship between 11-nor-9-carboxy-Δ9-THC deuterated internal standard response and percentage methanol in solution.



GRAPH 3.1B RELATIONSHIP BETWEEN ANALYTE DEUTERATED INTERNAL STANDARD RESPONSE AND PERCENTAGE METHANOL IN SOLUTION.



As seen in Graph 3.1A, the recovery of 11-nor-9-carboxy-Δ^9-THC was greatly improved by the addition of 20 % methanol. Furthermore, there were no negative effects on the recoveries of the other analytes of interest when the methanol concentration in solution is increased. Because of these results, a 600.00 ml sample of wastewater was used with an added 160.00 ml of methanol (20 % in solution).

The importance of the interaction of 11-nor-9-carboxy-Δ^9-THC with the particulate matter in the wastewater matrix cannot be stressed enough. No literature was found of the interaction of 11-nor-9-carboxy-Δ^9-THC with particulate matter present in wastewater. Thus, it is of utmost importance to pay attention to this finding, since skewed results can be obtained when care is not given to this important fact. In this study, it was shown that recovery of the analyte was adversely affected by the particulate matter. If the low level of deuterated internal standard spiked into the sample can be detected by the instrument, a scale is present by which the analyte concentration can be determined. As a result of the homogenization of the sample pool, recovery of 11-nor-9-carboxy-Δ^9-THC was adversely affected. Before the addition of methanol, no 11-nor-9-carboxy-Δ^9-THC analyte was detected after sample pool homogenization. Consequently, no deuterated internal standard was present to calculate a relative analyte response with. It should be asserted when using absolute concentrations or mean external calibration, large errors may be induced as

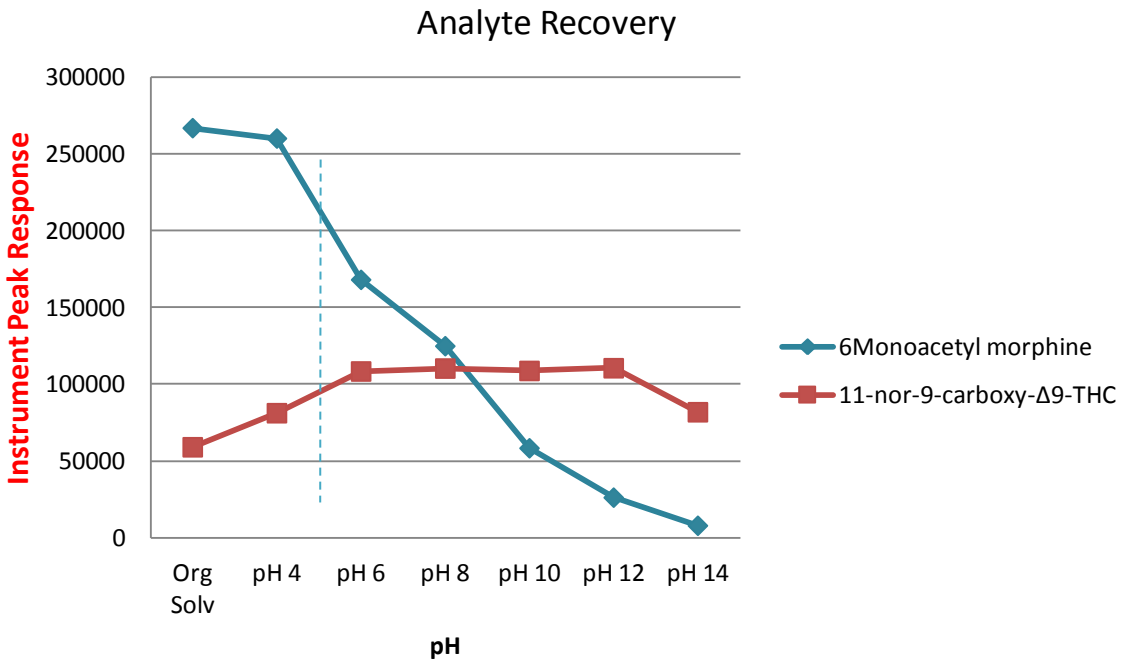
result of particulate interaction. Although a 100 % analyte recovery is not achieved, a partial reference is still present to be able to calculate the relative response when using deuterated internal standard. While analyte sample concentration is adversely affected and decreased the deuterated standard is also adversely affected and decreases. Because of the close molecular similarity of deuterated standard vs. analyte, the decrease of deuterated internal standard response is directly proportional to decrease in analyte response. In contrast to using deuterated internal standard, when employing mean external calibration and absolute response of analytes, no loss of analyte can be tolerated in order to produce accurate data.

In conclusion, the recovery of 11-nor-9-carboxy-Δ^9-THC was improved to where a considerable relative response could be measured using the current GCxGC/TOF-MS instrumentation. It should be noted that an equal, if not improved, response was measured for all other analytes, and the methanol had no adverse interactions hindering solid phase extraction.

3.2.1.2 pH ADJUSTMENT TO INCREASE 11-NOR-9-CARBOXY-Δ^9-THC RECOVERY

The experimental data showed that 11-nor-9-carboxy-Δ^9-THC yields only increased until a pH of 6. At a pH of 6 and above, a recovery yield plateau was evident, with no further increase in relative response observed. Conversely, the opposite was true for the opioids; at organic solvent and pH 4, no apparent difference in relative response for opioids were observed, while at pH 6 and above, the relative response for opioids were significantly diminished. As seen in the graph below, the optimum pH for maximum relative response of both 11-nor-9-carboxy-Δ^9-THC as well as 6MAM was at a pH of 5. At this pH an approximate 25% decline was observed for relative response of the opioids and a 75% increase was observed for 11-nor-9-carboxy-Δ^9-THC. It was also shown that at a pH of 6, an approximately 100% increase of 11-nor-9-carboxy-Δ9-THC was observed compared to a 50% decrease in opioid relative reponse. Since very low quantities of opioids are also present in the sample, an optimal recovery for both analytes 11-nor-9-carboxy-Δ^9-THC and opioids needed to be found. The lowest amount of opioid loss and the highest increase in recovery for 11-nor-9-carboxy-Δ^9-THC was found at pH 5.

GRAPH 3.2 ANALYTE INSTRUMENTAL PEAK RESPONSES WITH VARYING PH ELUTION SOLVENTS



The opioid analytes bound to the sorbent by means of ionic bonds required an increase in pH to completely break the ionic bonds. Due to 11-nor-9-carboxy-Δ^9-THC having a pK_a of 4.5, pH values above this led to increased quantities of ionized 11-nor-9-carboxy-Δ^9-THC in solution. As result of the increased polarity of the ionized 11-nor-9-carboxy-Δ^9-THC, reduced affinity for the divinylbenzene sorbent was observed and a higher recovery yield was obtained.

3.2.2 DERIVATIZATION INTERFERENCES

Some interferences occurred during derivatization and these will be discussed below.

3.2.2.1 PYRROLIDONIUM INTERFERENCES

PFPA was used as the derivatization agent for all of basic drug metabolites and benzoylecgonine. A significant interference was caused by a combination of different factors, one of which was the use of PFPA. The other factor causing interference was the use of Oasis™ MCX™ cartridges (Waters Corporation, Milford, MA, USA) for extraction. This cartridge, like the Phenomenex™ cartridge, uses a polystyrene divinylbenzene (PS-DVB) backbone. The difference between the two is that the Waters cartridge contains a pyrrolidone functional group that the Phenomenex™ cartridge does not contain. This is because a certain ratio of pyrrolidone to PS-DVB is used as a stabilizer during PS-DVB synthesis. [(1)] These N-vinyl pyrrolidone molecules are added via

postaddition methodology after the synthesis of the PS-DVB has started. [1] Thus, it is safe to assume that less than 100% of pyrrolidone molecules react to form N-vinyl pyrrolidone PS-DVB. If these pyrrolidone molecules are not removed by a cleaning procedure, they will be present as a by-product in the polymeric sorbent. These pyrrolidone molecules are the cause of the main interfering compound seen in this study.

N-vinyl pyrrolidone molecules are amphiphilic, meaning that they have affinity for both polar and non-polar solvents. [2] The nitrogen atoms contained within N-vinyl pyrrolidone are readily protonated to form N-vinyl-pyrrolidonium, which contains a positive charge (in ionised base form). [3] This influences the solubility of the N-vinyl-pyrrolidonium molecule in such a way that these molecules are released into the eluate upon elution of the analytes. The same comparison can be drawn between these pyrrolidonium molecules and any other basic analyte. When the sample is loaded at a low pH, the pyrrolidonium molecules are positively charged or protonated, thus forming ionic bonds with the sorbent's negatively charged sulfonic acid groups. As result of strong ionic bonds, the successive washings steps can only remove the pyrrolidonium molecules that are not bonded to the sorbent. When the elution solvent, at a pH of 12, is aspirated onto the Waters cartridge, these pyrrolidonium molecules are deprotonated as would be any basic analyte. The result is an unknown amount of un-protonated (free base) pyrrolidone molecules contained in the eluate.

As mentioned in Section 2.1.2.3.6, 10.00 ml of eluate is evaporated until a volume of about 1 ml is left. This step is necessary to remove all of the volatile ammonia as well as concentrate the sample for further work-up. The removal of ammonia is a necessity because the PFPA by-products will react with ammonia, causing the formation ionic bonds. The ammonia concentration decreases as a consequence of evaporation. The pH of the eluate solution also decreases as less ammonia is present. After samples are completely dried, the derivatization agent PFPA is added. Pentafluoro acetic acid is produced as a by-product during the reaction, which further decreases the pH of the solution. The additional decrease in pH favours protonation of the pyrrolidone molecules (ionised base) contained in the solution. Pyrrolidone molecules are not volatile enough to evaporate, and so will be present in the precipitate, and consequently, in the derivatization mixture. The decrease in pH assists in the protonation of pyrrolidone molecules to form pyrrolidonium molecules.

The cause of the main interfering compound is the interaction between the positively charged protonated pyrrolidonium molecules and the negatively charged pentafluoro acetic acid by-

product. An example of the interaction of these two compounds and the chemical properties of an ionic liquid can be best described by 1-Butyl-1-methylpyrrolidinium methyl carbonate (Sigma Aldrich Corporation, St. Louis, MO, USA). Both 1-Butyl-1-methylpyrrolidinium methyl carbonate and pyrrolidonium pentafluoro acetic acid have positively charged nitrogen atoms as well as negatively charged carboxylic acids. These coordinate with each other to form a viscous ionic liquid. Figure 3.1A contains an image of the Waters™ Oasis MCX™ sorbent backbone and Figure 3.1B shows the interfering ionic liquid structure.

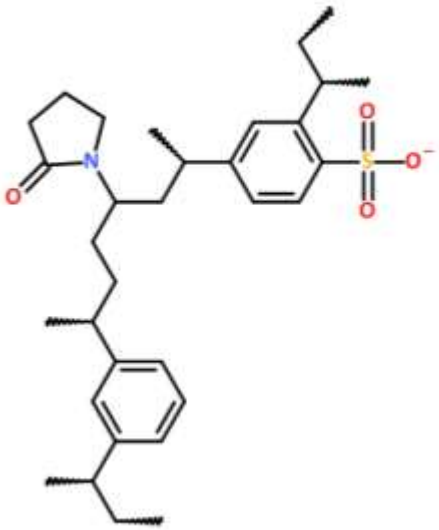


FIGURE 3.1A WATERS™ OASIS MCX™ WITH PYRROLIDONE MOLECULE

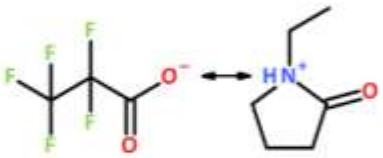


FIGURE 3.1B IONIC LIQUID CONTAINING PYRROLIDONIUM AND PENTAFLUORO ACETIC ACID.

Derivatization of the opioid and cocaine metabolites entailed a more intricate derivatization procedure. Firstly, as mentioned previously, the samples were dried until a residue was formed at the bottom of the GC vials. While these samples were drying, Dimethyl-aminopyridine (DMAP) was added as a catalyst. To do this, a 100.00 mg/ml solution of DMAP in dichloromethane was prepared and 100.00 µl of this solution was added to each opioid sample. A derivatization mixture containing propionic anhydride, PFPOH, ethylacetate, and acetic acid was used. The substitution of PFPA with propionic anhydride will be discussed at the end of this section under Catalyst Interference. After drying was complete, 80.00 µl of propionic anhydride was added, followed by 50.00 µl of PFPOH. Finally, 50.00 ul of the solvent ethylacetate was added to the vials. It should be noted that improved derivatization results were obtained by adding the ethylacetate solvent to

the mixture. Excluding the solvent caused the formation of unwanted derivatant remnants, specifically when using MAD as compared to conventional heating. Derivatization times were drastically reduced with no apparent degradation of compounds. [4] The derivatization mixture was vortexed for 30 seconds to allow the residue to completely dissolve. The opioids plus benzoylecgonine were derivatized in the microwave for a period of 4 minutes at 50 % power. Once more during derivatization the GC vials were positioned in a circle around the edge of the microwave glass plate. Propionic anhydride allowed for the addition of the propionyl group to the opioid functional groups, whereas the acid by-product produced by propionic anhydride—namely, propionic acid—acted as a catalyst to facilitate esterification of benzoylecgonine by PFPOH. The derivatization reaction was further aided by the catalyst DMAP, which has two functional proton acceptors, compared to only one proton acceptor by pyridine. DMAP interacts with the analytes' functional groups, decreasing the binding energy needed for propionic anhydride to facilitate a bond. This interaction is depicted in Figure 3.2.

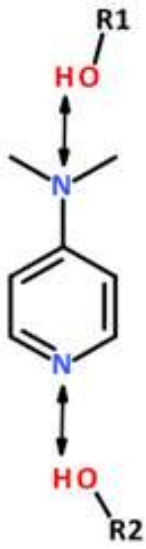


FIGURE 3.2 DMAP INTERACTION WITH ANALYTE

After the derivatization reaction was completed, the reaction mixtures were removed from the microwave enclosure and cooled to room temperature. At this point, the mixtures still contained abundant amounts of propionic acid, which would severely damage the GC column if injected. Thus, steps were taken to remove the propionic acid before injection onto the GC column. Due to the volatile nature of this acid, it was most convenient to dry the derivatization mixtures until the entirety of propionic acid had evaporated. Toluene was used as reconstitution solvent of the dried sample. Contamination of the inlet itself can cause split peaks, especially for dirty wastewater samples. Toluene showed the most focussed peak shape when loaded onto the column and

produced a good injection. Thus, after a dry residue of derivatized analyte had formed in the vials, the samples were reconstituted using 60.00 µl of toluene. At this point, the opioid plus benzoylecgonine samples were ready to be injected.

During the optimization of derivatization, a very important observation was made regarding the molar amount of propionic anhydride added to the reaction mixture. If a volume of propionic anhydride above 100.00 µl was added to the reaction mixture, the propionic acid released as by-product interacted negatively with all analytes containing ester functional groups. These bonds were hydrolysed by the strong acidic nature of the by-products. This occurred with all of the opioids as well as benzoylecgonine. Furthermore, a decrease in signal strength was observed for all analytes as the molar concentration of propionic anhydride increased. The catalyst DMAP acts as a proton acceptor but only to the extent of saturation. When saturation occurs, protons are released into solution, thus lowering the pH and hydrolysing the ester bonds.

3.2.2.2 Catalyst interferences

It should be noted that before propionic anhydride was used, PFPA was employed as derivatization agent. The drawback that resulted from using PFPA was the interferences caused by the catalyst DMAP and by the strong acid by-product pentafluoropropionic acid. Due to the pentafluoro group present in pentafluoropropionic acid, it can be characterized as exceedingly electronegative. Thus, the negatively charged acid will interact with the protonated form of DMAP. The result is the same ionic liquid as is produced with pyrrolidonium and pentafluoropropionic acid. This ionic liquid forms a viscous translucent residue preventing solvents from evaporating properly. When PFPA was substituted with propionic anhydride, the acid by-product produced was far less electronegative, and as result no (or a negligible amount of) ionic liquid interactions occurred.

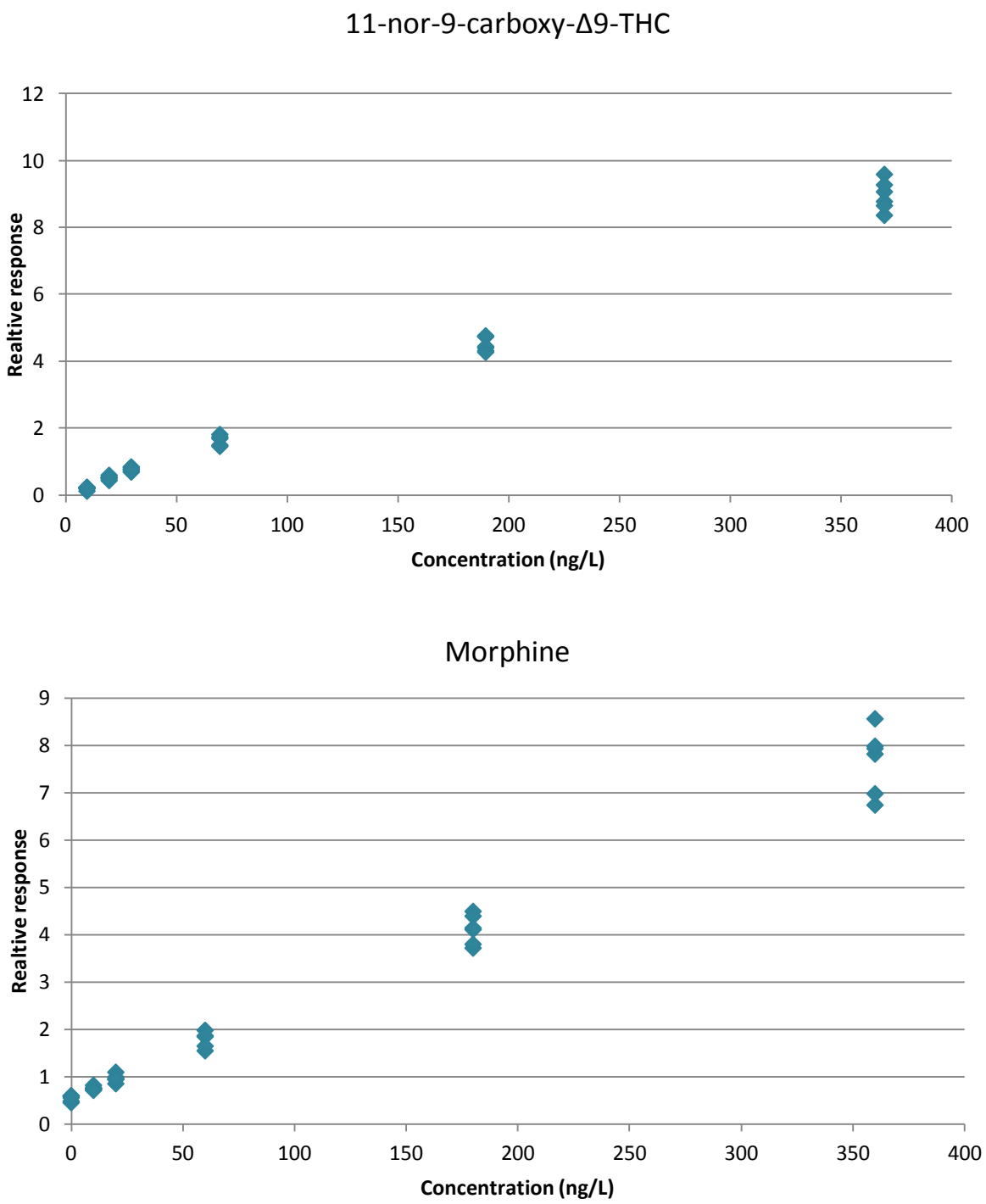
3.2.3 External calibration results

A full external calibration method validation was attempted, but due to enormous between day variations, the statistical model of standard addition was implemented. Some of these external calibration results will be discussed.

3.2.3.1 WEIGHTED REGRESSION

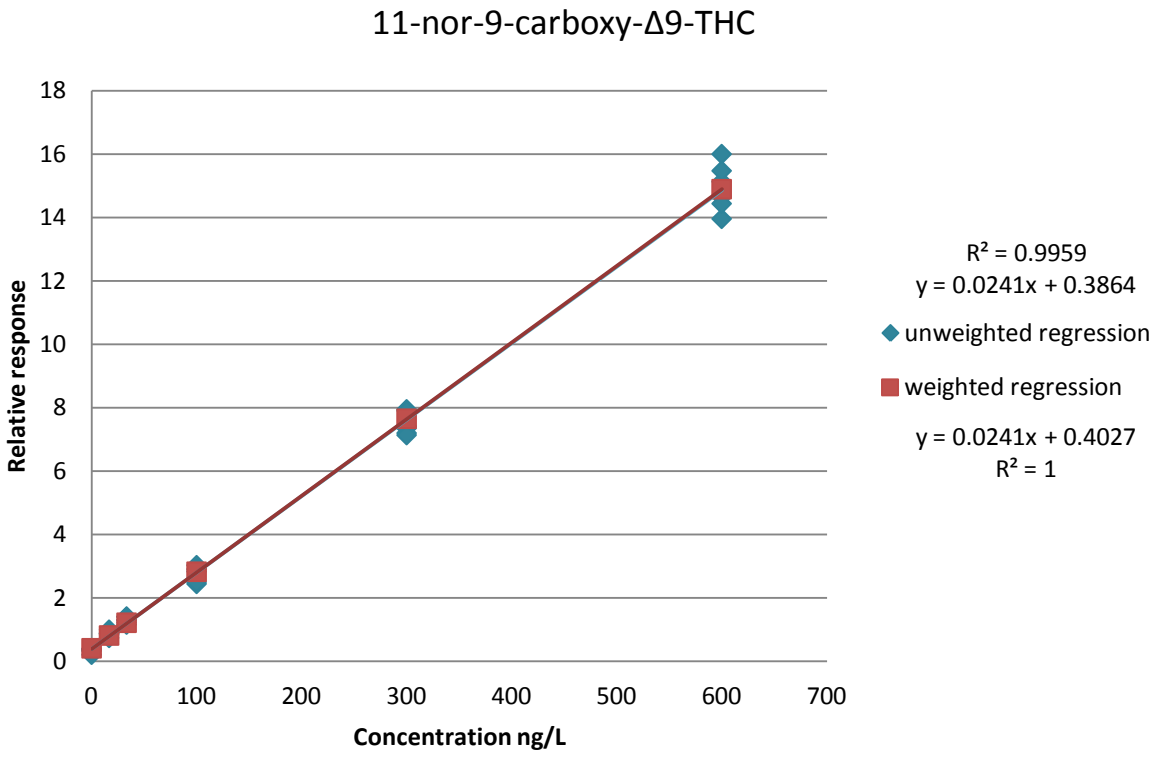
After a series of 6 replicates for each control level were analysed, the data was plotted. In the case of both 11-nor-9-carboxy-Δ^9-THC and morphine it can be seen from Graph 3.3 that heteroscedastic data is suspected. The y variance increases with an increase in x-axis concentration.

GRAPH 3.3 A VISUAL REPRESENTATION OF A HETEROSCEDASTIC DATA SET.

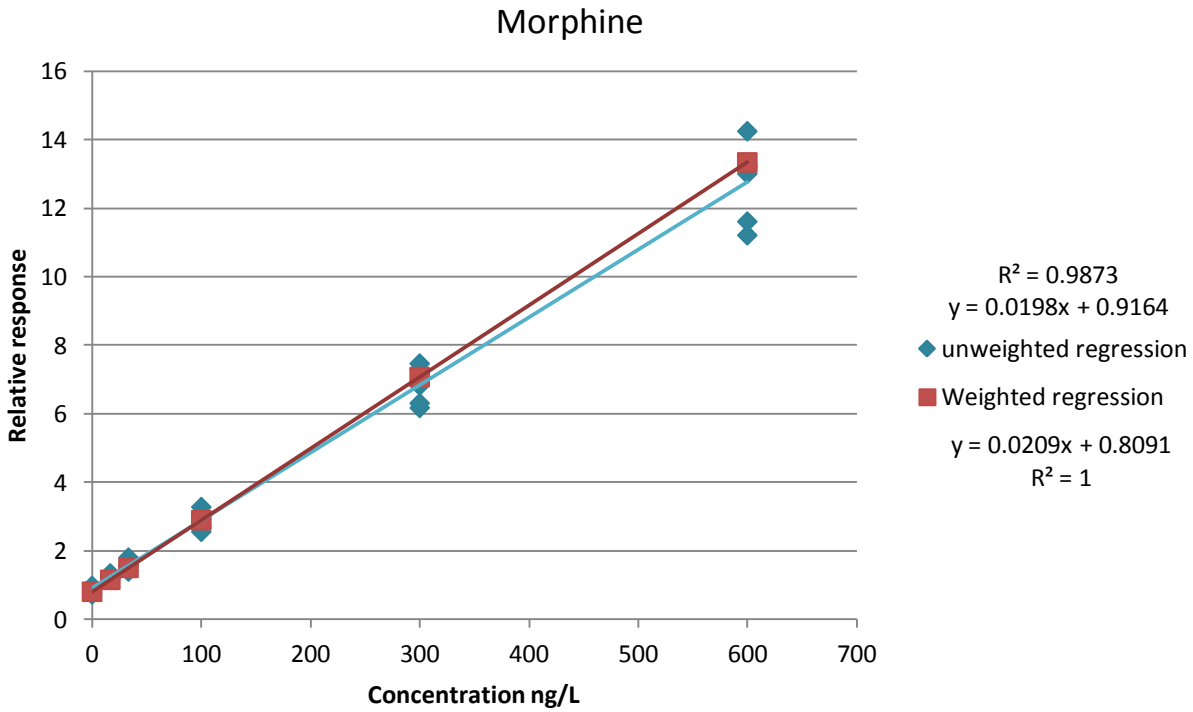


As seen from the curves above, the spread of the y-axis response increases as concentration increases. Thus, a weighted linear regression should be applied to these two analytes. Weighted regression lines were calculated for both 11-nor-9-carboxy-Δ^9-THC and morphine, along with the slopes and y-intercepts. A graphical representation of the un-weighted and weighted least squares regression lines for both analytes are shown below in Graph 3.4A and Graph 3.4B As for the rest of the analytes, homoscedasticity was suspected and normal least squares regression was applied.

GRAPH 3.4A COMPARISON OF WEIGHTED AND UN-WEIGHTED REGRESSION CURVES FOR 11-NOR-9-CARBOXY-Δ9-THC.



GRAPH 3.4B COMPARISON OF WEIGHTED AND UN-WEIGHTED REGRESSION CURVES FOR MORPHINE.

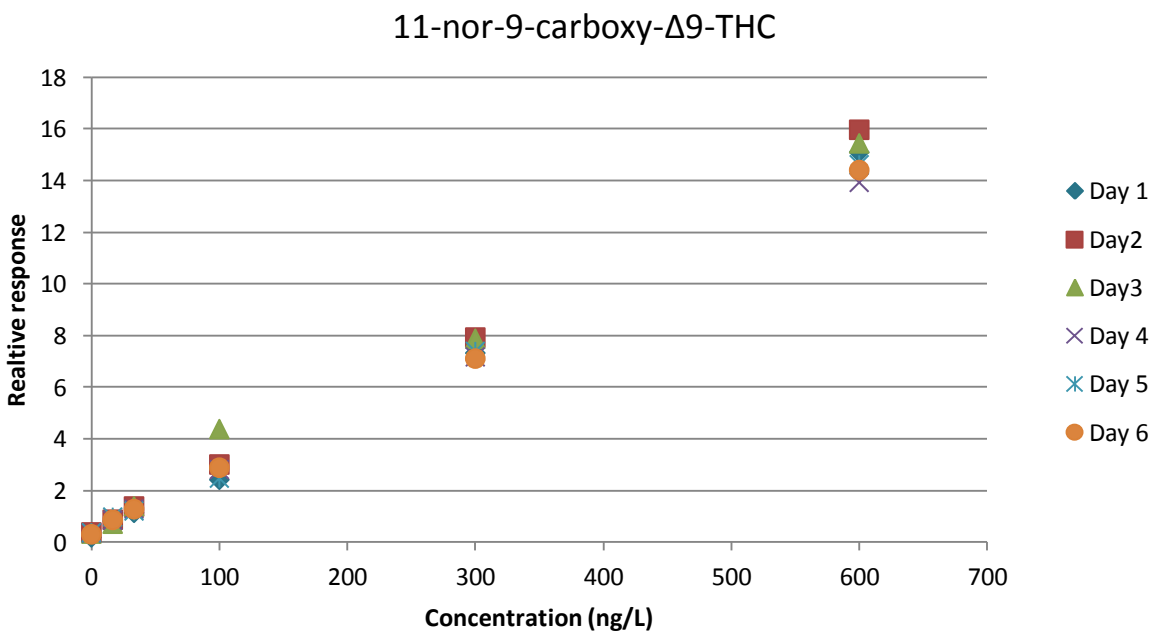


As seen from the Graph 3.4A, there is no apparent distinction between the weighted and un-weighted regression lines for 11-nor-9-carboxy-Δ^9-THC. However, upon further investigation of the equations for both types of regression fits, a difference in slope can be seen. A clearer difference can be seen between weighted and un-weighted regression lines of morphine, as seen in Graph 3.4B.

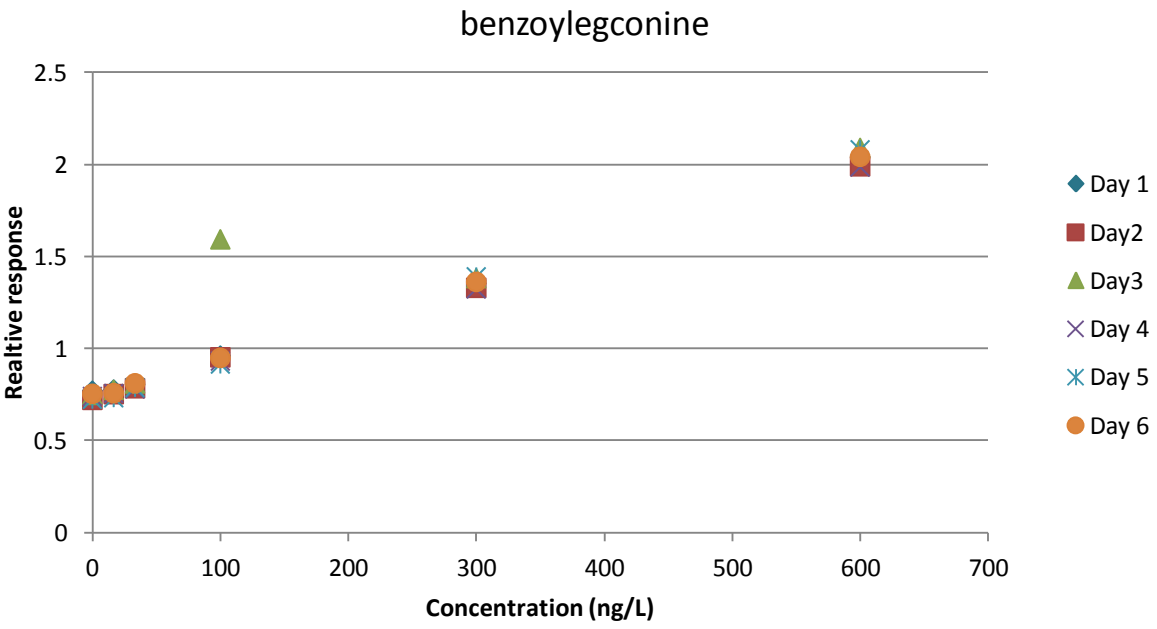
3.2.3.2 LINEARITY

The slope and intercepts for both 11-nor-9-carboxy-Δ^9-THC and morphine were calculated using the weighted regression equations in Section 1.3.3.9 (Weighted regression). For the rest of the analytes measured, an un-weighted least squares regression curve was employed, as explained in Section 1.3.3.8 (Regression). Equipped with the formula of a regression curve for each analyte, the residual $\hat{y}_i$-values could now be calculated and used in the identification of possible outlier values. The $\hat{y}_i$ residual of the suspected outlier value can be calculated from the fitted regression curve and compared to the $S_{y/x}$-value. If the calculated $\hat{y}_i$ residual of the suspected outlier value is 3 times greater than the $S_{y/x}$-value, the suspected data point will be rejected. The linearity results obtained are shown in Graphs 3.5A-E. The 4th concentration level for day 3 was tested for outlying data points using the $\hat{y}_i$ residuals and the $S_{y/x}$-values for all analytes, and is tabulated in Table 3.1

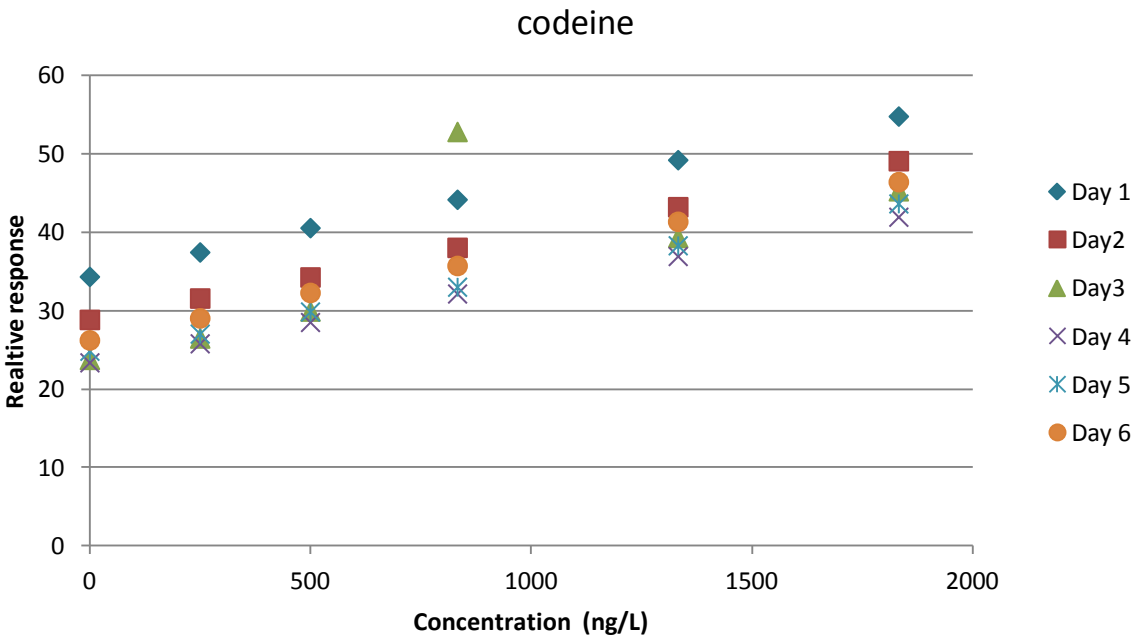
GRAPH 3.5A CALIBRATION DATA FOR 11-NOR-9-CARBOXY-Δ9-THC.



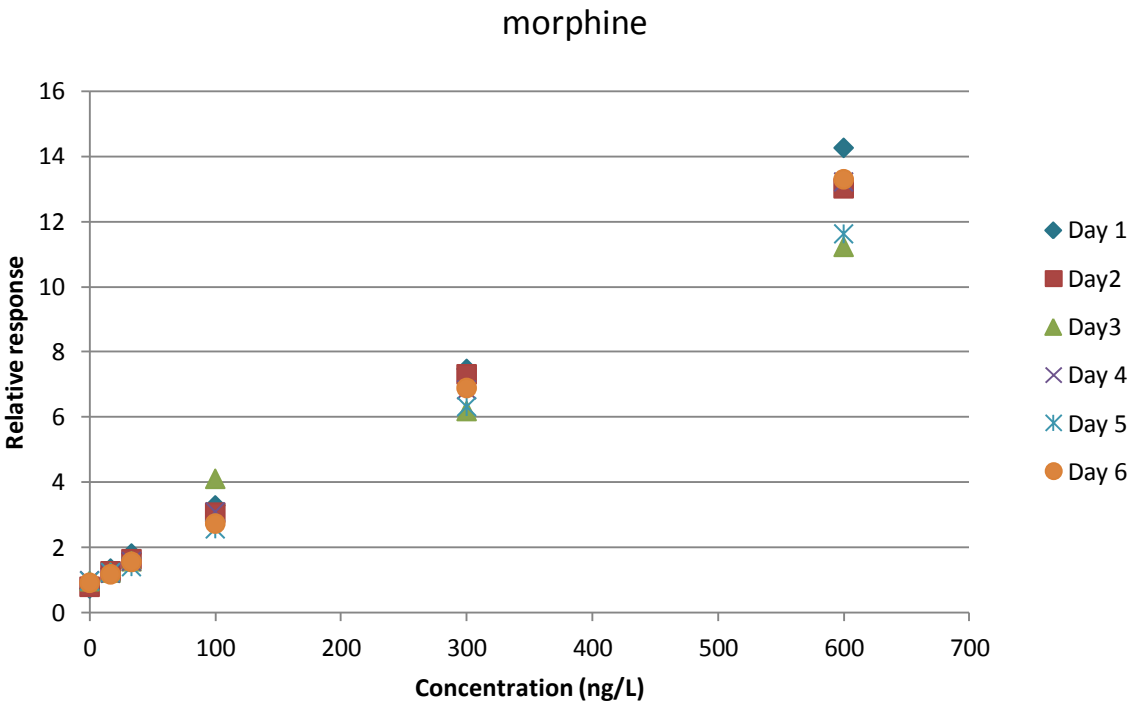
GRAPH 3.5B CALIBRATION DATA FOR BENZOYLECGONINE



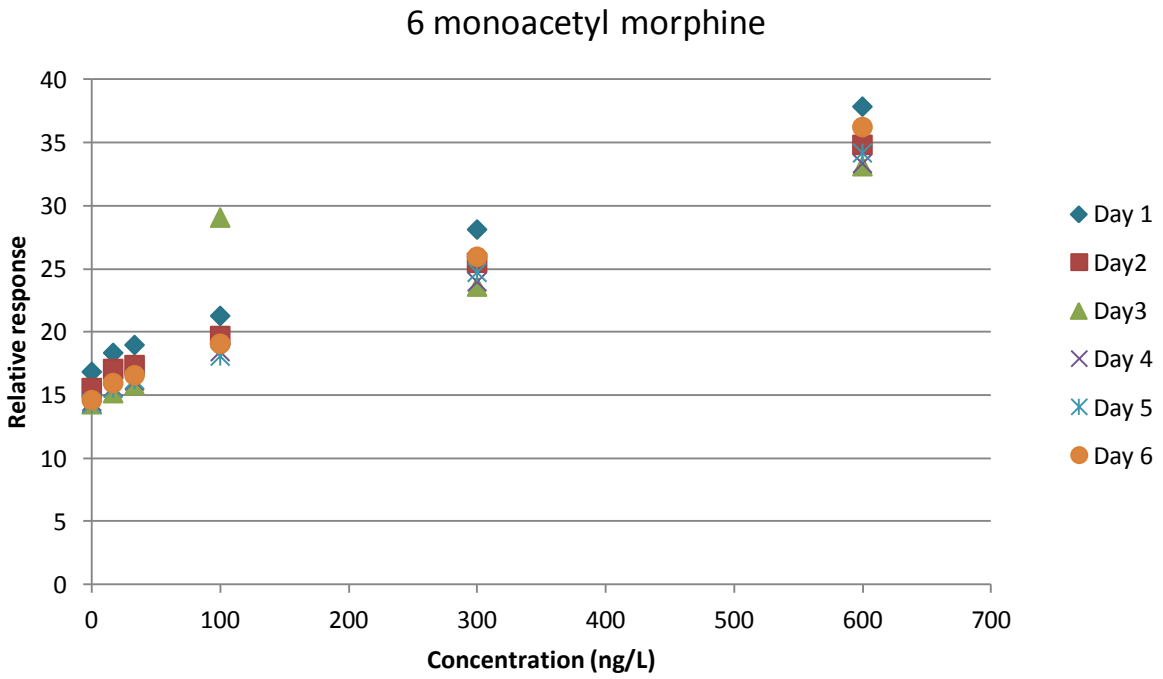
GRAPH 3.5C CALIBRATION DATA FOR CODEINE.



GRAPH 3.5D CALIBRATION DATA FOR MORPHINE.



GRAPH 3.5E CALIBRATION DATA FOR 6 MONOACETYL MORPHINE.



The 4th concentration level for day 3 was tested for outlying data using the $\hat{y}_i$ residuals and $S_{y/x}$-values for all analytes, and the results are tabulated in Table 3.1.

TABLE 3.1 COMPARISON OF $S_{Y/X}$ AND THE $\hat{Y}_I$ RESIDUAL FOR RESPECTIVE ANALYTES

	$S_{y/x}$	$\hat{y}_i$ residual	Times greater
11-nor-9-carboxy-Δ9-THC	0.349213	1.576443	4.514274
Benzoylecgonine	0.026728	0.650148	24.3246
Codeine	4.183497	16.72153	3.997022
Morphine	0.571779	1.199491	2.097823
6 Monoacetyl morphine	1.336589	10.2257	7.650594

As seen from the Table 3.1, the sample measured on day 3 at concentration level 4 is clearly invalid. All of the analytes mentioned, except for morphine, show a three times or greater $\hat{y}_i$ residual than their respective $S_{y/x}$. The exception to this criterion is morphine, which has a 2-times greater $\hat{y}_i$ residual than its $S_{y/x}$-value. Because of the clear evidence that the aforementioned data point has gross error associated with it, this data point will be classified as an outlier. Since all analytes originated from the same sample, it is logical to assume that the particular data point for morphine can also be classified as an outlier. Thus, although the $\hat{y}_i$ residual for morphine is only 2 times greater than the $S_{y/x}$-value, the sample data as a whole will be discarded.

Equation 1.8 was employed in acquiring an r-value for each of the respective analytes and the results are tabulated in Table 3.2.

TABLE 3.2 ESTIMATES OF THE GOODNESS OF FIT FOR LINEAR SLOPE REGRESSION.

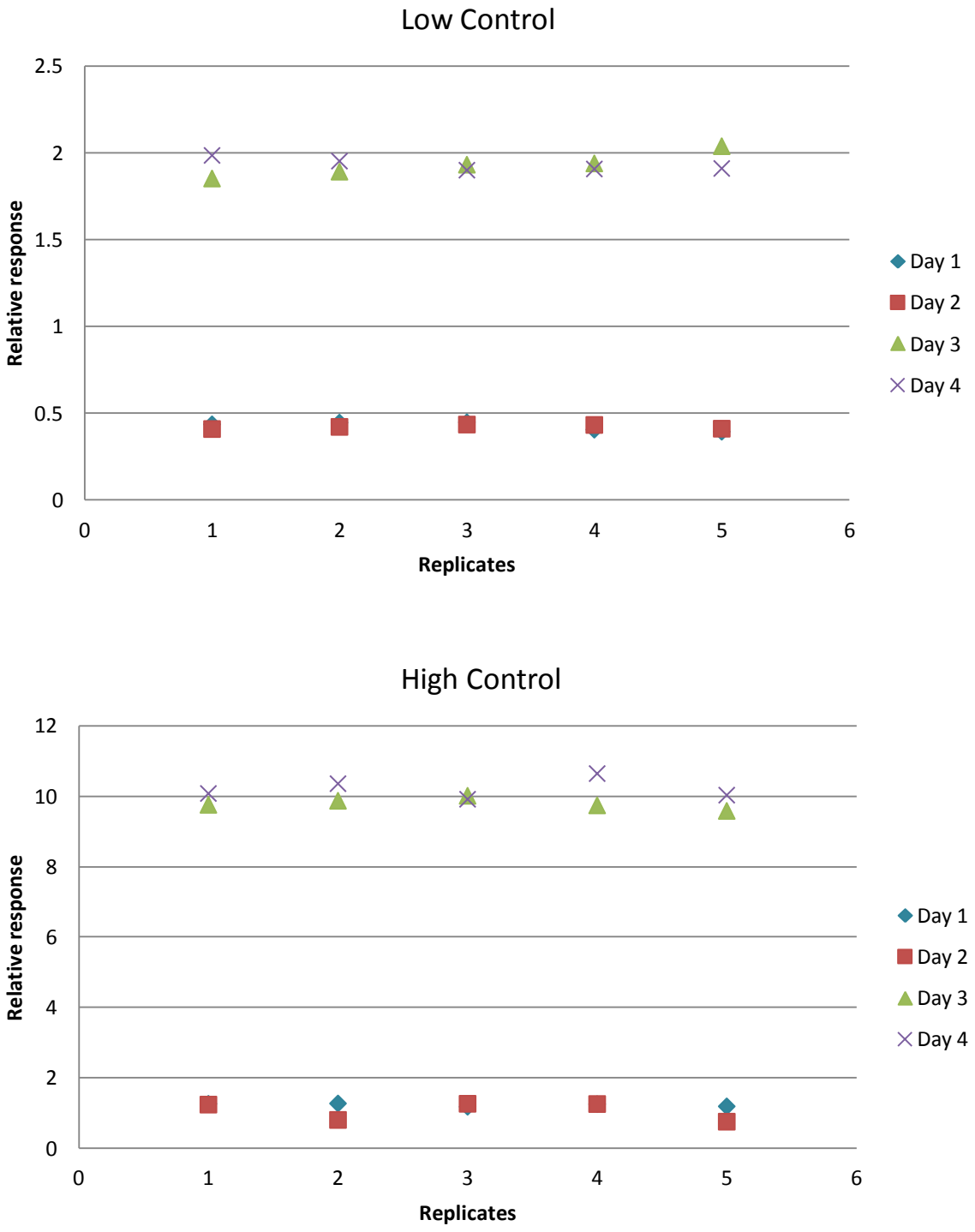
Analyte	Product moment correlation coefficient (r)
11-nor-9-carboxy-Δ9-THC	0.996
benzoylecgonine	0.996
codeine	0.745
morphine	0.987
6 monoacetyl morphine	0.967

The data in Table 3.2 reveal a clear linear response for all analytes except codeine. The codeine goodness-of-fit is affected by results obtained for sample preparation of Day 1. As seen from Graph 3.3C, an overall increase in relative response was witnessed for Day 1 of the codeine analyte. This phenomenon will be discussed in detail in next section. Irrespective of the included data for Day 1, weak linear relationship still exists, r≥0.75.

3.2.3.3 PRECISION

After plotting the precision data for 11-nor-9-carboxy-Δ^9-THC and benzoylecgonine, an atypical phenomenon was observed. This phenomenon was most pronounced with the benzoylecgonine data set and can be visualized in graph 3.6.

GRAPH 3.6 REPEATABILITY PHENOMENON MOST PRONOUNCED WITH BENZOYLECGONINE.



As can be seen from the graphs, the replicate repeats for both high and low control samples on Days 1 and 2 are substantially different from the replicates done on Days 3 and 4. This difference is significant, considering that the concentrations calculated from the regression line of benzoylecgonine have more than a 7-fold difference. The large between-day variation in repeatability data stemmed from 60 L wastewater pool used to obtain all validation data. The

repeatability experiments for Days 3 and 4 were conducted with the last available amount of pooled sample present in the container. In order to expel all of the container's contents, it needed to be decanted, and as a result of this, sediment from the bottom of the container was homogenized into the sample. Thus, it can be inferred that sediment or fine particulate present in the wastewater sample pool was responsible for the significant variation between the first two and the last two days. It is apparent that increased amounts of analytes are present in the settled particulate matter, in contrast to the solution, causing an increased calculated relative response of analytes. It is also evident that that the interaction between analytes and the particulate is more prevalent with certain analytes than with others.

3.2.4 Standard addition validation results

From the previous external calibration method it is evident that between day variations in matrix might impact error of measurement significantly. The statistical model of standard addition was implemented, due to enormous between day variations. For each individual measurement a calibration graph of 6 concentration points is obtained. As result of no replicate runs being employed in standard addition, statistical validation for each respective measurement must be done.

3.2.4.1 Linearity using standard addition

The linearity of each individual measurement can be seen in Appendix A at the end of this dissertation. The product moment correlation coefficient is displayed on each graph in order to produce a goodness of fit measure for each regression line.

3.2.4.2 Precision using standard addition

A precision value is obtained for each individual measurement for each analyte by using Equation 1.4. The percentage relative standard deviation (%RSD) is calculated by dividing standard deviation by the unknown concentration obtained through back extrapolation of the standard addition regression line multiplied by 100. A table showing the %RSD for each individual measurement can also be seen in Appendix A at the end of this dissertation.

3.2.4.3 LIMIT OF QUANTIFICATION (LLOQ) AND DETECTION (LOD) USING STANDARD ADDITION

LLOQ was determined by using %RSD. The limit of quantification is dynamic/changes for each individual measurement when using standard addition. Each of the measured analytes will in turn have their own limit of quantification if they adhere to a maximum of 20 %RSD. Thus if the unknown analyte concentration obtained through back extrapolation of the standard addition regression adheres to a maximum of 20 %RSD it will be classified as the LLOQ. A table showing the LLOQ for each individual measurement can be seen in Appendix A at the back of this dissertation. All LLOQ values adhered to the maximum 20 %RSD. The unknown concentration for the specific day would then be regarded as the LLOQ for that specific analyte.

LOD was assumed to be below the LLOQ, (LOD < LLOQ) since no replicate sample were analyzed a LOD cannot be calculated.

3.3 EPIDEMIOLOGY RESULTS AND WEEK WASTEWATER PROFILE

The week wastewater profile was determined by using the stipulated sampling procedure described in Section 2.1.2.1, to collect wastewater each day from 7am for 24 hours. Each of the hourly samples was pooled together to obtain a representative 24-hour sample. These samples were then analysed using the method described in Chapter 2. The results that were obtained are tabulated in Tables A1-5, and graphically represented with r-values and least squares regression equations in Graphs A1-5. The profiles are presented using the total quantity of an analyte metabolite detected with error indicated by error bars. The calculation estimating the number of doses was corrected for the amount of influent wastewater arriving at a specific sampling pool. Collected daily profile over one week raw data for each analyte are tabulated in the Appendix 1.

Six wastewater aliquots were prepared from the sample pool for each day, with each aliquot spiked with certified analyte standard and the sample pool spiked with deuterated internal standard. All aliquots contained the 5 analytes of interest. As mentioned in Chapter 2, a total of 6 wastewater solution aliquots were prepared for calibration, to accurately make use of the method of standard addition. The concentration ranges for the analyte standards spiked into the aliquots were:

- Benzoylecgonine and codeine: 250.00 ng/L, 500.00 ng/L, 833.33 ng/L, 1333.33 ng/L and 1833.33 ng/L
- 11-nor-9-carboxy-Δ9-THC, morphine, and 6 monoacetyl morphine: 16.67 ng/L, 33.33 ng/L, 100.00 ng/L, 300.00 ng/L and 600.00 ng/L.

Deuterated internal standard remained uniform at a concentration of 100.00 ng/600.00 ml of sample wastewater. After the collected samples were prepared and instrumental analysis was completed, manual integration allowed for peak areas to be measured. Consequently, a relative response could be calculated by dividing the analyte chromatographic peak area by the internal deuterated standard peak area. Following the determination of the relative response for each analyte, the data points were plotted, with concentration on the x-axis and relative response on the y-axis. The graphs for each analyte are presented in Appendix 1.

From each individual day, a least squares regression equation was obtained. Using this equation, the regression line was extrapolated and the x-intercept was calculated. The calculated x-intercept represents the unknown concentration of analyte present in the wastewater sample pool for each respective day of measurement. The unknown concentrations are presented in concentration per litre (ng/L), with error calculated by the statistical methods described in Chapter 1, Section 1.3.3.10. Data including each analyte and day of measurement, with the associated error present for a 95% confidence limit, are presented in Tables 3.3A-E.

TABLE 3.3A UNKNOWN CONCENTRATION AND 95% CONFIDENCE LIMIT FOR 11-NOR-9-CARBOXY-Δ9-THC

	Unknown concentration ng/L	% Error
Sunday Day 1	90.15	10.46
Monday Day 2	83.64	18.56
Tuesday Day 3	92.29	8.949
Wednesday Day 4	85.31	9.584
Thursday Day 5	81.35	13.93
Friday Day 6	68.07	15.99
Saturday Day 7	76.94	14.31

TABLE 3.3B UNKNOWN CONCENTRATION AND 95% CONFIDENCE LIMIT FOR BENZOYLECGONINE

	Unknown concentration ng/L	% Error
Sunday Day 1	465.31	7.46
Monday Day 2	378.80	7.92
Tuesday Day 3	305.46	13.29
Wednesday Day 4	378.66	9.50
Thursday Day 5	368.49	10.42
Friday Day 6	351.56	8.85
Saturday Day 7	671.18	4.37

TABLE 3.3C UNKNOWN CONCENTRATION AND 95% CONFIDENCE LIMIT FOR CODEINE

	Unknown concentration ng/L	% Error
Sunday Day 1	1725.06	1.42
Monday Day 2	1999.30	2.20
Tuesday Day 3	1194.60	3.51
Wednesday Day 4	2088.99	1.11
Thursday Day 5	2024.58	0.90
Friday Day 6	1257.87	2.95
Saturday Day 7	2930.57	0.97

TABLE 3.3D UNKNOWN CONCENTRATION AND 95% CONFIDENCE LIMIT FOR MORPHINE

	Unknown concentration ng/L	% Error
Sunday Day 1	58.15	14.86
Monday Day 2	32.89	22.13
Tuesday Day 3	45.57	20.03
Wednesday Day 4	39.16	23.63
Thursday Day 5	52.42	14.40
Friday Day 6	54.16	4.79
Saturday Day 7	53.43	15.57

TABLE 3.3E UNKNOWN CONCENTRATION AND 95% CONFIDENCE LIMIT FOR 6 MONOACETYL MORPHINE

	Unknown concentration ng/L	% Error
Sunday Day 1	498.09	1.62
Monday Day 2	438.23	2.63
Tuesday Day 3	255.26	1.39
Wednesday Day 4	446.80	2.09
Thursday Day 5	436.28	2.87
Friday Day 6	282.45	2.48
Saturday Day 7	621.23	1.01

Taking into account the ever-changing wastewater matrix and the inconsistency of the matrix composition, the comparable associated error for each analyte is modest. Additionally, because of the low concentration of analytes present in the matrix, associated error increases as the concentration of the analyte in the sample decreases, as seen from the data above. Morphine has the largest average percentage error because of it is the analyte with the lowest average concentration level. The daily profile over one week for each analyte with individual error is depicted in the graphs below. One notable fact is the total concentration of analyte present per day could not be back-calculated to the total amount of doses, without first having a measure of the amount of influent wastewater received at the plant. The week profile concentration plots (un-corrected for flow) for analyte in the samples are presented in Appendix 1.

To make an approximation of the amount of doses per day, the total volume of influent wastewater needed to be factored in. Thus, before the appropriate conclusions could be drawn, the total influent flow of the treatment plant needed to be calculated. Using this data the flow-corrected analyte totals were determined and are presented in the following section.

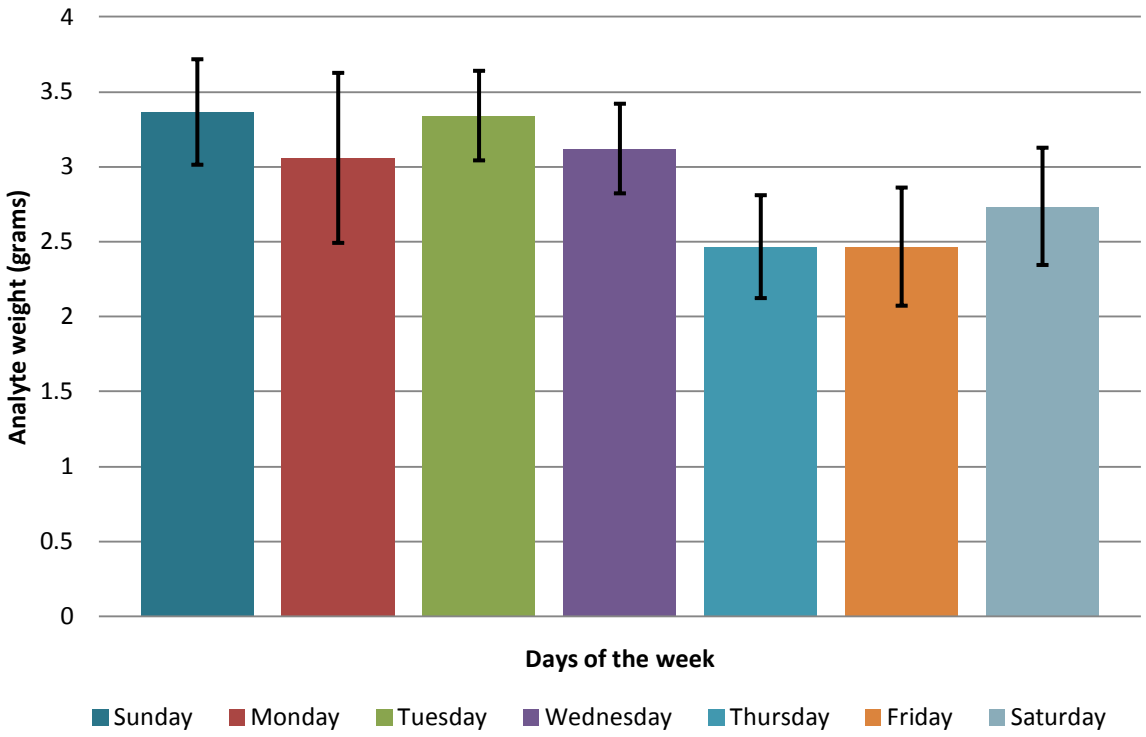
3.3.1 Influent total flow correction

In order to perform the back-calculation to estimate the total mass of analyte present in the wastewater for a specific day, the measure of the amount of wastewater influent was needed. There are different sedimentation pools removing solid organic waste from the wastewater matrix. At each of these respective pools a flow meter measures the influent flow into that specific pool. The influent flow is measured in 5-minute intervals in litres per second. After the 1-hour influent flow data has been plotted, the average flow for 24 hours is calculated and allocated to the respective 24-hour sample pool collected. From this data, the total analyte mass could be estimated since the concentration per litre had been determined. There are different sampling techniques that can be followed to improve the accuracy of estimation. Flow proportional sampling, as well as correlation of population size with yearly prescription pharmaceuticals use, are some of the ways that estimation of drug use can be improved. [5] However, with the available equipment and instrumentation, the aforementioned approach was the most accurate estimation that could be achieved. When all of the measured individual flow data for each sedimentation pool are added, a total influent quantity was calculated. The calculated total influent for each respective day was then correlated to the acquired analyte concentration. A total estimated mass of the measured metabolites was then calculated per day. From the total calculated influent

volume, the population served can also be calculated, since the average person utilizes approximately 130 L of water per day. [6] The population served by the Daspoort treatment plant will then vary from day to day as the calculated total influent flow volume per 24 hours is used to calculate the population size. Because the total influent flow differs between days, the analyte concentration per litre may vary, but the total analyte mass may stay constant. For example if the analyte concentration per litre is high for a specific day and the influent flow is decreased, the same total analyte mass will be achieved , as is also the case where the influent flow is high and the analyte concentration per litre is decreased. Thus, it is important to calculate the total mass of analyte corrected for flow in order to draw conclusions from the acquired data. It should be mentioned that the total amount of doses calculated is significantly more accurate than the estimate of population percentage consuming illicit substances. Although an average of 130 L per person was used, the influent volume may be attributed to areas other than residential. For example commercial as well as industrial metropolitan areas may contribute significantly more influent flow volume per capita inhabitants in the area. Population consumption percentages were only calculated to be able to draw some comparison between this and other studies. The following Tables A1-5 in Appendix 1 presents the concentration per litre as well as the total influent flow, from which the total analyte mass was calculated.

3.3.2. 11-NOR-9-CARBOXY-Δ9-THC PROFILE

GRAPH 3.7 GRAPHICAL REPRESENTATION OF TOTAL ANALYTE MASS PER DAY WITH ASSOCIATED ERROR FOR 11-NOR-9-CARBOXY-Δ^9-THC PROFILE

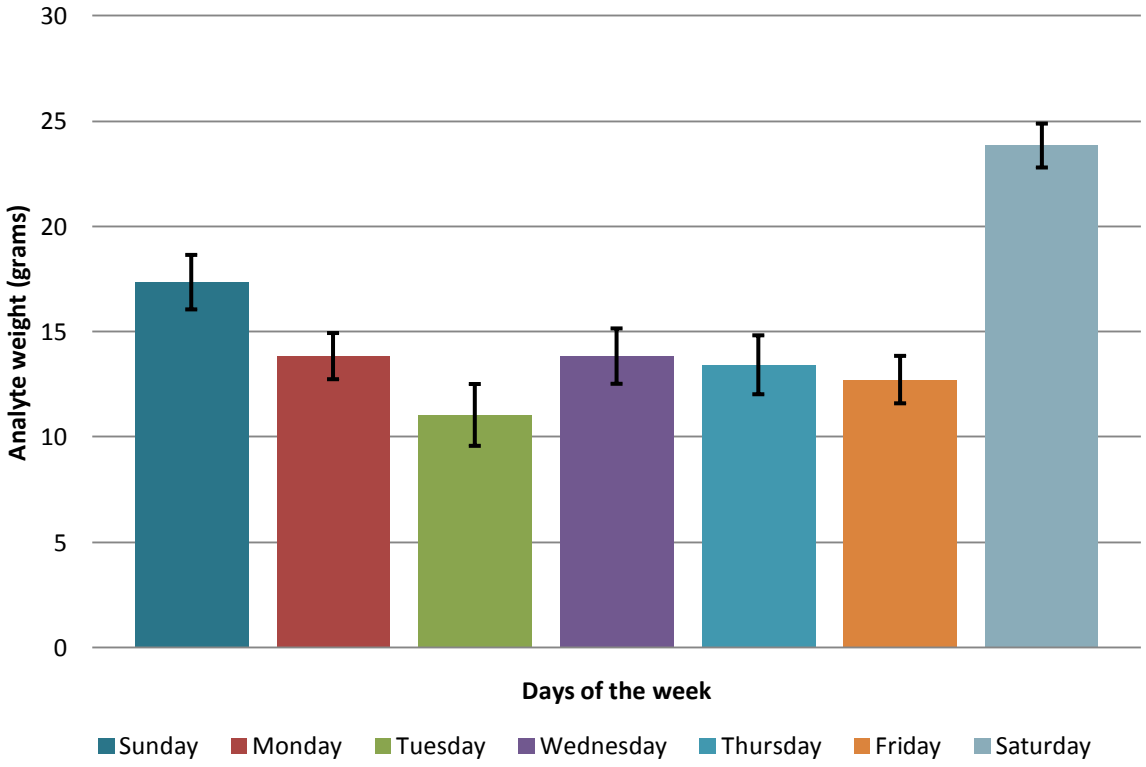


Starting with 11-nor-9-carboxy-Δ^9-THC, it is evident that the total mass of this analyte stays relatively constant from day to day. This constant trend would be expected as a result of the lipophilic nature of the metabolite in question. Considering renal metabolism, as explained in Chapter 1, all metabolism reactions have an inclination to increase the water solubility of compounds in order to be excreted via the renal microtubules. Due to the high lipophilicity of 11-nor-9-carboxy-Δ^9-THC, the excretion rate of this metabolite differs from other water-soluble molecules. The metabolite 11-nor-9-carboxy-Δ^9-THC is absorbed into human lipid cells. This absorption is due to the affinity of this metabolite for non-polar fat cells. As these lipid cells and 11-nor-9-carboxy-Δ^9-THC interact, the excretion rate of 11-nor-9-carboxy-Δ^9-THC is markedly decreased. The human body stores energy in the form of fat or lipid, and these energy stores are only consumed when energy reserves of other forms are low (e.g. glycogen and proteins). Because lipid reserves are only broken down and used for energy during physical exertion over a lengthened period of time, it is safe to assume that the excretion rate of 11-nor-9-carboxy-Δ^9-THC will stay fairly constant over time. As a result of this slow excretion and long half life of parent Δ^9-THC (24.9 to 34.5 hours) [7], a constant concentration profile in wastewater is expected as drug

users excrete small quantities of the analyte over a long period of time. When viewing the profiles, a fairly constant pattern can be observed, and care should be taken to account for the scale differences between Graphs 3.7-3.8.

3.3.3 BENZOYLECGONINE PROFILE

GRAPH 3.8 GRAPHICAL REPRESENTATION OF TOTAL ANALYTE MASS PER DAY WITH ASSOCIATED ERROR FOR BENZOYLECGONINE

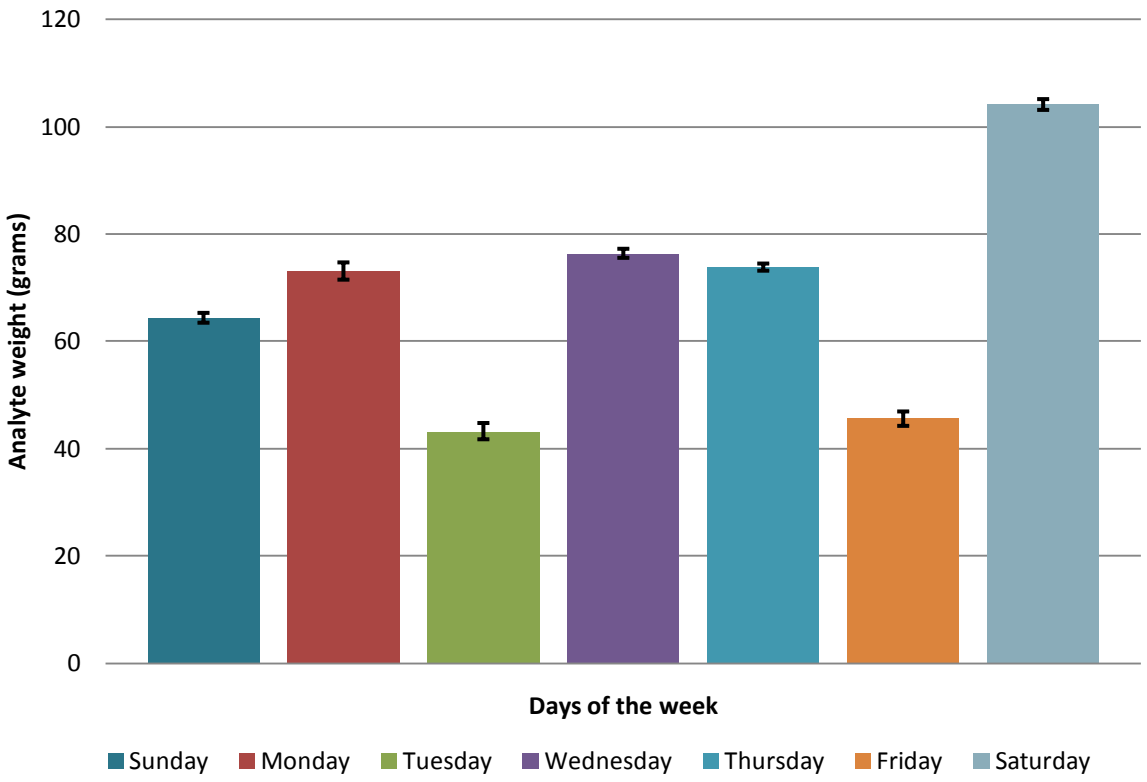


When viewing the total analyte mass profile of benzoylecgonine, one can recognize that a spike in concentration is observed during the weekend, on Saturday. An above average signal is also observed on the Sunday. Wastewater is transported via a pipeline from wherever it originates to the Daspoort treatment plant. According to the Daspoort treatment plant foreman, wastewater and excrement takes several hours to reach the plant. So it would be reasonable to speculate that a certain quantity of metabolites from drugs used on Friday would reach the treatment plant by Saturday morning. The half life for cocaine in urine is 4.1 ±0.9 hours [8]. Cocaine is metabolized over several hours and excreted only when the bladder of the subject is voided. The metabolism of drugs and the time frame for wastewater transport, support the hypothesis that Friday's drug use is likely represented in Saturday's wastewater sample. As seen from Graph 3.8, a significantly increased quantity of benzoylecgonine was observed in Saturday's wastewater, as well as a slightly

increased signal on the Sunday. Following upon the hypothesis stated above, it is conjectured that the analyte's parent compound, cocaine, was consumed to a larger degree on both Friday and Saturday; hence, the metabolite present in higher amounts on Saturday and Sunday.

3.3.4 CODEINE PROFILE

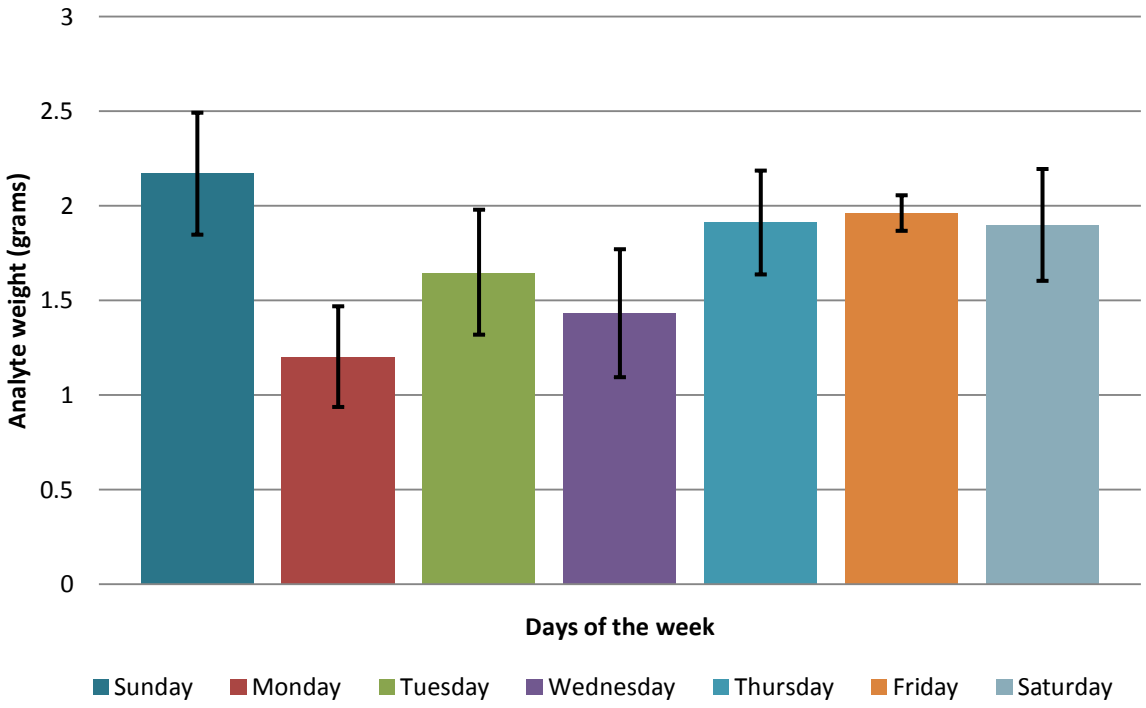
GRAPH 3.9 GRAPHICAL REPRESENTATION OF TOTAL ANALYTE MASS PER DAY WITH ASSOCIATED ERROR FOR CODEINE



From the week profile of codeine concentration, it is evident that the largest amount of codeine is consumed during the weekend as well as during the mid-week period. Since Tuesday has the lowest concentration of analyte, the hypothesis stipulates that the least amount of codeine was consumed during Monday. The highest quantity of codeine was consumed during Friday. It should be noted that no prescription is needed to obtain codeine in pharmaceutical form and it is readily available over the counter in South Africa. Codeine was added to the analytes of interest, since both codeine as well as 6 monoacetyl morphine (the marker for heroin use) gets metabolized to morphine. It is thus important to gauge whether the total morphine quantity is a result of codeine or heroin use.

3.3.5 MORPHINE PROFILE

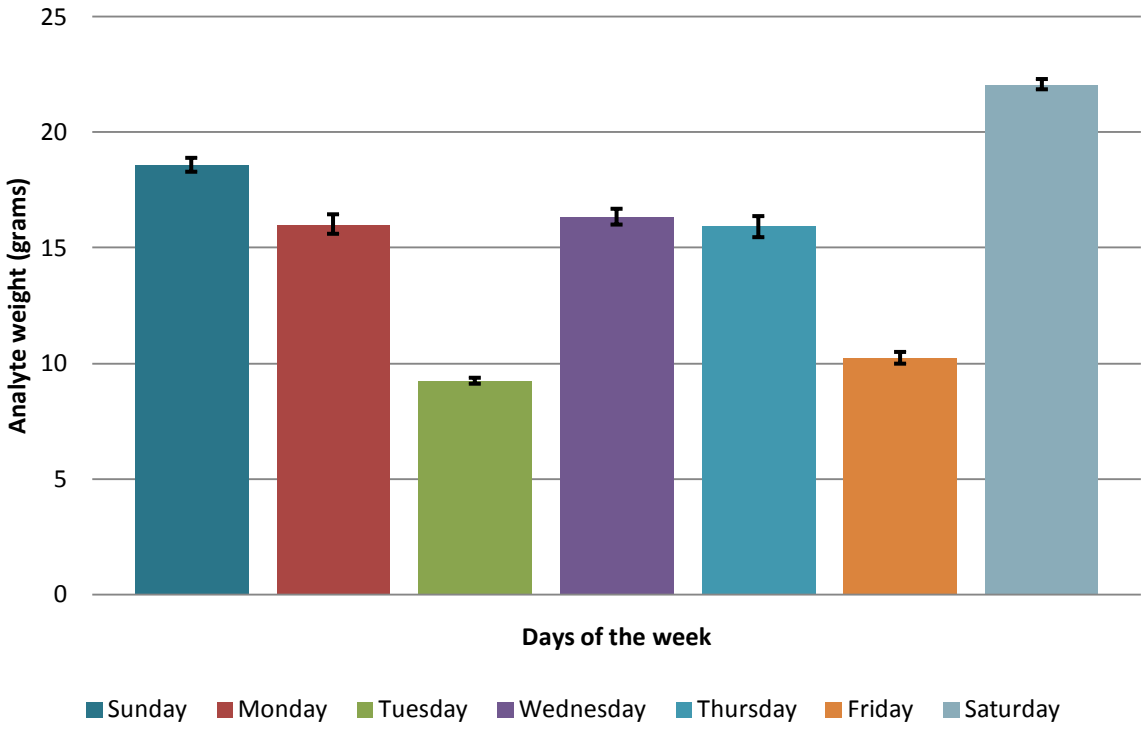
GRAPH 3.10 GRAPHICAL REPRESENTATION OF TOTAL ANALYTE MASS PER DAY WITH ASSOCIATED ERROR FOR MORPHINE



Although morphine is not classified as an illegal substance in South Africa, more stringent control measures are being followed in the distribution of morphine to the public. As result of these measures, it was expected that a lower concentration of morphine would be present in the wastewater influent. Compared to other analytes a decreased average concentration of morphine was present in the wastewater influent. Morphine has the single lowest concentration range of the analytes measured in this study. The morphine profile differed from codeine in that no apparent increase in morphine total mass was observed during the weekend. Based on the data, it can by hypothesized that morphine use declined at the beginning of the week profile and followed a somewhat equal distribution for the remaining days.

3.3.6 6-MONOACETYL MORPHINE PROFILE

GRAPH 3.11 GRAPHICAL REPRESENTATION OF TOTAL ANALYTE MASS PER DAY WITH ASSOCIATED ERROR FOR 6 MONOACETYL MORPHINE



This molecule is considered to be the primary marker for heroin abuse. Since heroin gets metabolized to 6MAM, and thereafter to morphine, the end product morphine is present after both heroin and codeine metabolism. Three different sources of morphine are present in the wastewater, all contributing to the total concentration. Firstly, administered morphine from hospitals and prescriptions get excreted as the parent compound and forms part of the total concentration of morphine. Secondly, both codeine and 6MAM get metabolized to morphine to a certain extent. The only metabolite present to distinguish between morphine, codeine and heroin use is the aforementioned compound, 6MAM. It is important, however, to pay attention to the experimental concentration ratios of these three metabolites. If, for example, the morphine concentration is high with no 6MAM present, a reasonable conclusion would be that the parent compound is morphine. If, however, codeine and morphine are present, the ratio of these metabolites could determine whether both morphine and codeine were ingested, or codeine alone. A codeine concentration higher than morphine would indicate codeine use, after which a quantity of the codeine was metabolized to morphine, hence, morphine's presence. It would be highly unlikely, however, that a morphine concentration higher than codeine could originate from

codeine use alone. As result of the very short half life of heroin (10-20 minutes), [9] both morphine and heroin is suspected to be present at different ratios in the excrement, depending on the time of excretion after ingestion. As a result of 6MAM being metabolized to morphine, the time after ingestion determines the progress of complete elimination of the parent compound. Thus, if a sample was taken long after heroin ingestion, the morphine concentration would be much larger than the 6MAM concentration, since 6MAM is metabolized to morphine. All of the above mentioned factors play a part in the estimation of opioid drug use.

3.3.6.1 Anomalous 6MAM profile

Taking all of the factors above into account, the anomalous profile plot for 6MAM is curious. When comparing the 6 MAM plot with codeine, although the concentrations differ, an astonishingly analogous profile was observed. It was expected that the average concentration of morphine would be large compared to 6MAM, since there are three different sources from which morphine might originate. This, however, was not true and a very large concentration of 6MAM compared to morphine was present in the collected wastewater samples. As mentioned previously, the similarity between the codeine and 6MAM week concentration profile is undeniable, and thus it may be hypothesized that codeine was somehow altered to produce the analyte in question, 6MAM. The only alterations necessary for this to occur are an acetylation reaction on the number 6 carbon and hydrolysis on the number 3 carbon. It is thus postulated that 6MAM was formed from codeine, resulting in the undeniably similar week profile plots. The codeine concentration present in the sample is of such profuse amounts that a small variation in the underlying 6MAM concentration may not be able to influence the plot. 6MAM is the marker for heroin use and it can be said with extreme certainty that this was the metabolite that was detected. What cannot be said with certainty is that this analyte represents heroin abuse in the population. As result of this opioid displaying a peculiar ratio compared to the rest of the opioid analytes, it is hypothesized that codeine may have been altered to form 6MAM by living organisms present in the wastewater. The error associated with 6MAM is also comparable to that of codeine, both being similarly small compared to the larger errors observed with other analytes. Comparing 6MAM and codeine, the only difference in profile was observed on Sunday, when relative codeine response was lower than 6MAM. This may indicate an increased amount of non-codeine-related 6MAM present in the wastewater on Sunday, and could be a sign of increased heroin abuse during Saturday.

The studies of Bernard *et al*. and Kimura *et al.* [10,11], have shown that bacterial enzymes, namely o-acyltransferases, are able to acylate hydroxyl groups. No literature was found reporting the phenomenon of codeine being acylated to form 6MAM. However, the possibility of this reaction occurring cannot be denied since these enzymes are already present in certain bacterial life forms. Further examination and experimentation regarding this subject is beyond the scope of this dissertation; however, future investigation could be undertaken as follows. A brief experimental setup could be used to see whether the bacterial life forms present in wastewater will acylate codeine to form 6MAM. Since both analytes of interest (codeine and 6MAM) are already present in wastewater, an experiment could be attempted by adding a large quantity of codeine deuterated internal standard. As a result of this deuterated standard not being present in the wastewater, endogenous codeine cannot interfere. The spiked internal standard would be homogenized and the sample stirred continuously for an extended period of time, after which the targeted analysis for an acylated form of the deuterated standard will be performed. This hypothesis of bacterial acylation can be tested using the wastewater matrix itself.

A wastewater sample pool was collected at the treatment plant each morning, and sample preparation started immediately after sample collection. No stability studies were performed since only half an hour transport time was incurred before sample preparation started.

3.3.7. Dosage quantity estimation

As mentioned previously, the total mass of analyte present in the wastewater each day was calculated. From this total mass, an estimation of the amount of doses consumed can be produced. It is, however, important to realize that only the amount of doses can be estimated and not the number of individuals using the drug, since one individual may consume multiple doses per day. Considering that the amount of doses is the end goal, the metabolism and product ratios need to be known. The metabolism of parent compounds and their corresponding reaction products have already been discussed in previous chapters, and the product ratios for each metabolite will be discussed in the following section.

3.3.7.1 Metabolism product ratios

Each of the parent compounds undergo certain reactions to form the metabolites that were measured in this study. The metabolic pathways of the products formed have been discussed in Chapter 2. Only the day with the highest amount of doses calculated will be used to draw

comparisons and calculate population statistics. References for the product ratios of each metabolite will now be discussed.

3.3.7.1.1 11-NOR-9-CARBOXY-Δ9-THC

Δ9-THC is metabolized to two main metabolites; namely, 11-hydroxy-Δ9-THC and 11-nor-9-carboxy-Δ9-THC. Since the latter compound is the metabolite measured in this study, the product yield percentage is required to estimate the number of doses consumed. The acidic metabolite, 11-nor-9-carboxy-Δ9-THC, is predominantly excreted in urine, whereas 11-hydroxy-Δ9-THC is principally found in faeces. [12] Since a large amount of data is available on urine analysis and less on faeces, it was decided that the urine-predominant 11-nor-9-carboxy-Δ9-THC would be targeted. The average dose of Δ9-THC needs to be considered before a back-calculation can be done. According to Huestis *et al.* [12], a low dosage Δ9-THC cigarette will contain an amount of 18mg active compound, while a high dosage of Δ9-THC will have a quantity of 34 mg. An experiment was conducted by Huestis *et al.* [12] confirming the product percentage ratio of 11-nor-9-carboxy-Δ9-THC after Δ9-THC was ingested. The amount of total Δ9-THC being metabolized to the 11-nor-9-carboxy-Δ9-THC amounted to 0.54±0.14 percent and 0.53±0.09 percent for low- and high-dose parent compound uptakes. [12] The high-dose figure, 0.53%±0.09 %, was used in order to estimate drug use from the measured metabolite quantity. This was done in order to obtain a conservative estimation of the lowest possible quantity of Δ9-THC ingested by the population. If the lowest possible number of doses is obtained, there can be no ambiguity that the studied population's individuals are consuming Δ9-THC.

The total analyte mass for each day is tabulated in Table 3.4. Using the metabolism data from the literature, the calculated mass of 11-nor-9-carboxy-Δ9-THC represents 0.53±0.09 % of the total Δ9-THC active compound mass ingested. [12] In accord with the decision to estimate the lowest amount doses cannabis consumed, the highest metabolism product percentage was employed. Thus, (0.53 %+0.09 %=0.62 %) was used to estimate the total Δ9-THC consumed since a high metabolism rate will ensure that a smaller total quantity of Δ9-THC is obtained through the calculation. It is also important to take the associated error into account for each measurement. The estimated population consumption of Δ9-THC was calculated in the following manner:

$$\boldsymbol{P_{tot}} = \frac{\mathbf{100}}{\mathbf{0.62}} \times (\boldsymbol{A_w}) \pm \boldsymbol{Error}\ \% \qquad \textbf{...Eq 3.1}$$

Where P_{tot} is the total amount of parent compound, and A_w is the total analyte mass. After the total quantity of parent compound was calculated, the amount of doses could subsequently be determined. P_{tot} is divided by the total amount of active parent compound present in one administered dose—in this case, 34 mg. [12]

It should be noted that the amount of doses may not be equal to the amount of population users, since individuals may consume more than one dose per day. Thus, only the amount of doses per day can be reported. The absolute minimal amount of doses consumed per day were calculated and are presented in Table 3.4.

TABLE 3.4 CALCULATED MINIMAL NUMBER OF DOSES CONSUMED PER DAY OF Δ9-THC

	A_w (g)	P_{tot} (g)	Doses	%Error
Sunday	3.36	542.43	15953.84	10.46
Monday	3.06	493.05	14501.41	18.55
Tuesday	3.34	538.55	15839.80	8.95
Wednesday	3.12	503.10	14797.16	9.58
Thursday	2.97	478.34	14068.82	13.93
Friday	2.46	397.54	11692.25	15.99
Saturday	2.73	440.90	12967.76	14.31

The calculated least significant difference (LSD) for Δ9-THC amounted to 998.31. If the amount of doses between comparable days differ more than the calculated LSD, the result is classified as being significantly different from one another with a confidence of 95 %. As seen in Table 3.4, the amount of doses consumed daily remained relatively constant, with 15,953 being the highest, on a Sunday. The amount of doses arranged from lowest to highest are shown below. If the doses do not significantly differ from one another they will be combined in brackets.

Friday < Saturday < (Thursday, Monday, Wednesday) < (Tuesday, Sunday)

The average population served by the plant was calculated by dividing the average influent flow volume (36,398,775.53 L) by the average water consumption of an individual (±130 L/day). [6] Thus, the estimated total population served by the Daspoort treatment plant amounted to 279,991. Since the total influent differs from day to day, a varying population quantity was obtained for each day of the week. The population served on Sunday amounted to 286,968 individuals. Taking the 10.46 % error into account, the total doses on Sunday amounted to 15953.84±1,669.14. If only one dose of Δ9-THC is allowed per person, the percentage of the

population consuming cannabis on Tuesday would equal 5.556±0.582. A study done by Zucatto *et al.* [13] at a treatment plant serving 1.25 million inhabitants reported a metabolite concentration of 18±2 g/day for Sunday. This figure, however, was reported with only one standard deviation and can therefore not be considered to be 95 % accurate, since a two times standard deviation is required to obtain a 95 % confidence interval. A concentration value of 18±4 g/day is obtained when a two times standard deviation error of 4 g or 22 % is employed. The total amount of doses calculated from the aforementioned Zucatto *et al.* [13] study equals 85,388.99±18,433.18 for a population size of 1.25 million. At this point, it can be seen that the total mass of analyte far exceeds that of South Africa, but the population served in Milan (where the Zucatto *et al.* study was performed) also far exceeds that of Daspoort. If only one dose is allowed per person and the estimated population statistic for cannabis use is calculated, Milan's drug consumption percentage would be 6.831±1.475. Compared to South Africa's percentage 5.692±0.509, Milan shows a 1.139 % larger quantity of estimated population cannabis use. It should be noted that the error measurement for the Milan population percentage could be as low as 5.356, which begs the question of whether Milan's usage may not be so different from South Africa's after all.

3.3.7.1.2 BENZOYLECGONINE

The next analyte that will be reviewed is benzoylecgonine, the metabolite is formed from the parent compound cocaine. The metabolic percentage of produced benzoylecgonine compared to the total parent compound consumed is 35-55 %; i.e., if cocaine is administered, 35-55 % of it will be metabolized to benzoylecgonine. [14] As was done with Δ9-THC, the highest metabolism percentage was employed to give the most conservative value for cocaine use and to ensure that the number of daily doses calculated represent a minimum possible usage. The highest metabolic percentage—55 %—was therefore applied. Dosages of 25 mg intravenous and 32 mg intranasal cocaine are classified as therapeutic amounts. [14] The 32 mg intranasal dosage quantity was used for the calculations—again, to ensure the absolute minimum possible number of estimated doses. The following equation was used to calculate total parent compound mass as well as estimate the amount of doses. The equation is the same as 3.1, except for the use of metabolic conversion percentage of cocaine to benzoylecgonine.

$$\boldsymbol{P_{tot} = \frac{100}{55} \times (A_w) \pm Error\ \%} \qquad \textbf{...Eq 3.2}$$

From equation 3.2, the total parent compound mass was calculated for each of the respective days. The total amount of doses was calculated by dividing the total mass by the 32 mg dosage mass. The calculated data are presented in Table 3.5.

TABLE 3.5 CALCULATED MINIMAL AMOUNT OF DOSES CONSUMED PER DAY OF COCAINE

	A_w (g)	P_{tot} (g)	Doses	%Error
Sunday	17.36	31.56	986.29	7.46
Monday	13.84	25.17	786.65	7.92
Tuesday	11.05	20.09	627.90	13.29
Wednesday	13.84	25.17	786.65	9.50
Thursday	13.43	24.42	763.24	10.42
Friday	12.73	23.14	723.28	8.85
Saturday	23.85	43.36	1354.98	4.37

The calculated least significant difference (LSD) for Cocaine amounted to 38.51. If the amount of doses between comparable days differ more than the calculated LSD, the results are classified as being significantly different from one another with a confidence of 95 %. A spike of total cocaine consumption is observed for Saturday as well as a slight increase for Sunday, with the total doses amounting to 986.29±59.24 for Sunday and 1354.98±73.56 for Saturday. The amount of doses arranged from lowest to highest. If the doses do not significantly differ from one another they will be combined in brackets.

Tuesday < Friday < (Thursday, Monday, Wednesday) < Sunday < Saturday

If one dose is allowed per person, the percentage of the population consuming cocaine is estimated at 0.344±0.0256 for Sunday and 0.496±0.0217 for Saturday. It should be noted, as explained earlier, these figures may reflect that cocaine was consumed during Friday and Saturday. In the study by Zucatto *et al.* [13], the metabolite mass present in Milan's wastewater reached 522±111 g and 394±60 g for Saturday and Sunday, respectively. Again these figures are reported with only one standard deviation. Using a 95 % confidence interval will yield the following for both quantities. For Saturday the A_w equalled 522±222 g corresponding to a 42.529 % error whilst the Sunday A_w equalled 394±120 g analogous to a 30.457% error. The total amount of doses calculated for both Saturday and Sunday are 29659.09±12613.64 for Saturday and 22386.36±6818.18 for Sunday. If as before only one dose is allowed per person the population

percentage responsible for the metabolite in question amounts to 2.373±1.009 for Saturday and correspondingly 1.791±0.545 for Sunday. From these calculated result of Milan, even at the lowest possible percentile for Saturday (1.364) and Sunday (1.246) these figures far exceeds the South African estimated cocaine use for both days. This informative comparison helps gauge the extent of drug use in South Africa (Pretoria west) compared to Milan.

3.3.7.1.3 CODEINE

The third analyte that will be discussed is codeine. As mentioned previously, codeine is not classified as illegal and is readily available over the counter in South Africa. It also adds to the aggregate morphine concentration present in the wastewater because about 2-3 % of codeine is metabolized into morphine. [15] Since codeine is predominantly excreted in its original form, the target analyte was codeine itself. An average codeine dosage contains approximately 60 mg. [14,15] Given the small amount of metabolism that occurs, the total analyte mass measured in the wastewater accounts for 97 % of the codeine consumed. Again, the conservative approach was followed in choosing the higher metabolism figure of 3 %, as to calculate the minimum number of doses present in the wastewater. Table 3.6 presents the calculated data. Equation 3.3 was used to calculate the total parent compound mass.

$$P_{tot} = 1.03 \times (A_w) \pm Error\,\% \qquad \textbf{...Eq 3.3}$$

TABLE 3.6 CALCULATED MINIMAL AMOUNT OF DOSES CONSUMED PER DAY FOR CODEINE

	A_w (g)	P_{tot} (g)	Doses	%Error
Sunday	64.35	66.29	1104.76	1.42
Monday	73.07	75.27	1254.43	2.20
Tuesday	43.21	44.52	741.92	3.51
Wednesday	76.38	78.67	1311.18	1.11
Thursday	73.81	76.02	1266.99	0.90
Friday	45.55	46.91	781.89	2.95
Saturday	104.13	107.25	1787.48	0.97

The calculated least significant difference (LSD) for codeine amounted to 10.46. If the amount of doses between comparable days differ more than the calculated LSD, the result is classified as being significantly different from one another with a confidence of 95 %. Although the absolute codeine concentrations measured were the highest for all analytes, a far higher total dose quantity per day was expected, due to the low metabolism and high therapeutic dose required,

The highest quantity of doses was measured on Saturday (1787 doses). The amount of doses arranged from lowest to highest are shown below. If the doses do not significantly differ from one another they will be combined in brackets. Because of the small error associated with codeine's analytical measurement all calculated doses are significantly different from one another.

Tuesday < Friday < Sunday < Monday < Thursday < Wednesday < Saturday

Comparing codeine concentration to the initially low concentration range of 11-nor-9-carboxy-Δ9-THC, it is interesting to note that the quantity of Δ9-THC (15,839) doses eclipsed that of codeine. It is thus very important to take note of the metabolic ratios of analytes before any assumptions can be made. When one dose per person was assumed, the percentage of the population that consumed codeine on Friday (leading to metabolite measurement on Saturday) was 0.6540±0.00633. Since no other studies were found that measured codeine in wastewater, no comparisons to other countries could be made

3.3.7.1.4 MORPHINE

Morphine is the product of both codeine and heroin metabolism, as well as being present in the wastewater as a result of morphine administration in hospitals and through prescription use. The morphine concentration in the wastewater is thus representative of three different sources. Since morphine is the final product of metabolism for both heroin and codeine, no product percentage needs to be applied. The entirety of morphine entering the metabolic pathway will be excreted as a glucuronide and further deconjugated bacteria. Thus, the mass determined is the original parent compound mass. It is impossible, however, to determine from which source the measured morphine originated. It would also be impossible to tell how far along the metabolism of both codeine and heroin has progressed in a subject. From the foregone statements it would then be impossible to determine the contribution of each of the sources to the total morphine mass. Since these contributions are not known the total amount of doses will be estimated without subtracting the unknown contribution from codeine and heroin. An error of unknown quantity will be expected, but no apparent way of estimating the number of doses accurately exists. The total number of doses for each day of the week, as well as the total analyte mass, are given in Table 3.7. The equation for calculating the total parent compound mass is simplified to the following:

$$\boldsymbol{P_{tot} = (A_w) \pm Error\ \%} \qquad \textbf{...Eq 3.4}$$

A typical adult dose of morphine consists of 5-20mg of either morphine hydrochloride, sulfate or nitrate. From this dosage range, the minimum amount of doses will be calculated using a 20 mg dose.

TABLE 3.7 CALCULATED MINIMAL AMOUNT OF DOSES CONSUMED PER DAY FOR MORPHINE

	A_w (g)	P_{tot} (g)	Doses	%Error
Sunday	2.17	2.17	108.46	14.86
Monday	1.20	1.20	60.11	22.13
Tuesday	1.65	1.65	82.43	20.03
Wednesday	1.43	1.43	71.58	23.63
Thursday	1.91	1.91	95.56	14.40
Friday	1.96	1.96	98.06	4.79
Saturday	1.90	1.90	94.92	15.57

The calculated least significant difference (LSD) for morphine amounted to 7.45. If the amount of doses between comparable days differ more than the calculated LSD, the results are classified as being significantly different from one another with a confidence of 95 %. The analyte morphine has the lowest average number of doses out of all targeted analytes. The most uncertainty is also associated with morphine as a result of the extremely low concentrations measured. The highest number of doses was seen on Sunday, with 108.46±16.12. Arranging the doses from lowest to highest as before:

Monday < Wednesday < Tuesday < (Saturday, Thursday, Friday) < Sunday

Again, one dose per person was assumed, giving a population percentage use of 0.0378±0.00562. When a comparison is drawn between Milan and Daspoort, a meaningful evaluation of the population percentage can be given. In the study by Zucatto *et al.* [13], the highest amount of total morphine mass was found to be 34±5 g/day on a Thursday. Again, the variance is only displayed as one standard deviation and the confidence interval needs to be increased in order to obtain 95% accuracy. After two standard deviations were applied, the mass was 34±10 g/day, with a 29.41 % error. The total amount of doses calculated for the Milan study was 1700±499.97 doses. It should be noted again that this value is for a plant serving 1.25 million citizens. If a population percentage is calculated as before, assuming one dose per person, the total percentage consuming morphine was: 0.136±0.03999. Comparing Milan and Daspoort, the morphine use in Milan exceeds that of Daspoort by ten times.

3.3.7.1.5 6 MONOACETYL MORPHINE

The final analyte targeted in this study was 6 monoacetyl morphine (6MAM) as a marker for heroin use. This was the most difficult analyte to draw conclusions about since its metabolic pathway is so different from the other analytes. As explained earlier, the molecule is a precursor to the end product of metabolism, morphine. Since 3 different sources contribute to total morphine mass, it would be unwise to use morphine as a marker for heroin use. The precursor 6MAM, however, is exclusively produced as a result of heroin metabolism. This then is the only marker available to verify heroin use. For all other drugs studied, the end product of the metabolic pathway was measured, compared to heroin, where the intermediary metabolite 6MAM was measured. In no way can a back-calculation using only 6MAM perfectly estimate the amount of heroin administered, since the time between administration and sampling is not known, nor is the amount of 6MAM that has been converted to morphine known. In addition, the fractions of morphine originating from each possible source are not known. Thus, there was no way to get an accurate estimate of doses. The only way to obtain a dose estimation was to take the 6MAM mass and make a direct estimation from this point, although a gross underestimation of the amount of doses was expected. The underestimation may be a consequence of an inaccurate estimated heroin mass, since 6MAM is further metabolized to morphine. Although the presence of 6MAM is indicative of heroin use, the hypothesis that acylation of codeine to 6MAM is a confounding factor. Due to these uncertainties, any estimation of doses would likely be more fiction than fact. While a total dose estimation was still attempted for interest's sake, all of the aforementioned factors should be kept firmly in mind. If the 6MAM mass is used for the calculation, a significant underestimation would occur, although this information might be useful in providing something by which to gauge the heroin use, compared to no estimation at all.

A therapeutic dose of heroin usually ranges from 5-10 mg. [14] It should be noted that even though the hypothesis states that 6MAM could be formed from codeine, this hypothesis will be regarded void for the following calculations, since nothing has been proven up to this point. It can be said with the utmost certainty that the analyte measured was 6MAM and this analyte corresponds to mass heroin use. Thus, for the purposes of calculations, the analyte mass of 6MAM was regarded as completely originating from heroin with no codeine contribution. As with the other drugs studied, the highest dosage was employed. The total parent compound mass was calculated by the following formula, assuming the 100% contribution of heroin to 6MAM.

$$\boldsymbol{P_{tot} = (A_w) \pm Error\ \%} \qquad \textbf{...Eq 3.5}$$

The calculated data are presented in Table 3.8.

TABLE 3.8 CALCULATED MINIMAL AMOUNT OF DOSES CONSUMED PER DAY FOR HEROIN

	A_w (g)	P_{tot} (g)	Doses	%Error
Sunday	18.58	18.58	1858.18	1.62
Monday	16.02	16.02	1601.70	2.63
Tuesday	9.24	9.24	923.50	1.39
Wednesday	16.34	16.34	1633.63	2.09
Thursday	15.90	15.90	1590.44	2.87
Friday	10.23	10.23	1022.76	2.48
Saturday	22.07	22.07	2207.28	1.01

The calculated least significant difference (LSD) for morphine amounted to 16.47, with a confidence of 95 %. As seen from the table above, the total 6MAM mass calculations in the wastewater sample for both Saturday and Sunday, yielded the highest estimated quantity of doses of heroin. As result of the small analytical error in measurement all of the doses between each day differ significantly from one another. Arranged from lowest to highest:

Tuesday < Friday < Thursday < Monday < Wednesday < Sunday < Saturday

When comparing total heroin doses with the other drugs of abuse, 6MAM produced the second highest dosage quantity of all the measured analytes, even though a perceptible underestimation had been made. No comparison could be drawn between Milan and Daspoort as a result of the analyte in question not being targeted by the Zucatto *et al.* [13] study. The total amount of doses for Saturday was 2,207.279± 22.227. It is curious to think that the amount of heroin doses would outweigh even prescription medicine like codeine. The high amount of heroin doses may be a result of a new local drug formulation popular among the low-income population, Nyaope. This drug formulation consists of low-grade heroin combined with cannabis and sometimes Antiretroviral (ARV) medication. This cheap, readily available, and widely used formulation may possibly be the rationale behind the increased heroin metabolite mass and consequently enlarged heroin dose numbers. When one dose per person was assumed, the percentage of the population using heroin on Saturday amounted to 0.8076± 0.00813.

As with all the discussed analytes, only the day with the highest amount of doses calculated was used to draw comparisons and calculate population statistics. The total amount of doses plus associated error is tabulated for each analyte and for each day. As mentioned, the population

statistic for each day was not given since this was done only for reference and one individual may consume more than one dose per day.

3.4 Conclusion

From the amounts of drugs of abuse metabolites present in the wastewater, it is evident that drug use in Pretoria-West (Daspoort) is a problem that should not be looked upon lightly. Moreover, the week profiles of the measured metabolites indicated certain time frames during which increases in drug use were likely. Armed with the statistical, instrumental, and sample work-up procedures, an apt method has been developed in this study for obtaining results which have not been produced in South Africa before. These results are not free from error, however, and further underestimation of drug levels occurs as result of the influent flow not originating from individuals alone, but rather from a combination of commercial, industrial and residential areas. Despite this, valuable information has been gained and important conclusions can be drawn from the data set. Comparing the GCxGC/TOF-MS method with other LC-MS methods as described in Chapter 1 the lowest quantification limit achieved was 32.89 ng/L since no LOD could be obatined. It should also be mentioned that expansion of the method to analyze additional target compounds would be more complex using the GC-MS derivatization method. Analyzing additional target compounds using an LC-MS analytical method would be less complex since no derivatization is usually required.

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4. CONCLUSION AND SUPPLEMENTARY RESEARCH

4.1 INTRODUCTION

During this study, the involvement of a numerous amount of factors contributed to the final result, including sample preparation, instrumental setup and measurement, and data analysis. The combination of parameters in a very specific manner resulted in a method capable of estimating the use of drugs of abuse in the population of Pretoria-West (Daspoort). Consequently, the concerns regarding pharmaceuticals and drugs of abuse residues present in wastewater can be addressed. The abuse of illicit compounds in South Africa has increased tremendously over the past years but data on drug use and the amounts consumed has been limited to data coming from law enforcement agencies, information from rehabilitation centres, and self-reported cases. The method developed and validated in this study provides a new avenue for investigating the drug abuse problem in South Africa.

4.2 METHOD APPLICATION

The method is particularly useful for investigating the drug use in an entire population without the need for personal interaction and contact with individual drug users. Many of the statistics available on drug use originate from questionnaires that may have large uncertainties associated with them, due to the possibility that drug users may not feel comfortable revealing their drug use habits, even on paper. The analysis of wastewater completely removes this element from the equation. A population produces wastewater sewage, which in turn needs to be treated by a treatment plant. This wastewater contains the metabolic products of the individuals served by the plant. Hence, the wastewater will also contain the metabolic products of the drug users residing in that population. Thus, through the analysis of wastewater, the drug use of the specific population can indirectly be determined without any invasive procedures or invasion of privacy. According to SACENDU, in 2009 a total of 9,751 individuals were admitted to rehabilitation centres across South Africa. [1] The total number calculated doses in Pretoria-West alone surpassed the total

number of patients admitted to rehabilitation centres. The SACENDU figures are the only South African data available by which to compare the results obtained in the current study. Comparisons between the Daspoort and Milan studies were presented in Chapter 3. The results obtained in this study support the notion that the abuse of illegal substances is an area of serious concern. The total parent compound mass measured in this study confirms the truth that the abuse of illicit compounds in Pretoria-West needs to seriously be addressed. This method provides a means of monitoring the drug use patterns of specific areas, as well as identifying localities with higher levels of drug use.

The developed method poses the possibility of acquiring vast amounts of epidemiological data never before available in South Africa. The patterns of drug use and its spread can be obtained, plotted and monitored, in order to observe trends and changes in the population. These trends can also be used to pinpoint localities with increased substance abuse profiles.

The problem of drug abuse can be addressed more effectively by law enforcement agencies when the appropriate and accurate data are available. Armed with the epidemiological data obtained using the method, a more focused approach can be employed by government agencies, since the types of substances being abused will be known, as well as the most relevant geographical areas. If, for example, a metropolitan area displays the increased abuse of one type of drug, law enforcement agencies can centralize their resources and energy on that specific area and drug class.

When epidemiological data is acquired on the illicit substance abuse patterns of metropolitan areas, this data can be publicized in order to inform the public. If the public is informed, actions can be taken by parents at home or by teachers at schools. A higher awareness of an illicit substance abuse problem translates to a more involved public, since the situation impacts all individuals in the specific area.

As the heading suggests, it is also important to employ the epidemiological data so that comparisons can be drawn between South Africa and other countries. It is important to be able to gauge the extent of a certain area's or country's substance abuse problem. Without an appropriate method for measurement, the degree of substance abuse cannot be gauged since there is no way to tell whether the measured substances are at levels warranting concern or at the lower end of the scale.

This study only focused on a small piece of a much larger puzzle. The method was used to measure a specific set of drug metabolites (11-nor-9-carboxy-Δ^9-THC, benzoylecgonine, codeine, morphine, and 6 monoacetyl morphine), but an abundance of additional research can be endeavoured utilizing the developed method. If true epidemiological data is the focus, a variety of individual treatment plants will need to be sampled from in order to determine a range of drug use patterns over geographical areas. Treatment plants from multiple cities can also be sampled to obtain substance abuse data for whole provinces and even further, whole countries. A larger array of metabolic analytes can be targeted in order to include other illicit substances. One example, especially relevant for South Africa, is the amphetamine class of drugs.

4.2.1. 6 Monoacetyl morphine anomalies

The anomalies seen in the 6MAM measurements should be studied further. The hypothesis that codeine may be transformed into 6MAM by bacterial acylation enzymes needs to be investigated. An amount of deuterated internal standard of codeine can be spiked into a wastewater pool and the bacterial life forms will then be allowed to interact with the spiked internal standard for a set period of time. A quantitative analysis can be conducted targeting labelled codeine as well as its metabolite, deuterated 6MAM. If deuterated 6MAM is present in the wastewater, it will prove the hypothesis that acylation of codeine contributes to the overall 6MAM concentration in wastewater.

4.3 Conclusion

Winston Churchill said, “Difficulties won are opportunities mastered.” All of the individual difficulties encountered in the development of a suitable method for analysing 11-nor-9-carboxy-Δ9-THC, benzoylecgonine, codeine, morphine, and 6MAM, led a final method that is accurate, repeatable, sensitive, and the methods results merit value. Since data of this type has not yet been published in South Africa, research using the method would contribute to the International research on drug abuse by adding a South African dataset of illicit substance abuse to compare to. The importance of such data in South Africa cannot be more highly stressed, since no figures of this kind have been available regarding the extent

of drug use in South Africa. It should be noted that the completed study is only the tip of a much larger field of research. The developed method is also apparently the first of its kind to employ gas chromatography instead of high pressure liquid chromatography. This is advantageous because not all laboratories have liquid chromatography instrumentation. Through challenges, experimentation, and perseverance, a piece of research was produced that can be used to improve the quality of life of South Africa's citizens.

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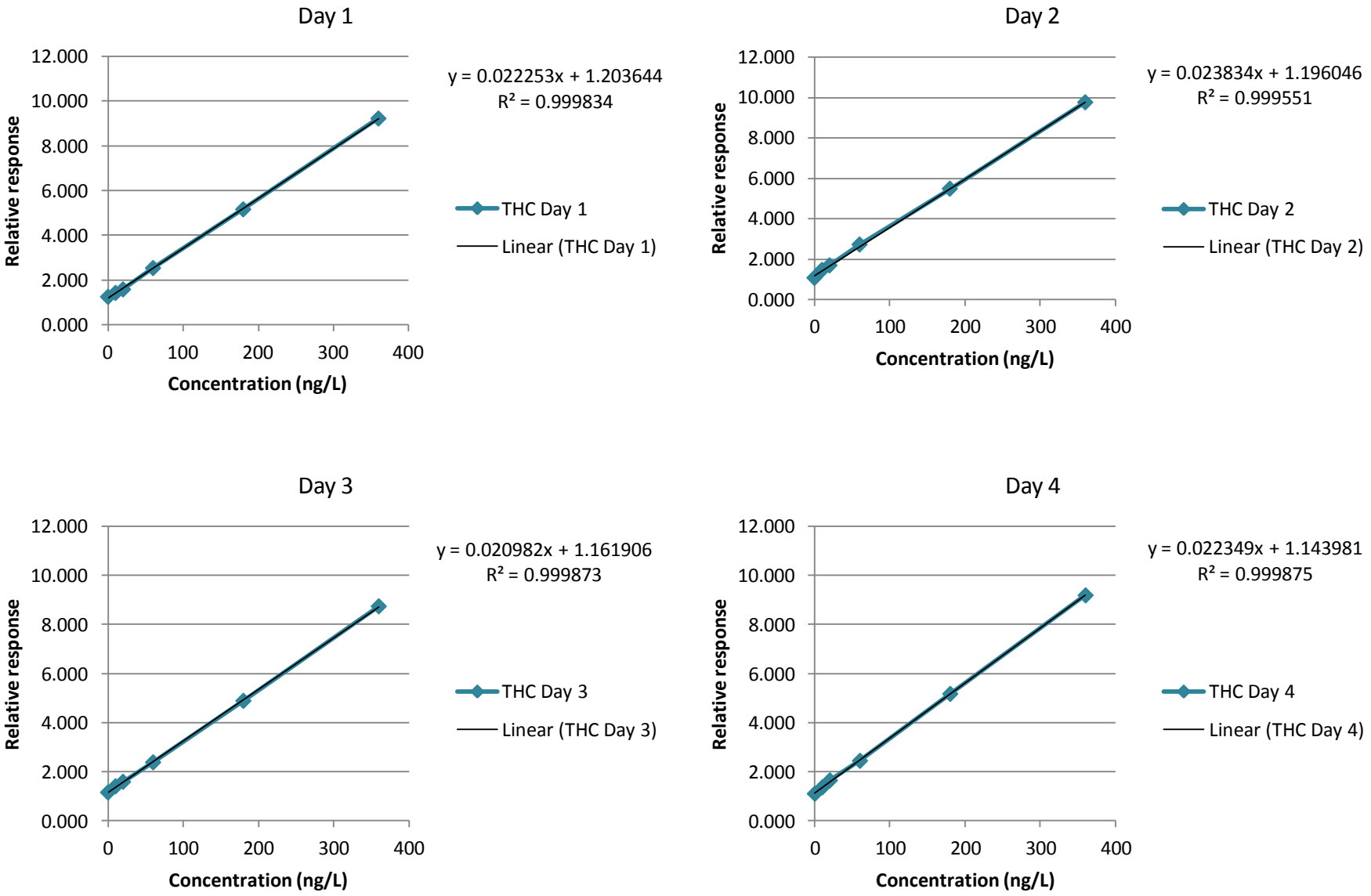
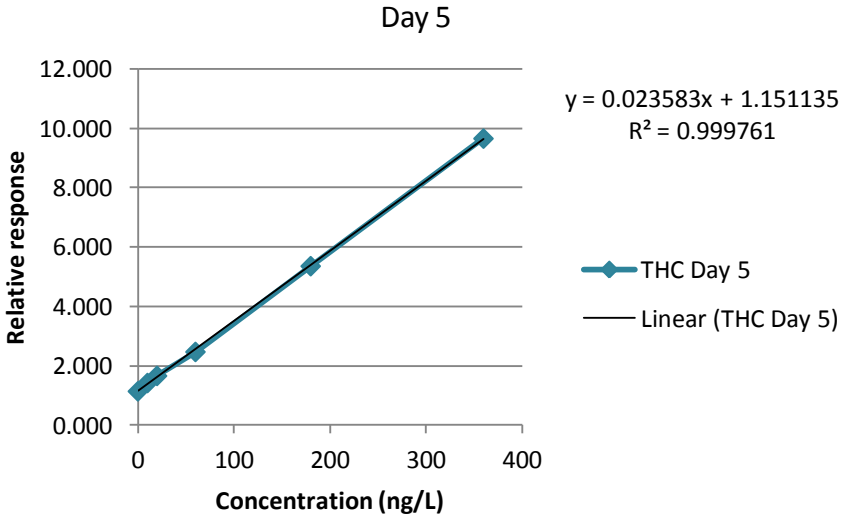
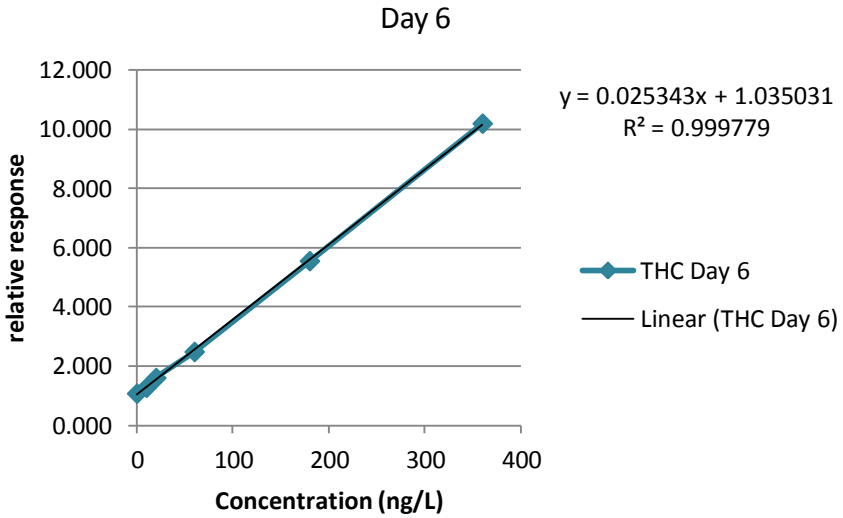
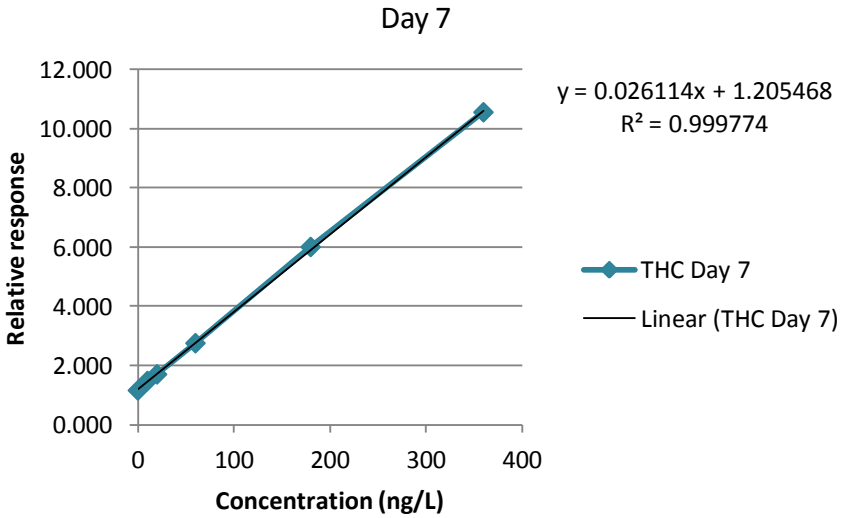
APPENDIX A

GRAPH A1 11-NOR-9-CARBOXY-Δ9-THC DAILY PROFILE PLOTS WITH LEAST SQUARES REGRESSION EQUATION AND PRODUCT MOMENT CORRELATION COEFFICIENT

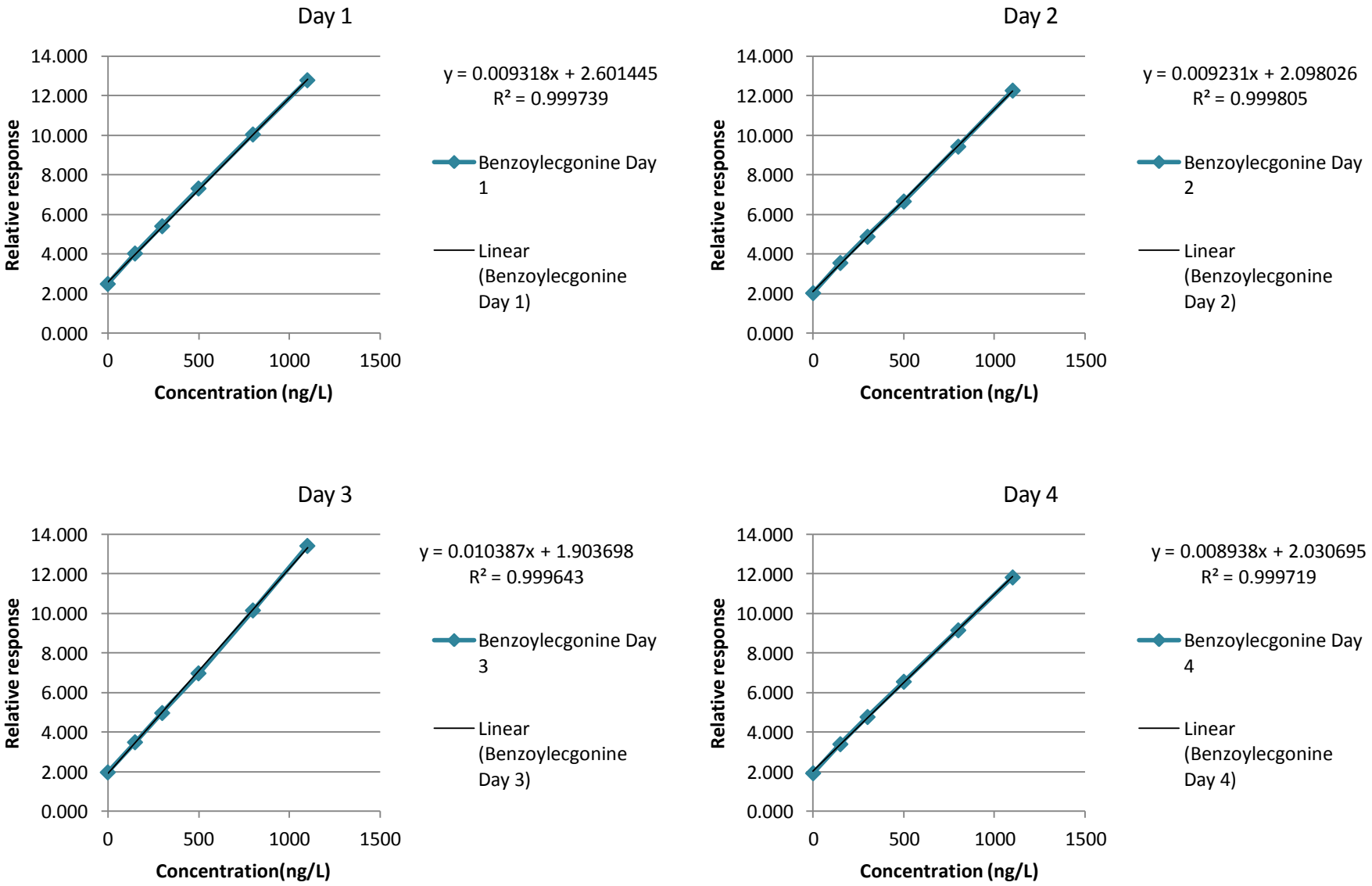


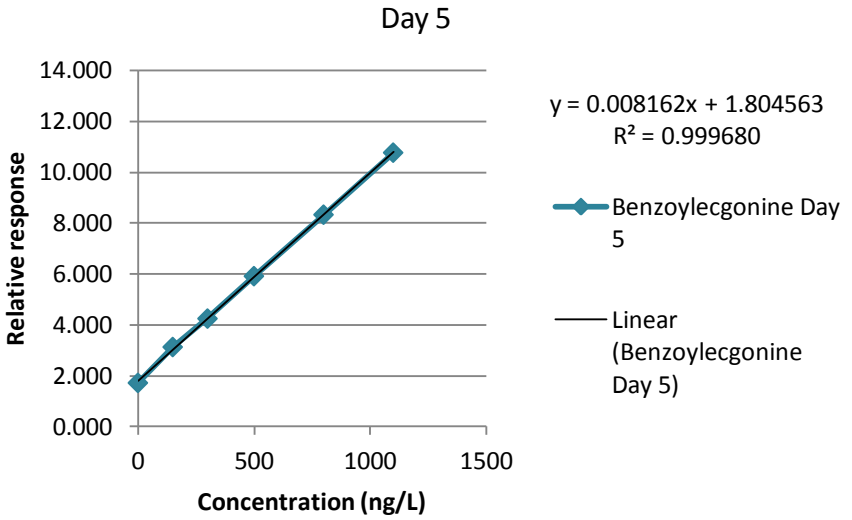


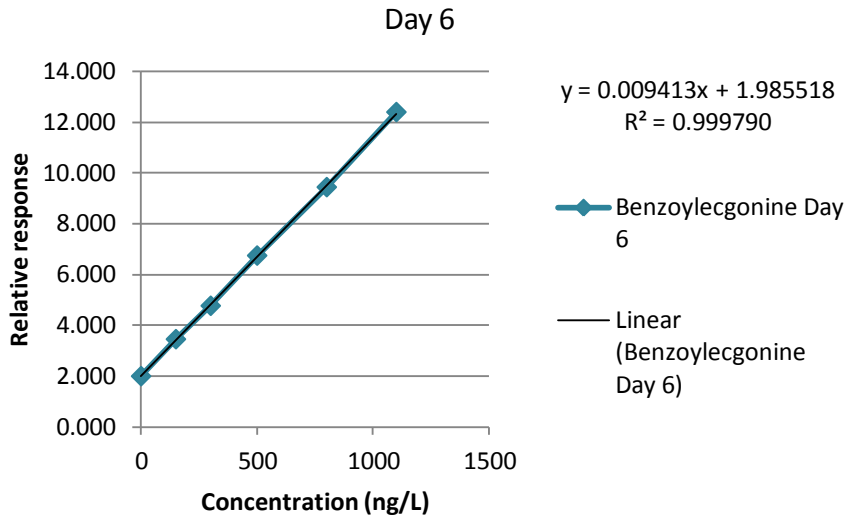


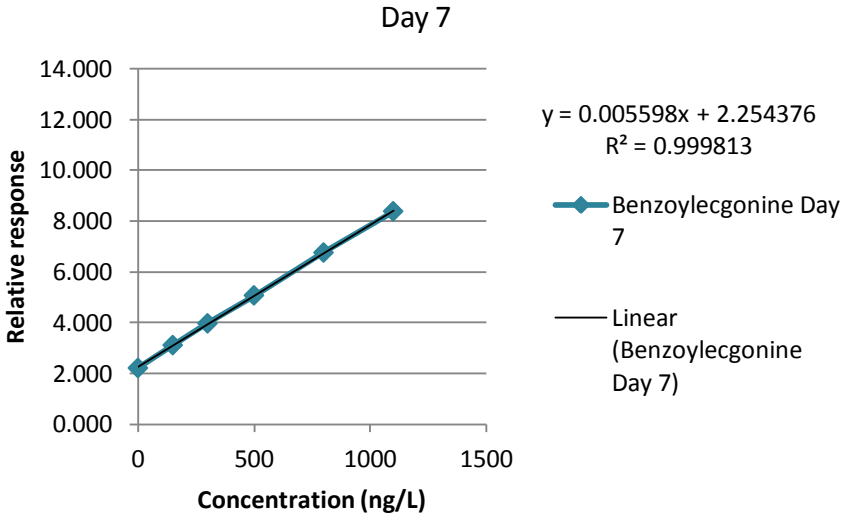


GRAPH A2 BENZOYLECGONINE DAILY PROFILE PLOTS WITH LEAST SQUARES REGRESSION EQUATION AND PRODUCT MOMENT CORRELATION COEFFICIENT

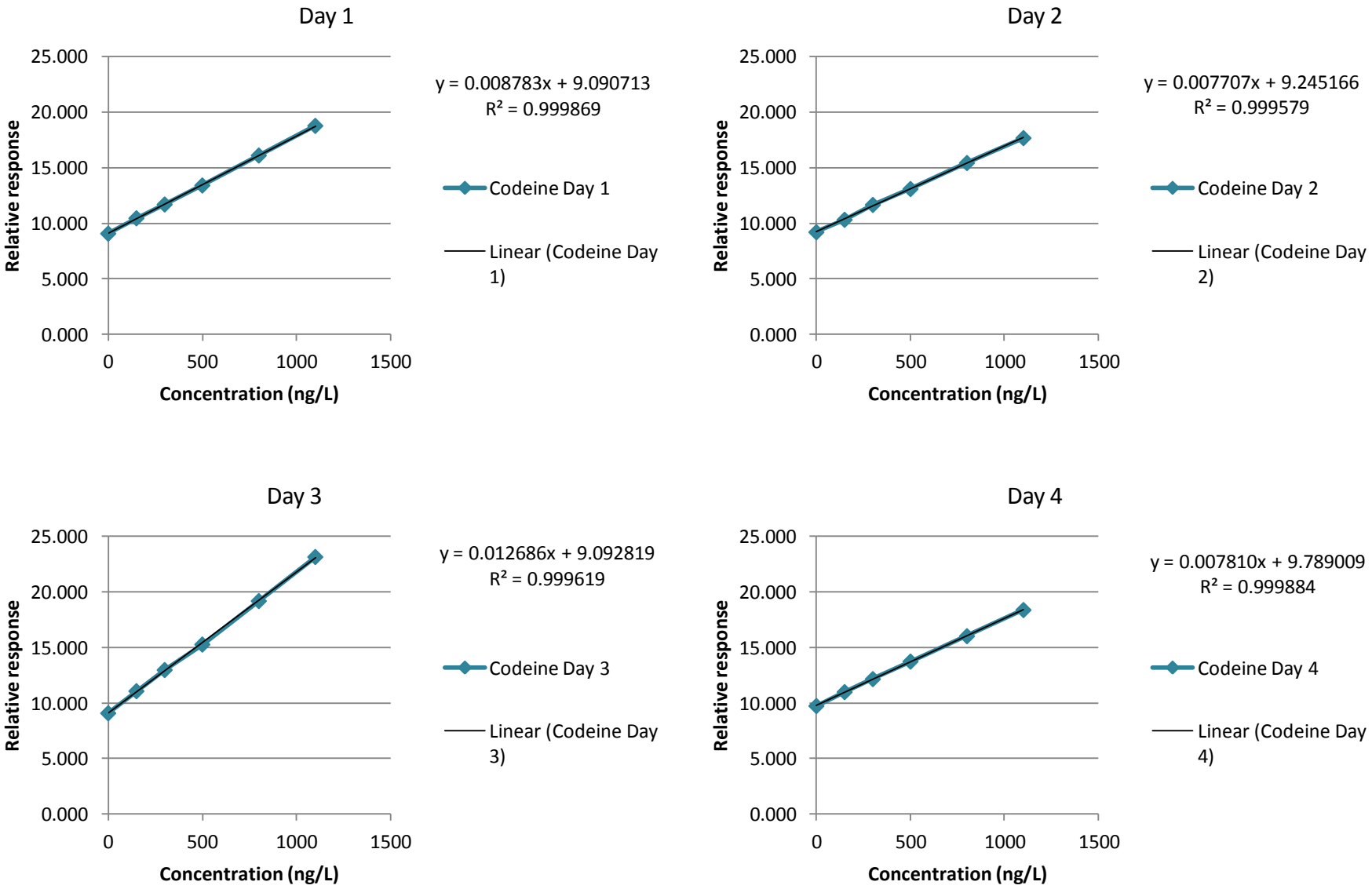


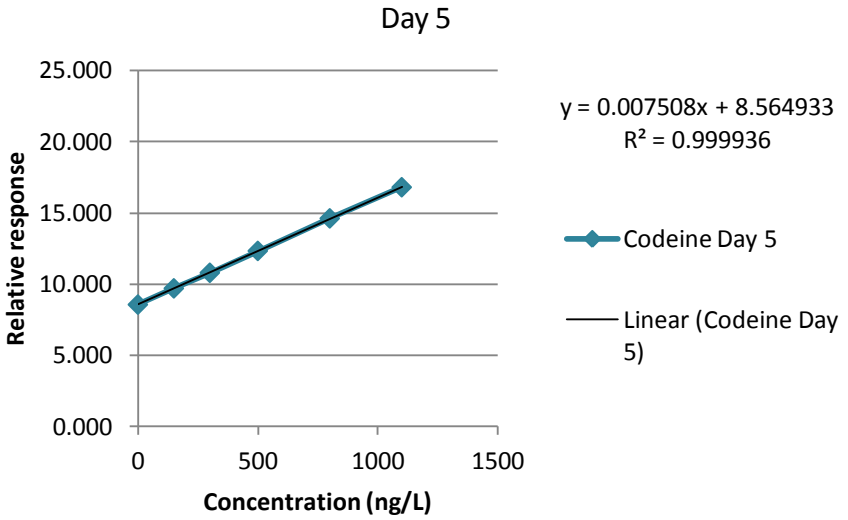


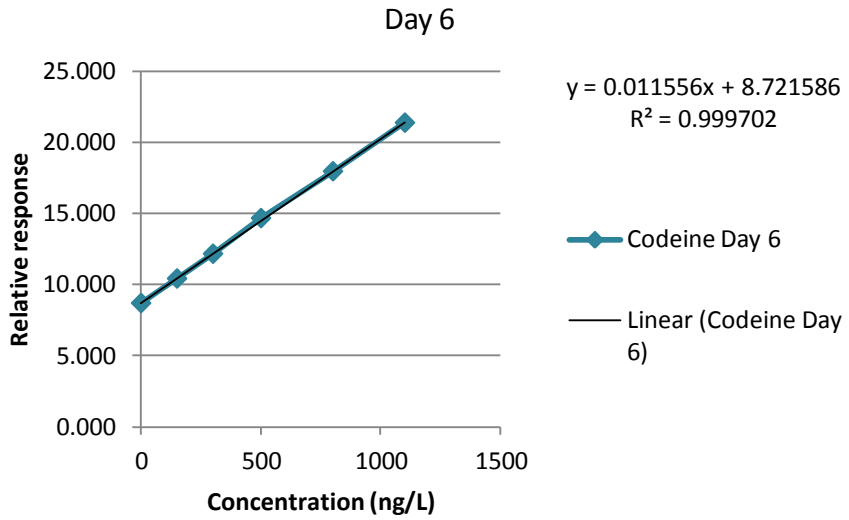


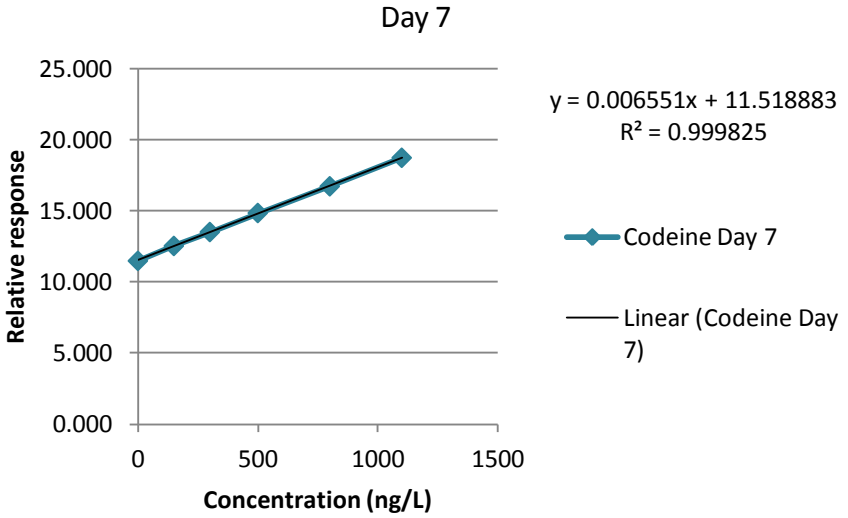


GRAPH A3 CODEINE DAILY PROFILE PLOTS WITH LEAST SQUARES REGRESSION EQUATION AND PRODUCT MOMENT CORRELATION COEFFICIENT

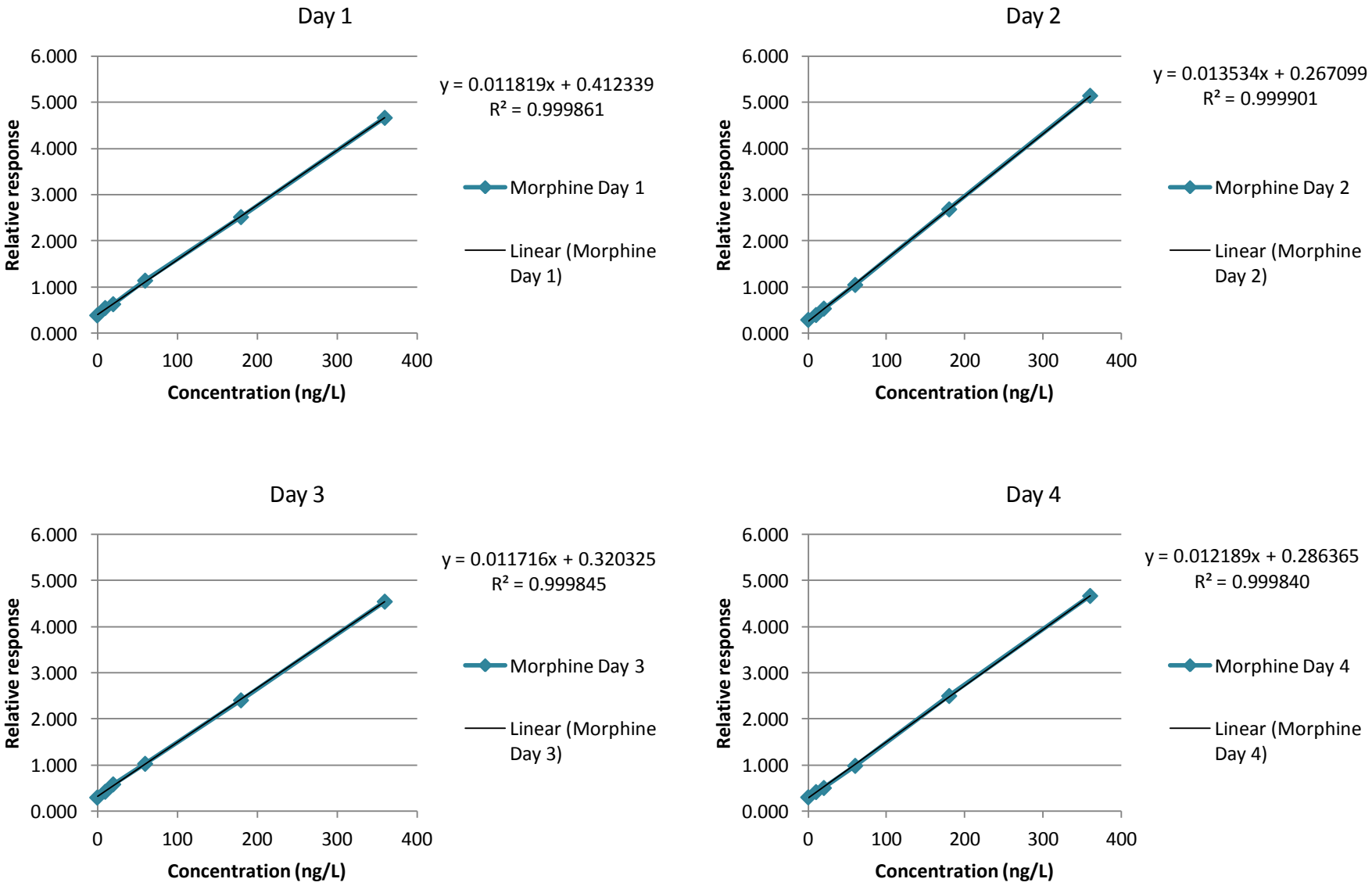


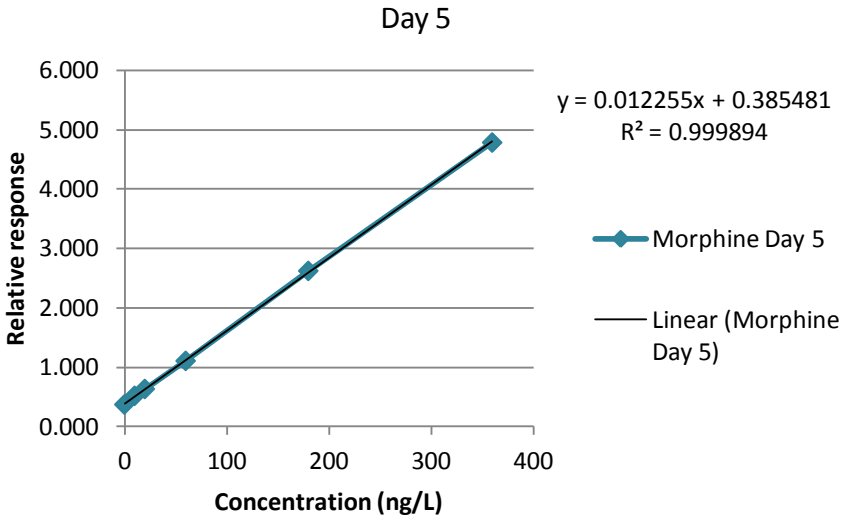


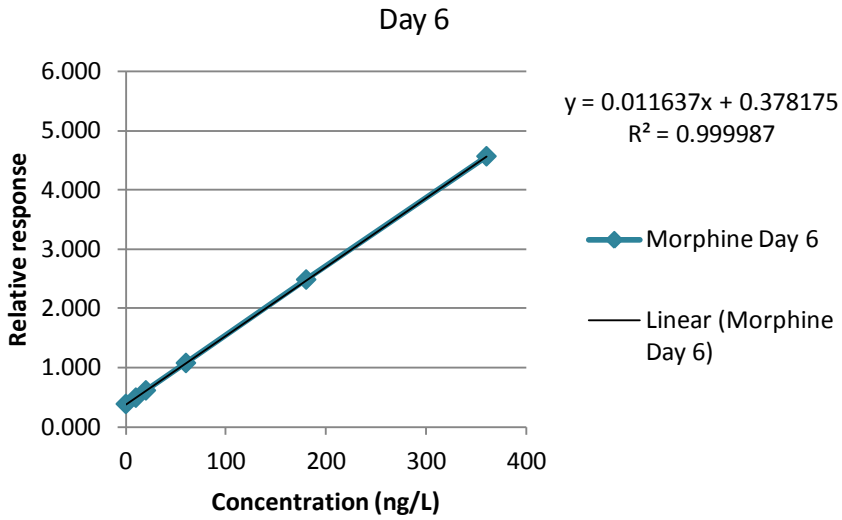


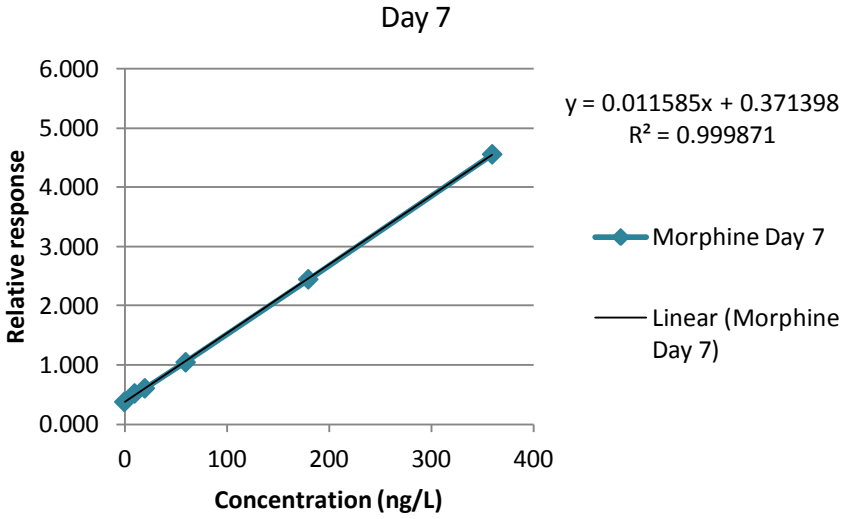


GRAPH A4 MORPHINE DAILY PROFILE PLOTS WITH LEAST SQUARES REGRESSION EQUATION AND PRODUCT MOMENT CORRELATION COEFFICIENT

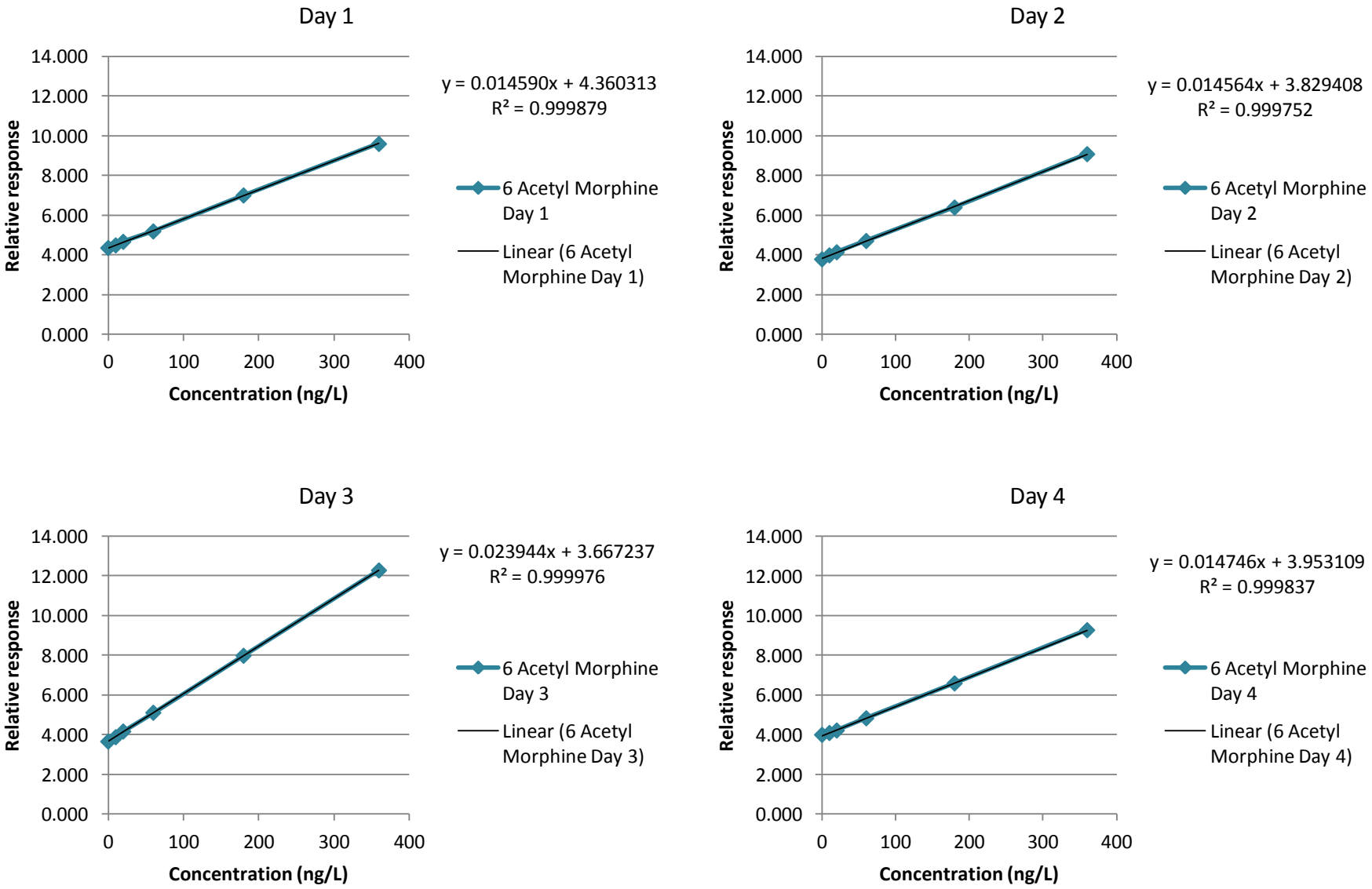


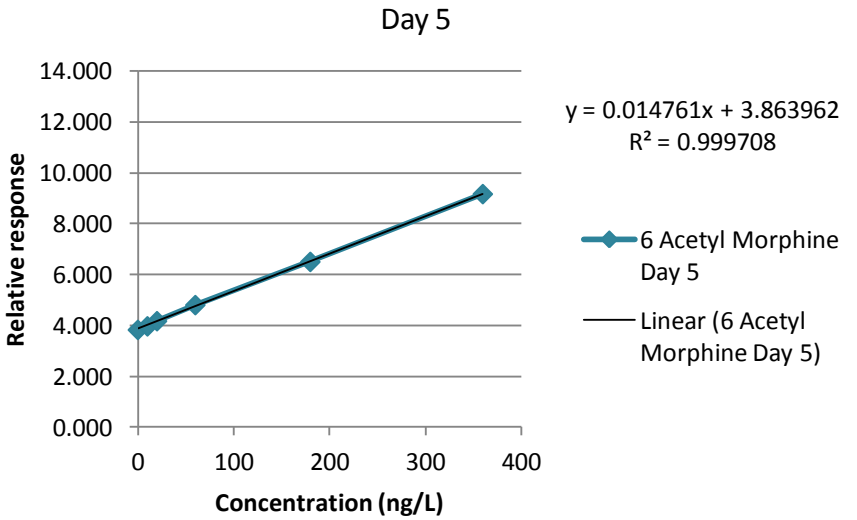


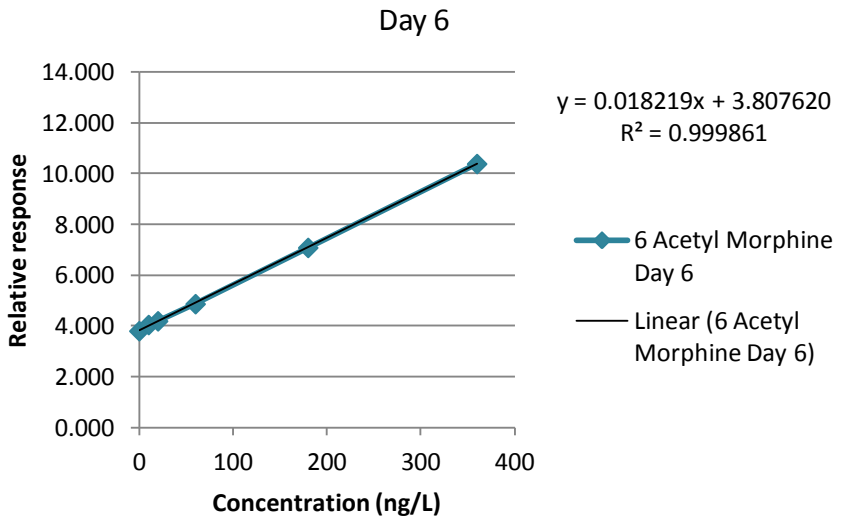


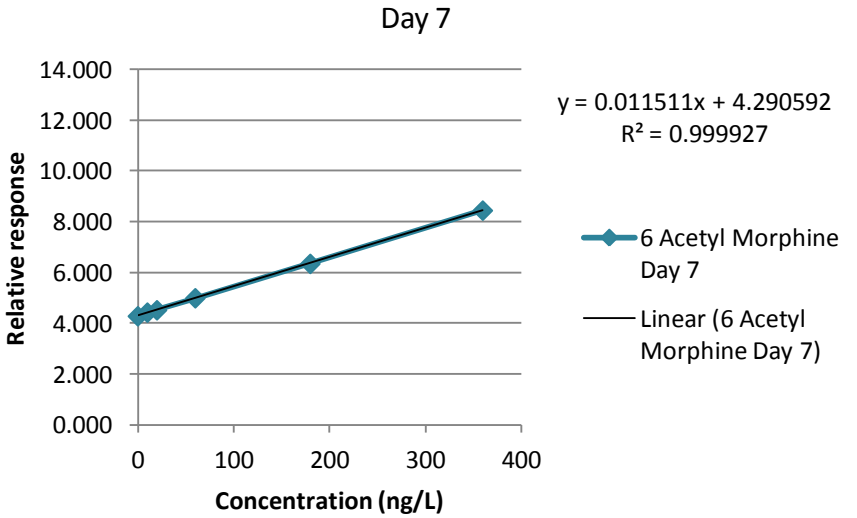


GRAPH A5 6-MONOACETYL MORPHINE DAILY PROFILE PLOTS WITH LEAST SQUARES REGRESSION EQUATION AND PRODUCT MOMENT CORRELATION COEFFICIENT

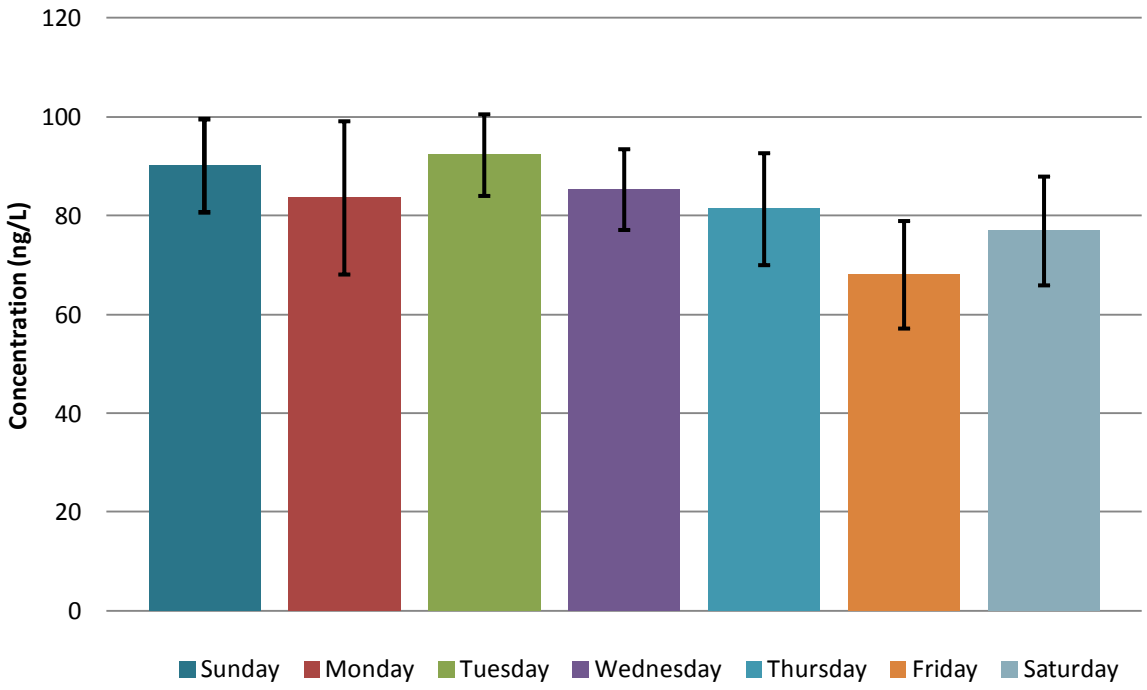




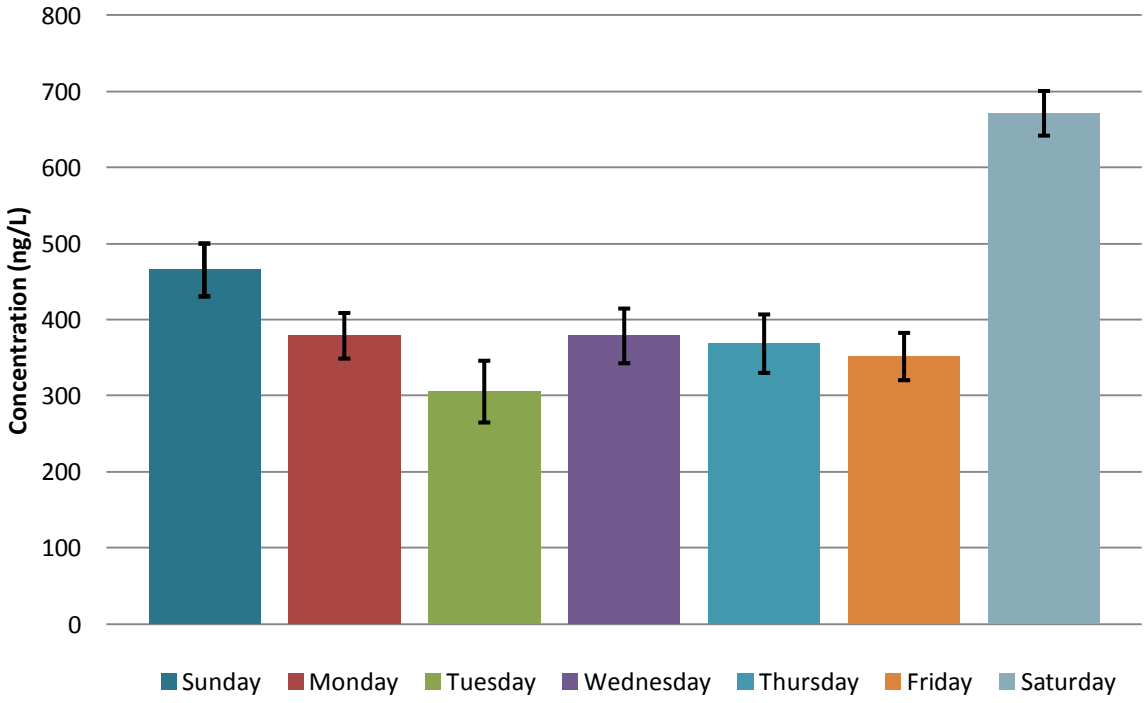




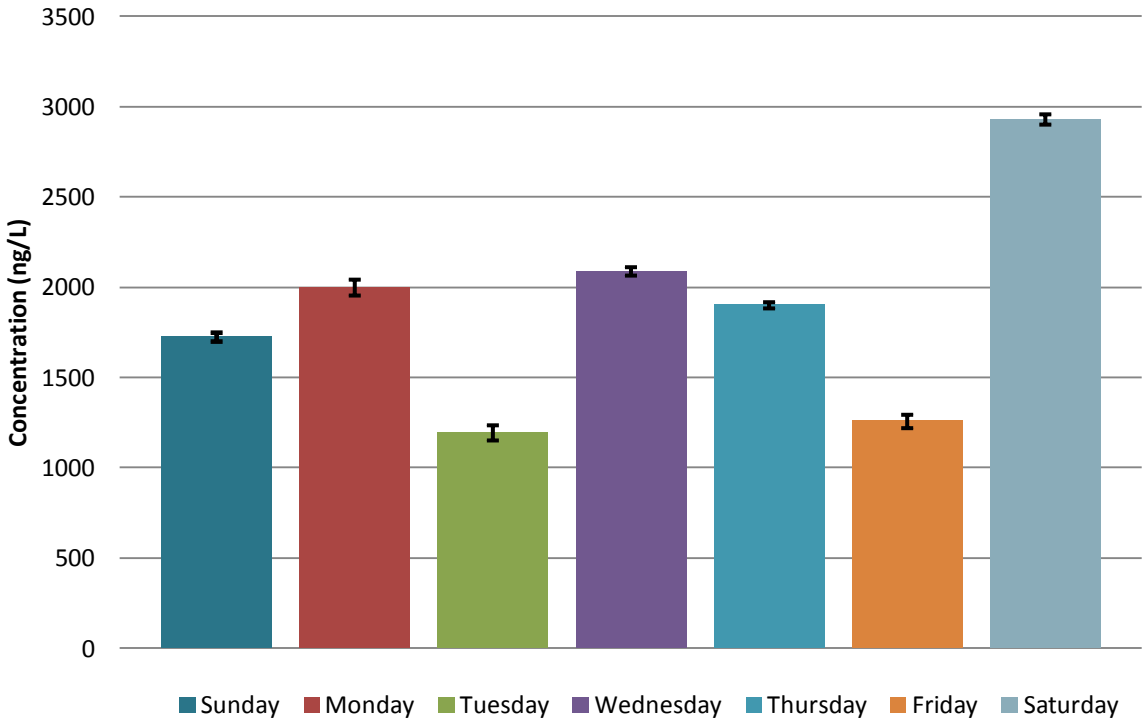
GRAPH A6.1 WEEK CONCENTRATION PROFILE AND PERCENTAGE ERROR OF 11-NOR-9-CARBOXY-Δ9-THC



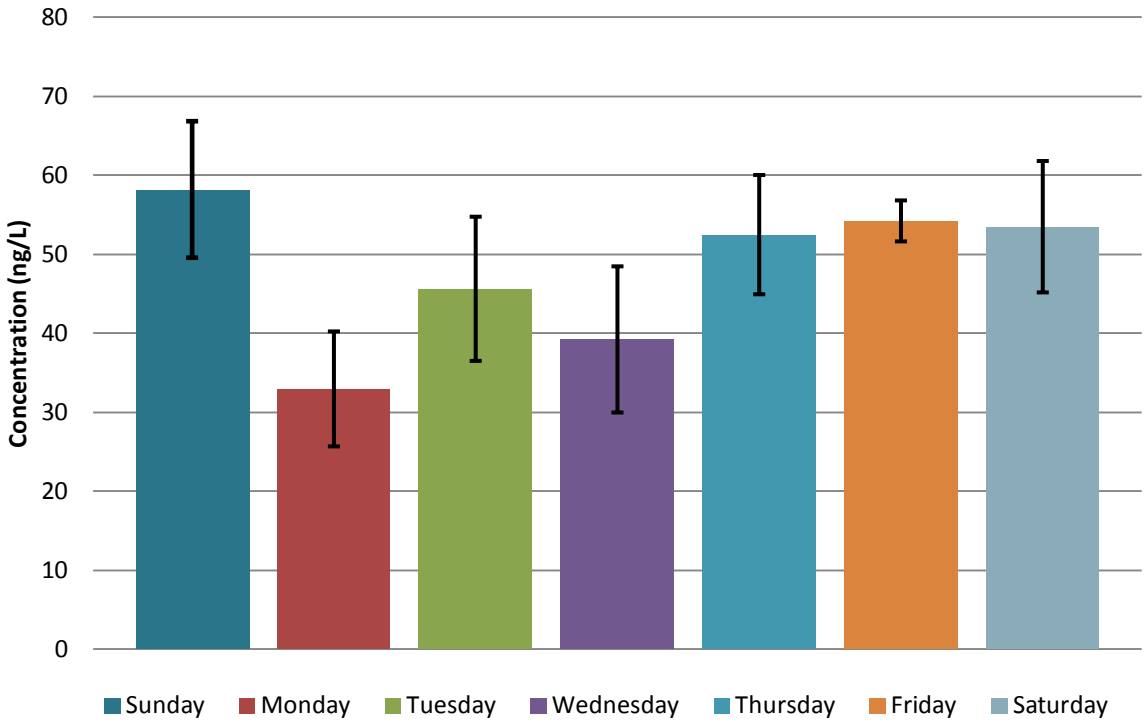
GRAPH A6.2 WEEK CONCENTRATION PROFILE AND PERCENTAGE ERROR OF BENZOYLECGONINE



GRAPH A6.3 WEEK CONCENTRATION PROFILE AND PERCENTAGE ERROR OF CODEINE



GRAPH A6.4 WEEK CONCENTRATION PROFILE AND PERCENTAGE ERROR OF MORPHINE



GRAPH A6.5 WEEK CONCENTRATION PROFILE AND PERCENTAGE ERROR OF 6 MONOACETYL MORPHINE

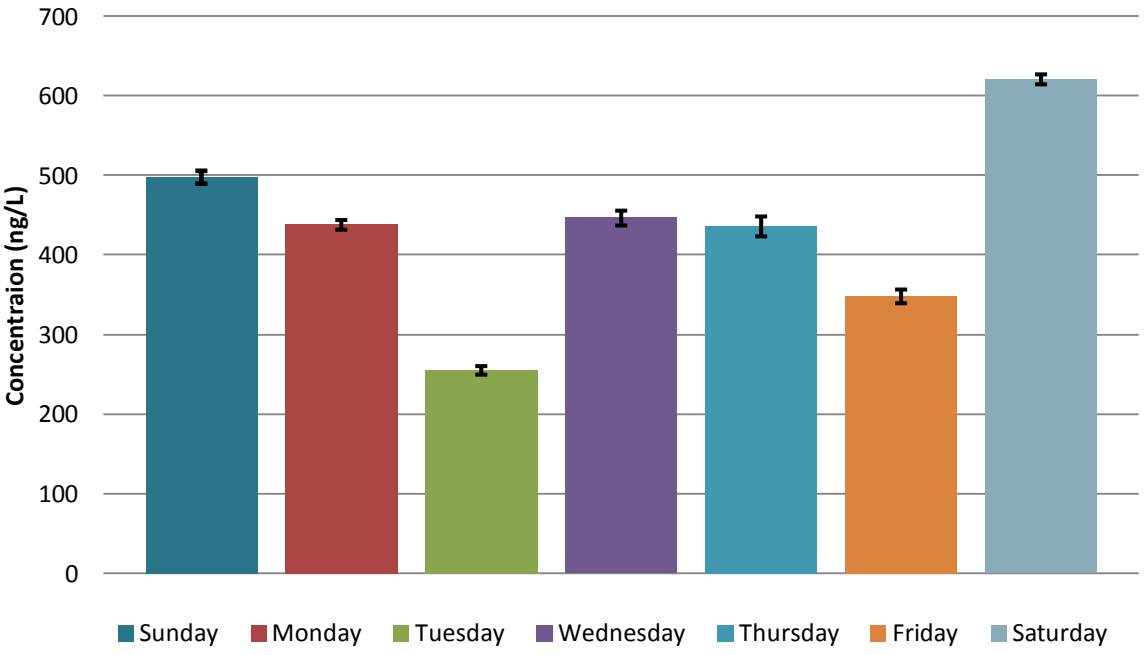


TABLE A1 CALCULATED TOTAL ANALYTE MASS WITH ASSOCIATED ERROR FOR 11-NOR-9-CARBOXY-Δ9-THC

	X Intcpt	Conc ng/L	Total Influent Litres	Total analyte mass (g)	%Error
Sunday	54.0891	90.1484	37305904.0269	3.3631	10.4623
Monday	50.1823	83.6372	36549467.5437	3.0569	18.5549
Tuesday	55.3763	92.2939	36178237.0976	3.3390	8.9496
Wednesday	51.1871	85.3119	36562797.8471	3.1192	9.5846
Thursday	48.8121	81.3534	36454603.2599	2.9657	13.9277
Friday	40.8409	68.0682	36209669.1271	2.4647	15.9878
Saturday	46.1618	76.9363	35530749.7779	2.7336	14.3124

TABLE A2 CALCULATED TOTAL ANALYTE MASS WITH ASSOCIATED ERROR FOR BENZOYLECGONINE

	X Intcpt	Conc ng/L	Total Influent Litres	Total analyte mass (g)	%Error
Sunday	279.1849	465.3082	37305904.0269	17.3587	7.4579
Monday	227.2805	378.8008	36549467.5437	13.8450	7.9220
Tuesday	183.2770	305.4616	36178237.0976	11.0511	13.2876
Wednesday	227.1979	378.6632	36562797.8471	13.8450	9.5000
Thursday	221.0932	368.4887	36454603.2599	13.4331	10.4212
Friday	210.9336	351.5560	36209669.1271	12.7297	8.8497
Saturday	402.7110	671.1849	35530749.7779	23.8477	4.3723

TABLE A3 CALCULATED TOTAL ANALYTE MASS WITH ASSOCIATED ERROR FOR CODEINE

	X Intcpt	Conc ng/L	Total Influent Litres	Total analyte mass (g)	%Error
Sunday	1035.0351	1725.0584	37305904.0269	64.3549	1.4237
Monday	1199.5804	1999.3006	36549467.5437	73.0734	2.2024
Tuesday	716.7601	1194.6002	36178237.0976	43.2185	3.5077
Wednesday	1253.3942	2088.9904	36562797.8471	76.3793	1.1063
Thursday	1214.7463	2024.5771	36454603.2599	73.8052	0.9003
Friday	754.7236	1257.8727	36209669.1271	45.5472	2.9466
Saturday	1758.3396	2930.5661	35530749.7779	104.1252	0.9690

TABLE A4 CALCULATED TOTAL ANALYTE MASS WITH ASSOCIATED ERROR FOR MORPHINE

	X Intcpt	Conc ng/L	Total Influent Litres	Total analyte mass (g)	%Error
Sunday	34.8878	58.1463	37305904.0269	2.1692	14.8599
Monday	19.7354	32.8923	36549467.5437	1.2022	22.1330
Tuesday	27.3408	45.5680	36178237.0976	1.6486	20.0330
Wednesday	23.4937	39.1562	36562797.8471	1.4317	23.6255
Thursday	31.4550	52.4250	36454603.2599	1.9111	14.3955
Friday	32.4976	54.1627	36209669.1271	1.9612	4.7915
Saturday	32.0585	53.4309	35530749.7779	1.8984	15.5718

TABLE A4 CALCULATED TOTAL ANALYTE MASS WITH ASSOCIATED ERROR FOR 6 MONOACETYL MORPHINE

	X Intcpt	Conc ng/L	Total Influent Litres	Total analyte mass (g)	%Error
Sunday	298.8561	498.0934	37305904.0269	18.5818	1.6167
Monday	262.9366	438.2276	36549467.5437	16.0170	2.6342
Tuesday	153.1589	255.2649	36178237.0976	9.2350	1.3922
Wednesday	268.0801	446.8001	36562797.8471	16.3363	2.0932
Thursday	261.7683	436.2805	36454603.2599	15.9044	2.8681
Friday	169.4725	282.4542	36209669.1271	10.2276	2.4830
Saturday	372.7384	621.2307	35530749.7779	22.0728	1.0070

TABLE A 5 METHOD OF STANDARD ADDITION STATISTIC RESULTS INCLUDING %RSD AND STANDARD DEVIATION (σ) WITHIN RUN SAMPLES.

	11-nor-9-carboxy-Δ9-THC				Benzoylecgonine				Codeine				Morphine				6MAM			
	σ	%RSD	LLOQ (ng/L)	Unknonwn conc. (ng/L)	σ	%RSD	LLOQ (ng/L)	Unknonwn conc. (ng/L)	σ	%RSD	LLOQ (ng/L)	Unknonwn conc. (ng/L)	σ	%RSD	LLOQ (ng/L)	Unknonwn conc. (ng/L)	σ	%RSD	LLOQ (ng/L)	Unknonwn conc. (ng/L)
Sunday	4.72	8.719	90.15	54.09	17.35	6.215	465.31	279.18	12.28	1.186	1725.1	1035.04	4.32	12.38	58.15	34.89	4.03	1.347	498.09	298.86
Monday	7.76	15.46	83.64	50.18	15.00	6.602	378.80	227.28	22.02	1.835	1999.3	1199.58	3.64	18.44	32.89	19.74	5.77	2.195	438.23	262.94
Tuesday	4.13	7.458	92.29	55.38	20.29	11.07	305.46	183.28	20.95	2.923	1194.6	716.76	4.56	16.69	45.57	27.34	1.78	1.16	255.26	153.16
Wednesday	4.09	7.987	85.31	51.19	17.99	7.917	378.66	227.20	11.56	0.922	2089.0	1253.39	4.63	19.69	39.16	23.49	4.68	1.744	446.80	268.08
Thursday	5.67	11.61	81.35	48.81	19.20	8.684	368.49	221.09	9.11	0.75	2024.6	1214.75	3.77	12	52.42	31.45	6.26	2.39	436.28	261.77
Friday	5.44	13.32	68.07	40.84	15.56	7.375	351.56	210.93	18.53	2.456	1257.9	754.72	1.30	3.993	54.16	32.50	3.51	2.069	282.45	169.47
Sunday	5.51	11.93	76.94	46.16	14.67	3.644	671.18	402.71	14.20	0.807	2930.6	1758.34	4.16	12.98	53.43	32.06	3.13	0.839	621.23	372.74