ANGLIA RUSKIN UNIVERSITY

Simultaneous Detection of Drugs of Abuse in Waste Water Using Gas Chromatography-Mass Spectrometry

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requirements of Anglia Ruskin University
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DECLARATION

I DECLARE THAT THIS RESEARCH IS MY ORIGINAL WORK EXCEPT WHERE REFERENCES AND ACKNOWLEDGEMENTS ARE MADE

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Psalms 139; Isaiah 26:3; Romans 8:28

ANGLIA RUSKIN UNIVERSITY

ABSTRACT

FACULTY OF SCIENCE AND TECHNOLOGY

DOCTOR OF PHILOSOPHY

SIMULTANEOUS DETECTION OF DRUGS OF ABUSE IN WASTE WATER USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Ellen Musili Mwenesongole

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Sewage epidemiology is increasingly becoming an alternative method of estimating drug usage and consumption patterns for a given population. With the constant emergence of new psychoactive substances such as cathinones and piperazines, versatile, reliable, specific and sensitive analytical methods are needed for their detection in complex matrices such as waste water. This thesis reports the development of an analytical method based on solid phase extraction, derivatization with pentafluoropropionic anhydride and analysis by gas chromatography-mass spectrometry for the simultaneous analysis of 29 illicit and therapeutic drugs of abuse.

All 29 drugs could be reliably identified in spiked waste water samples using selected ion monitoring and splitless injection. Recoveries for the majority of the drugs were above 70 %. Linearity varied based on the analyte but was assessed in the range 2.0×10^{-4} to 1.4 µg/mL. Intra-assay and intermediate precision of the instrument was determined at 0.005, 0.1 and 1.0 µg/mL, with the majority of relative standard deviations less than 10 %. Limits of detection and quantification for drugs such as amphetamine and methamphetamine were better than reported values for liquid chromatography-tandem mass spectrometry, a more commonly used technique.

Untreated 72 h composite waste water samples from Cambridge, UK, were analysed using a six-point standard addition curve. Eleven drugs of abuse were detected, including amphetamine, methamphetamine, butylone and 4-fluoromethamphetamine. The latter two having been detected for the very first time in waste water. Using the validated method, the consumption of heroin, ketamine, cocaine, methamphetamine and amphetamine, in Cambridge, UK, was estimated to be 399.4 ± 90.8, 2463.5 ± 182.5, 195.5 ± 95.4, 84.3 ± 59.1 and 38.9 ± 24.8 mg/day per 1000 inhabitants.

This is the first reported validated method for the detection of both classic drugs of abuse and new psychoactive substances in waste water using gas chromatography-mass spectrometry and derivatization with pentafluoropropionic anhydride.

Keywords: Drugs of abuse, gas chromatography-mass spectrometry, waste water, pentafluoropropionic anhydride, selected ion monitoring

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(All chemical structures, except where indicated, where drawn using ChemDraw Ultra 9.0.1 and graphs were produced using Microsoft Excel© 2010 and GraphPad Prism 4.03©).

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LIST OF ABBREVIATIONS

A: DRUGS AND INTERNAL STANDARDS

AMIT	Amitriptyline
AMP-d_6	Amphetamine-d_6
AMP	Amphetamine
BUTY	Butylone
BZP	Benzylpiperazine
CAT	Cathinone
3-CPP	3-chlorophenylpiperazine
COC-d_3	Cocaine-d_3
COC	Cocaine
DIAZ	Diazepam
EME	Ecgonine methyl ester
4-FMA	4-fluoromethamphetamine
3-FMC	3-fluoromethcathinone
2-FPP	2-fluorophenylpiperazine
4-FPP	4-fluorophenylpiperazine
KET	Ketamine
6-MAM	6-monoacetylmorphine
MAMP	Methamphetamine
MBDB	Methylbenzodioxolylbutanamine
MBZP	Methylbenzylpiperazine
MCAT	Methcathinone
MDMA-d_5	Methylenedioxymethamphetamine-d_5
MDMA	Methylenedioxymethamphetamine
MDPV	3,4-methylenedioxypyrovalerone
2-MeOPP	2-methoxyphenylpiperazine
4-MeOPP	4-methoxyphenylpiperazine
MEPH	Mephedrone
MOR-d_3	Morphine-d_3
MOR	Morphine
4-MPP	4-methylphenylpiperazine
PIP	Piperazine
3-TFMPP	3-trifluoromethylphenylpiperazine
4-TFMPP	4-trifluoromethylphenylpiperazine

B: GENERAL

AcAn:Pyr	Acetic anhydride:pyridine
ADHD	Attention deficit hyperactivity disorder
ATS	Amphetamine type stimulants
BOD	Biochemical oxygen demand
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
C	Confirmation
°C	Degree Celsius
$CHCl_3$	Chloroform
CF	Conversion factor
CI	Chemical ionisation
CIP	Chemical Investigations Program
COD	Chemical oxygen demand
CSEW	Crime Survey for England and Wales
CSJ	Centre for Social Justice
DC	Direct current
DEFRA	Department for Environment, Food and Rural Affairs
DH_2O	Deionised water
EA	Environment Agency
EC	European Commission
EDC	Endocrine-disrupting compound
EI	Electron impact ionisation
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EOC	Emerging organic contaminants
EQSD	Environmental Quality Standards Directive
ESI	Electrospray ionisation
etc.	Etcetera
EtOAC	Ethyl acetate
EtOH	Ethanol
EU	European Union
eV	Electron volts
FDA	Food and Drug Administration
GC	Gas chromatography
GC-ECD	Gas chromatography electron capture detector
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Liquid chromatography-tandem mass spectrometry
g	Grams
gr	Gravity
h	Hour
HCl	Hydrochloric acid
HDPE	High density polyethylene
HFBA	Heptafluorobutyric anhydride
HLB	Hydrophilic-lipophilic balanced
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
ICH	International Conference on Harmonisation
i.d.	Internal diameter
i.e.	That is
IPA	Isopropyl alcohol

K_{ow}	Octanol-water partition coefficient
L	Litre
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
M^+	Molecular ion
MCX	Mixed-mode strong cation exchange
MDA	Misuse of drugs act
MDR	Misuse of drugs regulations
mg	Miligram
MHRA	Medicines and Healthcare Regulatory Agency
min	Minute
mL	Millilitre
ms	millisecond
MS	Mass spectrometry (single quadrupole)
MS/MS	Tandem mass spectrometry (triple quadrupole)
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
m/z	Mass to charge ratio
N/A (NA)	Not applicable
N/Av	Not available
n	Number of samples
ND	Not detected
NH_4OH	Ammonium hydroxide
NIEA	Northern Ireland Environment Agency
NIST	National institute of standards and technology
ng	Nanograms
NPS	New psychoactive substances
OTC	Over the counter
PAR	Peak area ratio
PCPs	Personal care products
PE	Perkin Elmer
PES	Post extracted standard
PET	Polyethylene terephthalate
PFPA	Pentafluoropropionic anhydride
pg	Picogram
PhACs	Pharmaceutically active compounds
pK_a	Acid dissociation constant
PMT	Photomultiplier tube
POCIS	Polar organic chemical integrative samplers
PP	Polypropylene
PPCP	Pharmaceuticals and personal care products
Q	Quantifier
QC	Quality control
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RI	Retention index

RMS	Root mean square
RSC	Royal Society of Chemistry
RSD	Relative standard deviation
RT	Retention time
SEPA	Scottish Environment Protection Agency
SIM	Selected-ion-monitoring
SPE	Solid phase extraction
S/N	Signal to noise ratio
SPME	Solid-phase microextraction
SST	System suitability test
Std	Standard
StdDev	Standard deviation
TCPP	Tris (2-chloroisopropyl) phosphate
Temp	Temperature
TFAA	Trifluoroacetic anhydride
TIC	Total ion chromatogram
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TWA	Time-weighted average
UD	Underivatized
µg	Microgram
UHPLC	Ultra-high pressure liquid chromatography
UK	United Kingdom
UKWIR	United Kingdom Water Industry Research
UN	United Nations
UNODC	United Nations Office on Drugs and Crime
UV	Ultraviolet
v/v	Volume to volume
WADA	World Anti-Doping Agency
WFD	Water Framework Directive
WW	Waste water
WWTP	Waste water treatment plant

CHAPTER ONE

THEORETICAL BACKGROUND TO THE RESEARCH

The aim of this chapter is to present the research topic underpinning this study in the perspective of the wider area of water pollution and the global priorities in this field as well as the sample collection, pre-treatment, preparation and analytical techniques used. A background to waste water treatment in line with the regulations that govern it and the challenges due to emerging organic contaminants is also discussed.

1.1 WATER POLLUTANTS

The varied and thriving chemical industry has led to the increased production of pharmaceutical, petrochemical, agrochemical, industrial and consumer chemicals to meet growing demand (Deblonde, et al., 2011; Harrison, 2013). However, despite most of these compounds making a positive contribution to our daily lives, they have not been without controversy. The presence of these compounds, their by-products during manufacture and their degradation products in water has led to increasing public awareness as well as scientific interest and concern about their effects on aquatic life and the environment as a whole (Caliman and Gavrilescu, 2009; Wille, et al., 2012; Loos, et al., 2013). Key compounds of concern have traditionally included pesticides, polycyclic aromatic hydrocarbons, industrial chemicals and endocrine-disrupting compounds (EDCs) which are widely acknowledged as water pollutants (Mol, et al., 2000; Verenitech, et al., 2006; Boleda, et al., 2011; Tomsikova, et al., 2012; Luo, et al., 2014; Wilson, et al., 2014). EDCs have been associated with the disruption of hormonal activity, sexual development and reproductive function in aquatic organisms (Bayen, et al., 2013; Ribeiro, et al., 2014a). However, over the past two decades scientific interest in pharmaceutical and personal care products (PPCPs), including drugs of abuse, as emerging pollutants has steadily increased as evidenced by the increasing number of publications (Jjemba, 2008; Hogenboom, et al., 2009; Kummerer, 2009; Deblonde, et al., 2011; Bayen, et al., 2013; Petrie, et al., 2013; Luo, et al., 2014; Verlicchi, et al., 2012 & 2014; Zhang, et al., 2014). PPCPs consist of a wide variety of organic compounds emanating from human and veterinary pharmaceuticals, drugs of abuse and consumer products (Luo, et al., 2014). These are discussed further in section 1.3.4.

It is worth noting the definition of the terms 'contaminant' and 'pollutant', which are often used interchangeably. When a chemical is present in the environment with no evidence of harm it can be regarded as a contaminant. Where evidence of harm exists, the chemical can be regarded as a pollutant (Harrison, 2013). At a particular concentration relevant to the chemical activity, any chemical can cross over from 'contaminant' to 'pollutant' status (*ibid*). The effects of pollution on water include aesthetic (e.g. litter and smells), deoxygenation, disturbance of the pH balance and toxicity to aquatic and terrestrial organisms (Escher, et al., 2011). In various literature sources, PPCPs have interchangeably been referred to as emerging organic contaminants (EOCs) (Jurado, et al., 2012; Gilart, et al., 2014), emerging environmental pollutants (Kasprzyk-Hordern, et al., 2009c) and micropollutants (Jiang, et al., 2013; Luo, et al., 2014).

1.2 SUSTAINABLE WATER MANAGEMENT

With greater demand on water from domestic, agricultural and industrial use, fuelled by an unprecedented population growth, sustainable water management has become a key focus area among scientists and others. There is global consensus that better water management systems need to be put in place to ensure that water remains of good quality for human needs as well as for aquatic and terrestrial animals which rely on it (Royal Society of Chemistry, 2007). In the Royal Society of Chemistry's (RSC) summary report on sustainable water (RSC, 2007), chemistry has been shown to play an increasingly important role in helping to address some of the key challenges being faced in the management of water. This is because understanding the chemical nature and behaviour of pollutants in water can help bring about appropriate solutions into their management *(ibid)*. The chemical sciences have always played an important role in the treatment of water for consumption as well as in removing pollutants from waste water and industrial effluent. However, in order for solutions to be relevant, the ever changing nature and levels of pollutants in waste water need to be determined as well as their chemical and synergistic behaviour in the presence of other substances in water (Kummerer, 2009; Lopez-Serna, et al., 2010; Escher, et al., 2011; Bayen, et al., 2013). While there have been great advances in developing life cycle assessments for conventional man-made pollutants that pose a risk to aquatic systems, such as pesticides and EDCs, similar research into emerging contaminants, such as PPCPs, still has a long way

to go (Kasprzyk-Hordern, et al., 2007; Lopez-Serna, et al., 2010; Deblonde, et al., 2011; Bayen, et al., 2013). This is one of the key chemical science challenges as identified by the RSC (2007) report. One of the recommendations arising from this challenge is for funding bodies to prioritise research that seeks to identify and understand the behaviour of such emerging contaminants. Since the RSC report, there has been an accelerated emergence of funded research into emerging contaminants in various water sources (Jurado, et al., 2012) and in different parts of the world (van Nuijs, et al., 2011a; Thomas, et al., 2012; Luo, et al., 2014).

1.2.1 Policies Governing Sustainable Water Management

In Europe, sustainable water management is a concern for member countries of the European Union (EU). Hence the Water Framework Directive (WFD) (2000/60/EC) of the EU governs the quality of surface water through the Environmental Quality Standards Directive (EQSD) (2008/105/EC). The EQSD (2008) acknowledges the threat to the aquatic environment that chemical pollution poses as this ultimately affects ecosystems and human health. In this regard the EQSD identifies various priority substances that need to be regulated with regards to their discharge into surface water. These substances include chemicals such as anthracene and mercury and its compounds, which are toxic even at low concentrations and are carcinogens (EQSD, 2008; Harrison, 2013). The EQSD is focused on preventive action and lays the cost and responsibility of rectifying pollution on the 'polluter'. Another EU legislation complimenting the WFD is called Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (EC 1907/2006). REACH (2006) requires that all chemical manufacturers and importers present evidence of the environmental risk assessment of the chemical before it can be marketed. This is implemented through a registration system. Pharmaceutical products are not included under the REACH legislation as they are covered by separate registration procedures, such as the Medicines and Healthcare Regulatory Agency (MHRA) in the UK (MHRA, 2014).

In the UK, the waste water treatment processes are regulated by different agencies. England and Wales are managed by the Environment Agency (EA) (EA, 1994), the Scottish Environment Protection Agency (SEPA) is responsible for Scotland (SEPA, 1994), while Northern Ireland is managed by the Northern Ireland Environment Agency (NIEA) (NIEA,

2007). These agencies stipulate the quality of waste water from a waste water treatment plant (WWTP) before it is discharged into surface and ground water, based on regulations defined by the European Commission Council Directive concerning waste water treatment (91/271/EEC). In addition, a Chemical Investigations Program (CIP) was set up in 2009 by the UK Water Industry Research (UKWIR) and EA to investigate how efficient the current treatment processes are in removing some of the priority substances as stipulated by the EQSD. These priority substances have recently been expanded to include a few pharmaceutically active compounds (PhACs) such as steroid oestrogens and ibuprofen, which have traditionally not been targeted for analysis (Petrie, et al., 2013; UKWIR, 2013; Zenker, et al., 2014). However, the majority of PPCPs, including illicit drugs, are still not being routinely monitored in waste water and surface water and their effects on the aquatic and terrestrial ecosystems are not widely understood (Dong, et al., 2013; Zenker, et al., 2014).

1.3 WATER TREATMENT

One cannot mention sustainable water management without referring to water treatment plants. Water treatment plants cater for the treatment and purification of water for domestic use, treatment for industrial use or treatment of waste water emanating from these and other sources for discharge or reuse (Binnie & Kimber, 2009; Manahan, 2010). As samples for this research are sourced from a WWTP, more emphasis will be placed on this. However, treatment of water for domestic use will be briefly mentioned due to the link between the two.

1.3.1 Domestic Water Treatment

Sources of water for domestic use include rivers, lakes, streams, reservoirs, and groundwater (Gerba, et al., 2006; Harrison, 2013). Sources of contamination of these water bodies include natural organic and inorganic substances from the environment as well as man-made contaminants from industrial, waste water and run-off (Harrison, 2013). The main aim of treatment of water for domestic use is to remove microorganisms and chemical contaminants when present so as to produce water that is safe for human consumption (Binnie & Kimber, 2009). The treatment varies depending on the water source and supply needs but generally includes coagulation, flocculation, filtration and disinfection (Manahan, 2010). While treatment is effective in minimising the presence of

microorganisms and chemicals, studies have shown the presence of various drugs of abuse in domestic water (Boleda, et al., 2011). The main source of PhACs found in potable water is thought to be due to contamination from waste water effluent (Lopez-Serna, et al., 2010; Metcalfe, et al., 2010).

1.3.2 Waste Water and its Composition

Water from domestic use ultimately ends up at a WWTP where it undergoes various treatment processes. Historically, the aim of waste water treatment was to minimise disease and odour and protect potable water (Harrison, 2013). In present times, this aim has expanded to include the reduction in the concentration of organic matter and certain pollutants so as to produce an effluent that will pose minimal harm to human and animal health and the natural environment (Gerba & Pepper, 2006; Gomes, 2009). Over 11 billion litres of waste water, comprising mainly domestic and industrial waste, is treated in around 9000 WWTPs in the UK on a daily basis [Department for Environment, Food and Rural Affairs (DEFRA) 2012; Harrison, 2013]. Once waste water is treated, it is mainly discharged to surface waters such as rivers, streams, estuaries, the sea or ground water (EA, 1994; DEFRA, 2002; DEFRA, 2012). As water from these sources is ultimately re-used in one way or the other, the management of WWTPs forms part of sustainable water management (RSC, 2007). In this thesis and various other publications waste water flowing into a WWTP is interchangeably referred to as influent, raw and untreated waste water (van Nuijs, et al., 2011a; Castiglioni, et al., 2013 & 2014; Nefau, et al., 2013).

The composition of waste water varies widely depending on the source e.g. domestic, industrial, medical facilities, agricultural and run-off (Peirce, et al., 1998; Gerba & Pepper, 2006; Escher, et al., 2011; Lin, et al., 2014). The majority of WWTPs servicing large towns and cities treat combined waste water from these and other sources (Harrison, 2013). In this regard, the main constituents of waste water, aside from water itself, are nitrogenous compounds (e.g. proteins and urea), carbohydrates (e.g. sugars and starch), fats (e.g. cooking oil and soap), PPCPs, agricultural chemicals, metallic salts, heavy metals, grit and rocks (Manahan, 2010; Harrison, 2013).

1.3.3 Waste Water Treatment

Waste water treatment processes were originally developed to remove pollutants such as pathogens, nutrients, heavy metals, herbicides and pesticides (Jjemba, 2008). However, due to the ever changing composition of pollutants in waste water, treatment processes will need to be improved on to cater not only for traditional pollutants but also emerging ones such as pharmaceuticals (Caliman & Gavrilescu, 2009; Kummerer, 2009).

Generally waste water undergoes four main stages of treatment comprised of physical, chemical, and biological processes (Gomes, 2009). These are referred to as preliminary, primary, secondary and tertiary treatment in reference to increasing treatment levels (Gerba & Pepper, 2006). The intensity of treatment that waste water is subjected to mainly depends on the source, desired quality of the effluent and the capabilities of the sewage treatment plant (EA, 1995; RSC, 2007; Manahan, 2010). While the basic aims of each treatment level remains the same, the process of achieving this may vary from plant to plant depending on the technology used (Gomes, 2009).

1.3.3.1 Preliminary Treatment

The main aim of preliminary treatment is to remove large suspended solids such as rags, wood, glass and rocks as well as coarse solids such as grit (Jjemba, 2008). If not removed prior to secondary treatment, these items can cause damage and blockage to the network of pipes and pumps used to move waste water from one section to the other. Screens are typically employed to remove the larger solid objects while graters may also be used to reduce the size of some larger objects and facilitate their removal through subsequent treatment processes (Gomes, 2009). The objects removed during preliminary treatment are ultimately incinerated or disposed of at a landfill (Jjemba, 2008). Preliminary treatment is sometimes incorporated into the primary treatment stage (Gerba & Pepper, 2006; Manahan, 2010).

1.3.3.2 Primary Treatment

During the primary treatment stage, organic and inorganic suspended solids are removed by sedimentation while floating material is removed by skimming. A large portion of oil and grease is also removed during primary treatment (Jjemba, 2008; Gomes, 2009). Large sedimentation tanks are used and the settled solids, referred to as primary sludge, are

removed for further treatment. The primary sludge is subjected to digestion by anaerobic bacteria which help reduce its volume before disposal as solid waste (Jjemba, 2008).

1.3.3.3 Secondary Treatment

The main aim of the secondary treatment stage is the removal of dissolved organic components and colloidal constituents (Pepper, et al., 2006). Microorganisms found in waste water rely on oxygen to degrade the organic matter and this results in the reduction of the oxygen content in water which can have detrimental effects to aquatic life that rely on oxygen for various biological processes (Manahan, 2010). This is referred to as the biochemical oxygen demand (BOD). The key focus during secondary treatment is therefore to reduce the BOD by limiting the organic components that are available for decomposition by microorganisms in waste water. This is achieved mainly by using aerobic microorganisms (mainly bacteria) in the presence of added oxygen (Gomes, 2009). The microorganisms oxidize the organic matter until the BOD is within acceptable levels (Manahan, 2010). Sedimentation tanks are also used during secondary treatment but the 'sediment' comprises of microorganisms and is referred to as biological sludge. The biological sludge is normally processed further in combination with primary sludge (Gomes, 2009). Additional suspended solids, not removed during primary treatment, are also removed during secondary treatment (Gerba & Pepper, 2006).

1.3.3.4 Tertiary Treatment

Waste water normally undergoes tertiary treatment when more specific components need to be removed to meet specific criteria such as for drinking water (Pepper, et al., 2006; Manahan, 2010). During this stage, components such as nitrogen, phosphorus, heavy metals and pathogens are removed. Although mentioned separately, tertiary treatment processes are sometimes incorporated into primary or secondary treatment processes or used instead of secondary treatment (Gomes, 2009). A combination of biological and chemical processes are used to remove the components as well as improve the aesthetic aspects such as odour and colour before the effluent is released into surface and ground water or reused (EA, 1994; Manahan, 2010). Advanced filtration using granular or membrane filters, oxidation using chlorination or ozonation, photodegradation using ultraviolet (UV) radiation, electrolysis and aeration are some of the processes employed during tertiary treatment (Jjemba, 2008: Binnie & Kimber, 2009).

Waste water that has undergone primary, secondary or tertiary treatment is referred to as effluent or treated waste water (Hedgespeth, et al., 2012; Loos, et al., 2013; Nefau, et al., 2013).

1.3.4 Pharmaceuticals and Personal Care Products (PPCPs) in Waste Water

Although only a few PPCPs are being monitored in waste water effluent (section 1.2.1), this appears to be a step in the right direction in recognising the changing nature of emerging pollutants. PhACs being detected in waste water and surface water are from diverse therapeutic classes such as antibiotics, analgesics, hormones, beta-blockers, and antidepressants (Dong, et al., 2013; Zenker, et al., 2014). Table 1.1 lists the PhACs monitored by the CIP (section 1.2.1) due to their high levels being detected in waste water and surface water and potential ecotoxicological effects (Petrie, et al., 2013; Zenker, et al., 2014).

Table 1.1: Pharmaceutical compounds and quality criteria as monitored by the Chemical Investigations Program, UK (UKWIR, 2013).

Pharmaceutical Compound	Application	CIP (UK) Quality Criteria (µg/L)
Diclofenac	Anti-inflammatory	1.0×10^{-2}
Erythromycin	Antibiotic	1.0×10^{-2}
Ethinyloestradiol	Natural oestrogen	1.0×10^{-4}
Fluoxetine	Antidepressant	1.0×10^{-2}
Ibuprofen	Anti-inflammatory	1.0×10^{-2}
Oestradiol	Natural oestrogen	1.0×10^{-3}
Oestrone	Natural oestrogen	3.0×10^{-3}
Ofloxacin	Antibiotic	1.0×10^{-2}
Oxytetracycline	Antibiotic	1.0×10^{-2}
Propanolol	Anti-anxiety, hypertension	1.0×10^{-2}

PhACs, by definition, include drugs legally manufactured for therapeutic purposes (e.g. Ibuprofen), drugs with no known legal therapeutic use (i.e. illicit drugs such as ecstasy) as well as legally manufactured drugs which are being abused (e.g. diazepam) [Moffat, et al.,

2011b; United Nations Office on Drugs and Crime (UNODC), 2014]. The latter two categories are collectively referred to as drugs of abuse in this thesis to distinguish from drugs being used solely for therapeutic purposes. The term 'abuse' in the phrase 'drugs of abuse' refers to the deliberate use of a substance for non-medicinal purposes to produce psychoactive effects (altering of alertness, mood, perception or behaviour) or intoxication (King, et al., 2013). Sources of PhACs found in waste water include manufacturers, hospitals, and households (Kummerer, 2009; Philips, et al., 2010; Behera, et al., 2011; Luo, et al., 2013).

Although not the focus of this research, personal care products (PCPs) are normally grouped and investigated together with PhACs (Hedgespeth, et al., 2012; de Garcia, et al., 2013; Zhang, et al., 2014). Categories of PCPs include parabens, antiseptics, surfactants, UV filters and polycyclic musks (Stuart, et al., 2012; Loos, et al., 2013; Luo, et al., 2013). Table 1.2 lists some PPCPs and their concentrations as detected in untreated and treated waste water from urban areas.

Table 1.2: Concentrations of pharmaceuticals and personal care products reported in urban waste water.

PPCP	Application	Concentration in Influent (µg/ml)	Reference
Ibuprofen	Anti-inflammatory	0.373	Verlicchi, et al., 2012
Paracetamol	Anti-inflammatory	0.143	Hedgespeth, et al., 2012
Caffeine	Stimulant	0.150	Baker & Kasprzyk-Hordern, 2013
Amitriptyline	Anti-depressant	0.001	Baker & Kasprzyk-Hordern, 2013
Amphetamine	Stimulant	0.003	Baker & Kasprzyk-Hordern, 2013
Acesulfame	Sweetener	2.500	Loos, et al., 2013
TCPP	Flame Retardant	0.021	Loos, et al., 2013
Bisphenol A	Plasticizer	0.001	Sun, et al., 2014
Benzoylecgonine	Cocaine metabolite	0.001	Gilart, et al., 2014

TCPP = Tris (2-chloroisopropyl) phosphate

1.3.4.1 Pharmaceutically Active Compounds (PhACs)

The focus of this research is on PhACs and more emphasis will be placed on them from this point on. Compared with pesticides and EDCs, the environmental risk assessments and toxicological profiles in the aquatic and terrestrial environment are not fully

established for the majority of PhACs (Deblonde, et al., 2011; Zenker, et al., 2014). However, some research groups suggest low ecotoxicological risk to aquatic and terrestrial organisms due to the dilution factor of waste water effluent entering surface water or as a result of photodegradation (Gros, et al., 2010; Wang & Lin, 2014) while others report an associated ecotoxicological risk (Escher, et al., 2011; Stuart, et al., 2012; Zhang, et al., 2014). These risks can reportedly lead to development of antimicrobial resistance, reduction in plankton diversity, an impact on human embryonic development, feminization of certain aquatic organisms and possible bioaccumulation of certain pharmaceuticals (Kumirska, et al., 2011; Stuart, et al., 2012; Loos, et al., 2013; Zenker, et al., 2014; Zhang, et al., 2014). In this regard, the ecotoxicological effects on aquatic organisms such as algae, *daphnia* and certain fish have been reported (Zenker, et al., 2014; Zhang, et al., 2014). There are also published reports on the ecotoxicological effects of non-steroidal anti-inflammatory drugs (NSAIDS) on terrestrial animals e.g. diclofenac and its effect on the declining vulture populations in Pakistan (Oaks, et al., 2004) and the detection of flunixin in sheep's wool which also has possible ecotoxicological effects (Richards, et al., 2011). However, as ecotoxicological data is available for less than 10 % of the prescribed PhACs, it is thus evident that more work still needs to be done to fully understand their potential risk to the environment (Bayen, et al., 2013; Zhang, et al., 2014). This incorporates developing analytical methods to enable the detection of these PhACs in the environment, such as in waste water, and developing toxicological risk assessments to determine concentration levels that pose a risk (Escher, et al., 2011). Although a PhAC may be present at a low concentration and not exhibit any toxicological effects individually, its unknown synergistic effect in the presence of other compounds in a complex mixture, such as waste water, is still cause for concern (Loos, et al., 2013; Wang & Lin, 2014). In addition, concentration levels that may not be harmful to terrestrial life may be harmful to aquatic organisms and hence any toxicological risk assessments need to account for the effects on different types of organisms. PhACs are also known to undergo degradation and transformation processes within the sewage system, which may have different ecotoxicological effects to the parent drug. These processes are described in the next section.

1.3.4.2 In-Sewer Degradation and Transformation of Pharmaceuticals

There are various factors that affect the presence and levels of PhACs within the sewerage system between their points of excretion and sampling. Some factors are linked to the characteristics of a particular WWTP and these include the treatment processes (section 1.3.3), hydraulic retention time (HRT), pH, temperature and composition of the waste water. The HRT is the time allowed for biodegradation and sorption and varies depending on the compound and loading rate at the WWTP (Luo, et al., 2014). Other factors, such as the chemical structure, polarity, half-life, pK_a (acidity) and pK_{ow} (hydrophobicity) are based on the physico-chemical properties of the drug. Collectively, all these factors affect the natural attenuation of a compound in waste water and surface water (Luo, et al., 2014; Zenker, et al., 2014). Natural attenuation processes include physical processes (e.g. dispersion, dilution and sorption), biological processes (e.g. biodegradation and biotransformation) and chemical reactions (e.g. photodegradation, interchange between different enantiomeric forms, interaction with other compounds within the matrix (Baker & Kasprzyk-Hordern, 2013; Wang & Lin, 2014; Zhang, et al., 2014).

These transformation and degradation processes can affect both quantitative and qualitative aspects of the compounds, such as a reduction in the concentrations of compounds below their limits of detection or potential transformation of one compound that is already present in the waste water into the target analyte e.g. methamphetamine into amphetamine (Reid, et al., 2014a; Thai, et al., 2014). Therefore, in-sewer transformation and degradation of compounds forms a major limitation of the sewage epidemiological approach since the final detected concentration may be an under- or over estimation of the original concentration present in the matrix before the degradation processes (Zuccato, et al., 2008; Lai, et al., 2011; van Nuijs, et al., 2011b; Castiglioni, et al., 2013). In this regard, various studies have been conducted to try and understand these attenuation processes, including the stability of drugs of abuse during various storage conditions (van Nuijs, et al., 2012; Bijlsma, et al., 2013a; Senta, et al., 2014).

However, it should be noted that the longer the compound is exposed to the conditions in the WWTP, especially during the treatment process, the higher the possibility of it

undergoing the various attenuation processes. This in turn affects the efficiency of removal of the PhACs from waste water before it is discharged into surface water (Stuart, et al., 2012; Zhang, et al., 2014). For instance, samples collected at the inlet of the WWTP would have spent less time exposed to the natural attenuation processes than those which have been collected after completion of the treatment process. Therefore, concentrations of some PhACs will be found at higher levels in untreated waste water than in treated waste water or surface water (Hernandez, et al., 2011; Baker & Kasprzyk-Hordern, 2013; Nefau, et al., 2013). This is discussed further in section 1.3.4.3. This also implies that as a result of attenuation processes, the parent drug may not be detected in waste water (treated and untreated) or surface water, but its degradation products may be present and more stable and hence may also be used for quantification. In this regard, some research groups have investigated degradation products of PhACs in the aquatic environment with the aim of determining their environmental fate (Bijlsma, et al., 2013a; Rodayan, et al., 2014). On the other hand, the degradation products could also have negative ecotoxicological effects different from that of the parent compound and hence may need to be included in any ecotoxicological risk assessments (Wang & Lin, 2014).

1.3.4.3 Removal of Pharmaceuticals during Waste Water Treatment

Although not specifically targeted during waste water treatment, published studies have shown that a reasonable amount of these PhACs are removed from waste water as a result of current treatment processes (Postigo, et al., 2010; Behera, et al, 2011; Luo, et al., 2014; Sun, et al., 2014). However, the various attenuation processes discussed in section 1.3.4.2 affect the removal of PhACs from a WWTP leading to a wide variation in removal rates between different compounds and also within the same compound (Postigo, et al., 2010; Hedgespeth, et al., 2012; Luo, et al., 2014). The type of treatment processes at the WWTP also plays a crucial role in the efficiency of removal of PhACs (Hedgespeth, et al., 2012; Bayen, et al., 2013; Kumirska, et al., 2013). This has led to various published investigations into the efficiency of the treatment processes in removing PhACs and results vary widely depending mainly on the physico-chemical properties of the PhAC and the waste water treatment process used (Gros, et al., 2010; Postigo, et al., 2010; Behera, et al., 2011; Baker & Kasprzyk-Hordern, 2013; Gilart, et al., 2014; Luo, et al., 2014). Therefore, removal rates can range from 20 % to 100 % (Table 1.3), which still constitutes only partial removal, and these drugs persist in treated waste water which ultimately gets

discharged into surface water (Huerta-Fontela, et al., 2008; Boleda, et al., 2009; Kasprzyk-Hordern, et al., 2009c; Postigo, et al., 2010; Bayen, et al., 2013; Pal, et al., 2013; Patrolecco, et al., 2013; Zhang, et al., 2014).

Table 1.3: Removal efficiency of pharmaceuticals at waste water treatment plants.

Pharmaceutical Compound	Removal Efficiency (%)
Amphetamine	52-99[1]
Ketamine	80[1]
Methamphetamine	44-99[1]
Methylenedioxymethamphetamine	50-99[1]
Diclofenac	30-100 [2]
Ofloxacin	20-99[2]
Salbutamol	20-99 [2]
Ibuprofen	97.9[3]
Paracetamol	100[3]
Caffeine	97[3]

[1]Huerta-Fontela, et al., 2008; [2]Gros, et al., 2010; [3]Lacina, et al., 2013

More specifically, drugs of abuse have been shown to persist in treated waste water and hence their detection in surface water can mainly be attributed to discharge of treated waste water and untreated influent into surface waters (Jones-Lepp, et al., 2004; Postigo, et al., 2008a; Boles & Wells, 2014). Drugs of abuse can also be directly disposed into the aquatic system leading to a minimal or major contribution to the final concentration (Postigo, et al., 2008a; Kasprzyk-Hordern, & Baker, 2012; Emke, et al., 2014). Although concentrations of drugs of abuse detected in surface waters are low (ng/L), their potential risk to human and environmental health cannot be dismissed (Postigo, et al., 2010; Jurado, et al., 2012; Repice, et al., 2013). Some of the surface water these drugs are present in is ultimately treated for drinking water (potable water) and if the drugs are not efficiently removed, they can persist in the potable water (Boleda, et al., 2009 & 2011; Baker & Kasprzyk-Hordern, 2013; Luo, et al., 2014). It is worth mentioning here that in Boleda's (2009 & 2011) findings, the majority of drugs of abuse and their metabolites

studied were completely eliminated from the potable water by the more stringent treatment processes with some only occurring at ultratrace levels (below 3 ng/L).

However, the long term effects of unwitting consumers drinking low level drug concentrations are still unknown, especially when multiple drugs are present in the potable water (Vazquez-Roig, et al., 2013). Since they are increasingly being regarded as emerging pollutants, it is therefore necessary to not only determine the levels of these drugs in waste water but to also understand their behaviour. Individual behaviour may differ from synergistic behaviour when combined with other chemicals in waste water (Repice, et al., 2013; Wang & Lin, 2014). Although determining the full life-cycle assessment on these drugs of abuse and their potential harm to aquatic and terrestrial life is out of the scope of this study, it is hoped that the findings presented will make a positive contribution to overall research in this field. Detecting their levels in waste water and determining any potential adverse effects will need a wide variety of analytical methods, including those presented in this research, to suit different applications and budgets.

1.3.4.4 Drugs of Abuse and Sewage Epidemiology

Although some PhACs were recognised as environmental contaminants in the 1970s, widespread scientific interest in them occurred mainly from the mid-1990s coinciding with development of more sensitive analytical techniques (Jones-Lepp, et al., 2004; Farre, et al., 2012). On the other hand, drugs of abuse have only recently been targeted for investigation as environmental contaminants post 2000 (Postigo, et al., 2010; Daughton, 2011; Repice, et al., 2013). As mentioned in section 1.3.4.3, PhACs have been detected in waste water influent and effluent as well as surface water. The rationale behind this is that once drugs are consumed, they are processed by the body and released as parent drug and metabolites, mainly through urine and faeces, into the sewage system (Repice, et al., 2013). Once at the WWTP, they are subjected to various treatment processes as discussed in section 1.3.3. However, as already inferred, these treatment processes are not always 100 % efficient when it comes to removing PhACs and hence these substances persist even in treated waste water. Several studies have been conducted around the globe to detect the levels of pharmaceutical substances in waste water and surface water (Berset, et al., 2010; Metcalfe, et al., 2010; Irvine, et al., 2011; de García, et al., 2013;

Boles & Wells, 2014). Although initially suggested by Daughton (2001b), investigations into the presence of PhACs in treated and untreated waste water was taken a step further in 2005 (Zuccato, et al., 2005) when measured levels of cocaine and its metabolites were used to estimate its consumption by a population serviced by a particular WWTP. Since then various research groups from different countries (e.g. Australia, Belgium, Canada, Finland, Italy, Spain, Switzerland, UK, USA) have investigated drugs of abuse in waste water and surface water (Gheorghe, et al., 2008; Loganathan, et al., 2009; Metcalfe, et al., 2010; Zuccato, et al., 2011; Hernandez, et al., 2011; Irvine, et al., 2011; Baker & Kasprzyk-Hordern, 2013; Burgard, et al., 2013; Emke, et al., 2014; Ort, et al., 2014; Vuori, et al., 2014). The main difference between these groups has been the classes of drugs of abuse investigated, the approaches to sampling and sample preparation, the type of validation carried out and the interpretation of results. With such data gathered from different countries or at different times of the year, comparisons can be made regarding usage patterns of drugs of abuse in different locations, at different days, months or seasons (Reid, et al., 2011; van Nuijs, et al., 2011a; Zuccato, et al., 2011; Thomas, et al., 2012; Burgard, et al., 2013). Hence, the phrase 'sewage epidemiology' was developed to refer to the process of using analytical tools to extract targeted biological indicators from waste water in order to gather specific epidemiological information (Daughton, 2011). Drugs of abuse were the first substances to be analysed in this manner.

Sewage epidemiology, as a means of estimating the consumption of drugs of abuse, compliments other forms of data such as criminal and medical records, drug monitoring, drug seizures, consumer interviews and population surveys (González-Mariño, et al., 2010; van Nuijs, et al., 2009d; Castiglioni, et al., 2014). In many cases, the drug consumption figures obtained from sewage epidemiology closely match figures obtained from social-epidemiological studies (Reid, et al., 2011; Lai, et al., 2013b; Ort, et al., 2014). Hence, comparisons between the different forms of data can be made in order to obtain a better understanding of the trends in the use of drugs of abuse (Reid, et al., 2012). One major advantage of the sewage epidemiology approach over socio-epidemiological methods is the production of real-time data since results from sewage can be obtained within hours or days while socio-epidemiological studies take longer (González-Mariño, et al., 2010; Khan & Nicell, 2011; Prichard, et al., 2014). The sewage epidemiological approach has even been adopted by the European Monitoring Centre for Drugs and Drug

Addiction (EMCDDA) as a feasible method for estimating community drug consumption (EMCDDA, 2008 & 2014b). Although sewage epidemiology is anonymous and generalised, it can also be applied to more localised situations such as a waste water stream from a prison (Postigo, et al., 2011), a college (Burgard, et al., 2013), a music festival (Lai, et al., 2013c), a neighbourhood (Boles & Wells, 2014*)* or even after a major sporting event (Berset, et al., 2010; Gerrity, et al., 2011) or holiday period (van Nuijs, et al., 2011b; Lai, et al., 2013a) to determine any spatial and/or temporal trends in drug usage. However, ethical and legal aspects of localised sampling needs to be carefully considered and addressed prior to any research work being undertaken (Boles & Wells, 2014; Castiglioni, et al., 2014; EMCDDA, 2008 & 2014b; Prichard, et al., 2014).

There are increasing collaborations between research groups in the area of sewage epidemiology to better understand the behaviour of these drugs in waste water individually (stability), collectively (drug-drug interactions) and their effect on aquatic wildlife. Further research into separating chiral compounds, thereby enabling the better estimation of illicit drug consumption versus therapeutic use of amphetamine type stimulants (ATS), as well as between consumption versus direct disposal into the sewer system, has also been investigated (Kasprzyk-Hordern & Baker, 2012; Emke, et al., 2014; Ribeiro, et al., 2014b). Illicit drugs in sewage sludge have also been investigated but not to a large extent (Kaleta, et al., 2006). As treated sludge is increasingly used for agricultural purposes, it could be worth investigating for the presence of PhACs and their potential effect on the environment. The presence of PhACs in groundwater has also been investigated due to the reliance on groundwater as a potable water source in some areas (Juradao, et al., 2012; Stuart, et al., 2012).

The most commonly abused drugs in the UK and the selection of drugs for this research are discussed in the next section.

1.4 DRUGS OF ABUSE

As mentioned in section 1.3.4, PhACs can be considered to comprise therapeutic drugs as well as drugs of abuse. Most drugs of abuse are restricted in use, possession or supply, by law, due to the potential harmful effects on the user and others. They are, therefore, referred to as controlled substances. The majority of drugs of abuse are either illegally

manufactured in clandestine laboratories or diverted from legitimate sources (Cole, 2003; White, 2005; King, et al., 2013; EMCDDA, 2014a).

In the UK, drugs are primarily controlled by the Misuse of Drugs Act 1971 (MDA, 1971) and the Misuse of Drugs Regulations 2001 (MDR, 2001). The MDA, 1971 controls the unauthorized possession and supply of drugs and stipulates penalties for their misuse while the MDR, 2001, controls the legitimate use, distribution and sale of drugs (MDR, 2001; Cole, 2003). However, in spite of restrictions on their possession, supply, manufacture and use, the abuse of drugs has not abated. Instead, the use of drugs of abuse has been increasingly fuelled by an enterprising and prevalent illegal drug manufacturing industry (King, et al., 2013; EMCDDA, 2014; UNODC, 2014). The most commonly abused drugs are derived from plants (e.g. cannabis, cocaine) but synthetic drugs (e.g. cathinones, synthetic cannabinoids and synthetic opioids) and semi-synthetic drugs (e.g. heroin) pose just as much of a social problem (Dickson, et al., 2010b; EMCDDA, 2014a; Reid, et al., 2014b). These are contextualised with regard to usage in North America and Europe in the following section.

1.4.1 Commonly Abused Drugs

As discussed in section 1.4, the regulation of drugs of abuse has not stopped their ever increasing recreational use. According to the UNODC (2014) report, the worldwide estimate of people aged 15-64 years who used drugs of abuse in 2012 lies between 162 and 324 million. In Europe, cannabis is the most widely abused drug followed by cocaine and drugs in the opioid and ATS groups (EMCDDA, 2014a). According to the Crime Survey for England and Wales (CSEW, 2014), the most commonly abused drugs in England & Wales among 16-59 year olds in the 2013/2014 period, in decreasing order of consumption, are cannabis, cocaine, and ecstasy. This equates to 8.8 % of the population (i.e. 2.7 million people) in this age range having taken an illicit drug. In terms of amounts of drugs seized in Europe, cannabis is the most prevalent (81 %), followed by cocaine (9 %), heroin (4 %), amphetamine (3 %), ecstasy (2 %), with methamphetamine and lysergic acid diethylamide both at 1 % each (EMCDDA, 2014a). The majority of these seizures occurred in Spain and the UK (*ibid*). Consistent or declining trends in heroin and cocaine use in the major markets (North America & Europe) are negated by increasing and/or consistent misuse of new psychoactive substances (NPS) and prescription drugs

such as benzodiazepines and barbiturates, a noticeably growing health and social problem in a number of developed and developing countries (UNODC, 2013).

1.4.2 Selection of Drugs for this Research

The selection of target drugs and metabolites included in this research was, therefore, based on a) the most commonly abused drugs in Europe and England and Wales according to the CSEW (2014) and EMCDDA (2014a) reports, respectively, b) published findings from toxicological and sewage epidemiological studies (van Nuijs, et al., 2011a; Helander, et al., 2014; Pal, et al., 2013; Baker, et al., 2014), and c) emerging drugs of abuse (i.e. NPS) as indicated in the UNODC (2013 & 2014) and EMCDDA (2014) reports. Most data obtained from published sewage epidemiological studies has been on classic drugs of abuse such as cocaine, amphetamine, and ecstasy and therapeutic drugs such as morphine, diazepam, ibuprofen (Reid., et al.,2011; van Nuijs., et al., 2011; Thomas., et al., 2012). Although classic drugs of abuse were also included in this research, the emphasis was on NPS such as piperazines, cathinones and ketamine, since they are increasingly being abused and little sewage epidemiological data is available for them (Meyer, et al., 2010; Corkery, et al., 2012; Chen, et al., 2014; Helander, et al., 2014; Reid, et al., 2014a; UNODC, 2014). Classic illicit drugs of abuse such as cocaine, amphetamine, methylenedioxymethamphetamine (MDMA) and therapeutic drugs of abuse such as morphine and diazepam were added for comparative purposes and to determine if their levels would be much higher than the NPS due to their historically more prevalent use (CSEW, 2014; EMCDDA, 2014; UNODC, 2014).

Table 1.4 lists the drugs and metabolites investigated in this research and internal standards used. The compounds belong to a wide range of chemical classes, namely cocainics, phenylethylamines, piperazines, cathinones, opiates, benzodiazepines, tricyclic antidepressants and dissociatives. For purposes of this thesis, classification of the drugs is based on the system adopted by the UNODC (2013) report. In addition, some drugs are referred to using their more commonly used terms, such as heroin for diacetylmorphine and MDMA or ecstasy for methylenedioxymethamphetamine (Cole, 2003; Baker & Kasprzyk-Hordern, 2013; Nefau, et al., 2013; Ostman, et al., 2014). However, as a note, the common terms normally refers to illegally manufactured drugs which may contain other drugs and additives while the structural name denotes the drug standard as

obtained from licensed suppliers. Further background to the drug classes investigated in this research is provided in Appendix II.

Table 1.4: Drug and internal standard structure, empirical formula and molar mass (g/mol).

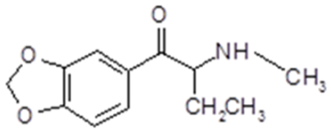
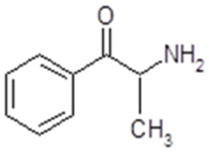
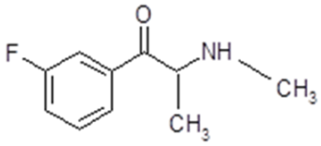
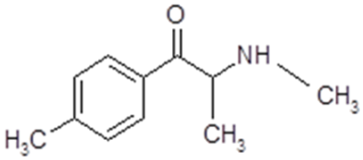
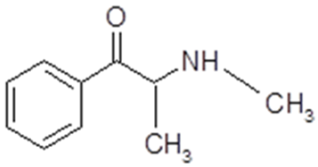
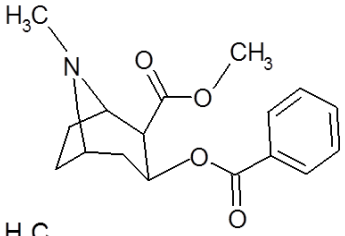
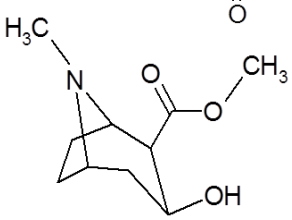
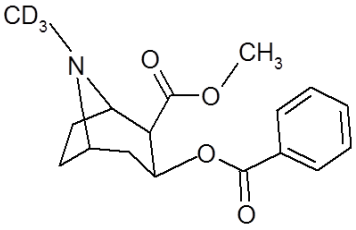
DRUG	STRUCTURE	EMPIRICAL FORMULA	MOLAR MASS (g/mol)
CATHINONES			
Butylone		$C_{12}H_{15}NO_3$	221.3
Cathinone		$C_9H_{11}NO$	149.7
3-Fluoromethcathinone		$C_{10}H_{12}FNO$	181.2
Mephedrone		$C_{11}H_{15}NO$	177.2
Methcathinone		$C_{10}H_{13}NO$	163.2
COCAINICS			
Cocaine		$C_{17}H_{21}NO_4$	303.2
Ecgonine methyl ester		$C_{10}H_{17}NO_3$	199.3
Cocaine-d_3		$C_{17}H_{18}D_3NO_4$	306.2

Table 1.4 *cont'd*: Drug and internal standard structure, empirical formula and molar mass (g/mol).

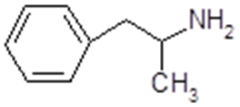
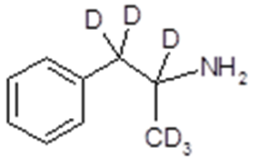
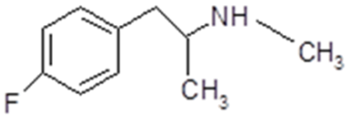
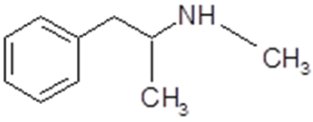
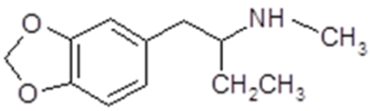
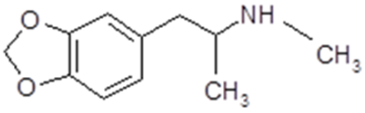
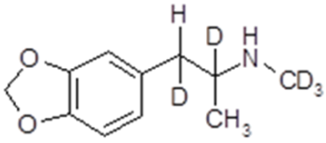
DRUG	STRUCTURE	EMPIRICAL FORMULA	MOLAR MASS (g/mol)
PHENYLETHYLAMINES			
Amphetamine		$C_9H_{13}N$	135.2
Amphetamine-d_6		$C_9H_7D_6N$	141.2
4-Fluorometh-amphetamine		$C_{10}H_{14}NF$,	167.2
Methamphetamine		$C_{10}H_{15}N$	149.2
Methylbenzodioxolyl-butanamine		$C_{12}H_{17}NO_2$,	207.3
Methylenedioxy-methamphetamine (Ecstasy)		$C_{11}H_{15}NO_2$	193.0
Methylenedioxy-methamphetamine-d_5		$C_{11}H_{10}D_5NO_2$	198.0

Table 1.4 *cont'd*: Drug and internal standard structure, empirical formula and molar mass (g/mol).

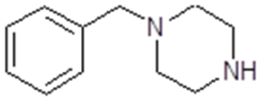
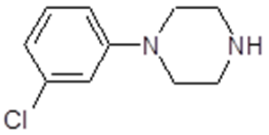
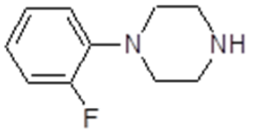
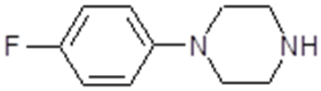
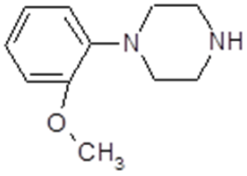
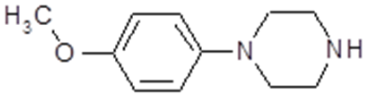
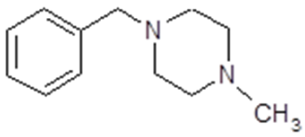
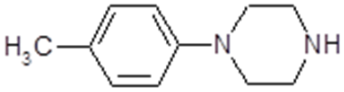
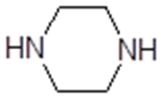
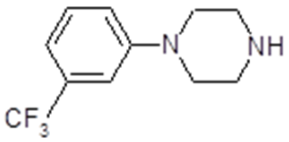
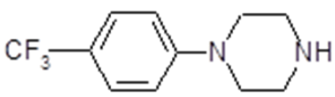
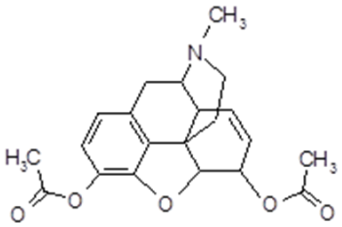
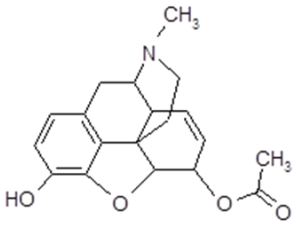
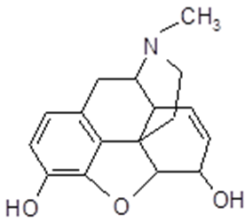
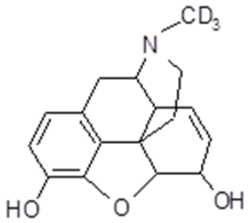
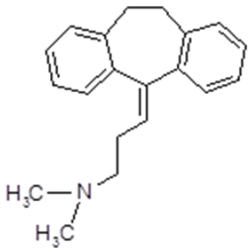
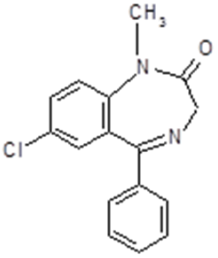
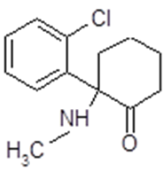
DRUG	STRUCTURE	EMPIRICAL FORMULA	MOLAR MASS (g/mol)
PIPERAZINES			
Benzylpiperazine		$C_{11}H_{16}N_{2,}$	176.3
3-Chlorophenyl-piperazine		$C_{10}H_{13}ClN_2$	196.5
2-Fluorophenylpiperazine		$C_{10}H_{13}FN_2$	180.2
4-Fluorophenylpiperazine		$C_{10}H_{13}FN_2$	180.2
2-Methoxyphenyl-piperazine		$C_{11}H_{16}N_2O$	192.3
4-Methoxyphenyl-piperazine		$C_{11}H_{16}N_2O$	192.3
Methylbenzylpiperazine		$C_{12}H_{18}N_2$	190.3
4-Methylphenyl-piperazine		$C_{11}H_{16}N_2$	176.3
Piperazine		$C_4H_{10}N_2$	86.1
3-Trifluoromethyl-phenylpiperazine		$C_{11}H_{13}F_3N_2$	230.2
4-Trifluoromethyl-phenylpiperazine		$C_{11}H_{13}F_3N_2$	230.2

Table 1.4 *cont'd*: Drug and internal standard structure, empirical formula and molar mass (g/mol).

DRUG	STRUCTURE	EMPIRICAL FORMULA	MOLAR MASS (g/mol)
OPIATES			
Diacetylmorphine (Heroin)		$C_{21}H_{23}NO_5$	369.4
6-Monoacetylmorphine		$C_{19}H_{21}NO_4$	327.4
Morphine		$C_{17}H_{19}NO_3$	285.1
Morphine-d_3		$C_{17}H_{16}D_3NO_3$	288.1
BENZODIAZEPINES, DISSOCIATIVES AND TRICYCLIC ANTIDEPRESSANTS			
Amitriptyline		$C_{20}H_{23}N$	277.4
Diazepam		$C_{16}H_{13}ClN_2O$	284.7
Ketamine		$C_{13}H_{16}ClNO$	237.7

1.4.2.1 New Psychoactive Substances (NPS)

NPS, incorporating 'designer drugs', became prevalent in the late 1980s and are based on various adaptations of the phenylethylamine backbone (Figure 1.1). These include addition of a methylenedioxy ring moiety (e.g. MDMA), addition of fluorine (e.g. 4-fluoromethamphetamine) and addition of a ketone group (e.g. methcathinone) (Shulgin & Shulgin, 1991; Julien, 2005; Santali, et al., 2011; UNODC, 2013).

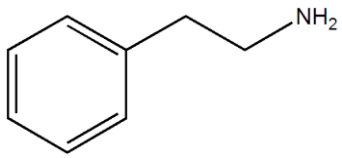


Figure 1.1: Structure of phenylethylamine.

NPS have been marketed as 'legal highs' to mislead the general public into thinking that they are safe and legitimate to consume, and as 'bath salts', 'research chemicals' or 'plant food' to mislead the authorities (Measham, et al., 2010; Corkery, et al., 2012; Khreit, et al., 2013; UNODC, 2013; EMCDDA, 2014a). However, since NPS tend to be based on pharmacological or structural properties of classic illicit drugs such as amphetamine or ecstasy, they are proving to be just as harmful as the legislated drugs on which they are based (Corkery, et al., 2012; UNODC, 2013). Aryl substituted piperazines (e.g. benzylpiperazine), cathinones (e.g. mephedrone) and ketamine have increasingly become key members of this group of NPS (UNODC, 2013; Chen, et al., 2014). The majority of illegal production of NPS (except ketamine) occurs in clandestine laboratories in Europe and Asia and since the slight modification in chemical structure makes the drugs 'unlegislated' on an international basis, they keep one step ahead of the legalisation that more commonly abused drugs are subjected to (Zuba, 2012; UNODC, 2013). Ketamine used illegally is mainly diverted from legitimate sources. While many NPS are now legislated, new drugs with slight chemical modifications (to circumvent the legislation) are being reported weekly, making it difficult for the relevant authorities to take the necessary social intervention or legal measures (Favretto, et al., 2013; EMCDDA, 2014a; UNODC 2013 & 2014).

Table 1.5 lists some NPS (mainly cathinones and piperazines) that have been investigated in published sewage epidemiological studies. Other NPS investigated include synthetic cannabinoids and ATS analogues (Reid, et al., 2014b).

Table 1.5: New psychoactive substances investigated in waste water.

NEW PSYCHOACTIVE SUBSTANCE	COUNTRY	REFERENCE
BZP	Australia, UK	Baker & Kasprzyk-Hordern, 2013 a & b; Lai, et al., 2013b; Chen, et al., 2014
Cathinone, 2-MeOPP	Norway	Reid, et al., 2014 a & b
Ketamine	Belgium, Canada, China Sweden, Taiwan, UK	Lin, et al., 2010 & 2014; Baker & Kasprzyk-Hordern, 2013 a & b; van Nuijs et al., 2013; Yargeau, et al., 2013; Khan, et al., 2014; Östman, et al., 2014
MBDB, MCAT, 3-TFMPP	Australia, UK	Baker & Kasprzyk-Hordern, 2013 a & b; Chen, et al., 2014
MDPV	Australia, Belgium, China, Finland	van Nuijs et al., 2013; Vuori, et al., 2013; Chen, et al., 2014; Kankaanpää, et al., 2014; Khan, et al., 2014
Mephedrone	Australia, Belgium, China, Norway	Lai, et al., 2013b; van Nuijs et al., 2013; Chen, et al., 2014; Khan, et al., 2014; Reid, et al., 2014b
Methylone	Australia, Finland, Hong Kong	Lai, et al., 2013b; Chen, et al., 2014; Kankaanpää, et al., 2014

BZP = Benzylpiperazine; MBDB = Methylbenzodioxolylbutanamine; MCAT = Methcathinone; MDPV = 3,4-Methylenedioxypyrovalerone; 2-MeOPP = 2-Methoxyphenylpiperazine; 3-TFMPP = 3-Trifluoromethylphenylpiperazine

The number of identified NPS in the European Union rose from 166 in 2009 to 251 by the end of 2012 and 348 in 2013 (UNODC 2014). These included synthetic cannabinoids, substituted phenylethylamines and those outside the common chemical groups (EMCDDA, 2014a). According to the CSJ (2013) report, NPS are entering the UK market at a rate of one drug per week and are used more often than classic drugs of abuse. In addition, the misuse of ketamine has doubled since 2006. Therefore, due to their increasing usage, NPS have recently started to be included in sewage epidemiological studies as shown in Table 1.5. However, the NPS listed in Table 1.5 were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and not all studies detected the target NPS in waste water. To the author's knowledge, NPS have not been analysed by gas chromatography–mass spectrometry (GC-MS) for sewage epidemiological purposes.

1.4.3 Linking Drug Metabolism and Sewage Epidemiology

In order to link the amount of target drugs detected in waste water with their consumption, it is important to understand their fate once administered into the human body. Drugs of abuse are mainly administered into the body through oral or intravenous means, or by inhalation (Julien, 2005; Levine, 2006; Drummer, 2011). Once administered, the drug is absorbed and distributed to different sites around the body through the vascular system in order to have an effect (Jjemba, 2008). Some of these sites include organs that help with its elimination from the body such as the kidneys, liver and lungs (Drummer, 2011). Most drugs undergo metabolism in the liver and are ultimately excreted from the body mainly by the kidneys into urine (Drummer & Wong, 2013). Other metabolic processes also result in excretion from the body by other means such as sweat, faeces and exhalation. Some of the excreted fractions are referred to as metabolites (Drummer, 2011). Therefore, the majority of the administered dosage of a drug is eliminated in metabolite form. However, the parent drug can also be excreted at various rates depending on the drug (Jjemba, 2008).

Whilst the main aim of metabolism is to facilitate the excretion of the drug, it can also alter the pharmacological action of the drug thereby resulting in pharmaceutically active or inactive metabolites (Drummer, 2011; Drummer & Wong, 2013). The metabolic process in the body is mainly conducted through two phases referred to as phase I and phase II metabolism (Levine, 2006; Drummer, 2011). During phase I metabolism the drug is chemically modified through processes such as oxidation, hydroxylation, hydrolysis, dealkylation or reduction (Levine, 2006; Drummer & Wong, 2013). Some drugs only become pharmacologically active after phase I metabolism. For example, the tricyclic antidepressant amitriptyline only becomes active once converted to nortriptyline as a result of phase I metabolism (Drummer, 2011).

The metabolites formed in phase I are further modified by conjugation reactions during phase II metabolism. These phase II metabolites are more water-soluble than the phase I metabolites and hence more amenable to elimination from the body. Common conjugation reactions include glucuronidation and sulphation but due to the prevalence of glucose in biological systems, glucuronidation is the most prevalent phase II process (Levine, 2006; Drummer, 2011). One advantage of the glucuronidation process is that it

reduces the pharmacological and biological activity of a drug and hence potentially minimises the harmful effects these metabolites could cause to aquatic systems (Jjemba, 2008). There are a few exceptions to this, such as morphine-6-glucuronide which is more active as an analgesic than morphine (Levine, 2006; Drummer & Wong, 2013). However, it is reported that glucuronide conjugates are deconjugated in waste water back to the parent drug due to the activity of glucuronidase enzymes present in faecal bacteria (Melis, et al., 2011; van Nuijs, et al., 2011a; Senta, et al., 2014). This releases the more pharmacologically active form of the metabolite which poses potential risk factors to the aquatic system and hence the concern regarding these emerging pollutants (section 1.3.4.2).

By the direct measurement of the metabolites or parent drug excreted in urine or faeces through the sampling of waste water from a WWTP, an estimate of the consumption of the parent drug can be made (Daughton, 2011). However, it is preferable to measure the metabolites where possible since measurement of the parent drug alone does not necessarily indicate consumption, and in some cases the parent drug may not survive the metabolic process (e.g. heroin). The parent drug may also be present as a result of illegal dumping or direct discharge from a manufacturing facility (Fick, et al., 2009; Phillips, et al., 2010; Baker, et al., 2014). For instance, cocaine detected in waste water could be from direct disposal through the sewer system, while detection of one of its main metabolites, benzoylecgonine (BZE) or ecgonine methyl ester (EME), would indicate consumption (Zuccato, et al., 2005; van Nuijs, et al., 2009b). As a number of metabolites can be produced by a single drug due to the various mechanisms involved in metabolism, only one or two key metabolites need to be targeted for analysis (Drummer & Wong, 2013). The choice of metabolite selected for analysis depends on the aim of the analysis, its stability in the matrix and during analysis, the type of analytical method and the intended application of the results (forensic, work-place testing, and statistics). For waste water analysis, it makes sense to target the major metabolites which are eliminated at relatively high excretion rates. Since the drugs occur at trace levels in waste water, these major metabolites would have a higher chance of detection compared to metabolites eliminated only at very low excretion rates.

1.4.4 Linking Drug Detection Levels with Consumption

In order to estimate the consumption of the target drug (mg/day) from the concentration (ng/L) detected in waste water, a knowledge of the percentage of the dosage of the target drug excreted as the main metabolite is required. Using cocaine as an example, the following information is required (van Nuijs, et al., 2009a; Castiglioni, et al., 2014):

a) The flow rate of the waste water at the WWTP (L/day). This is used to convert concentrations (ng/L) of one of the main metabolites of cocaine i.e. ecgonine methyl ester (EME) into daily mass loads (mg/day).

b) The relative amount of cocaine excreted as its main metabolite EME. This is required to back-calculate from mass loads into a total amount of cocaine consumed (mg).

c) The relative molar mass of cocaine and EME.

d) The population served by a particular WWTP.

Equation 1.1 is then used to obtain drug consumption data in mg per day;

$$\text{cocaine consumption (mg/day)} = \text{EME (ng/L)} \times \text{flow rate (L/day)} \times 1000 \times 3.4 \qquad \text{Equation 1.1}$$

The conversion factor (CF) is obtained from the ratios of the molar masses of cocaine and EME, taking into account the percentage dose of cocaine excreted as the metabolite i.e. 45 % (Equation 1.2). The choice of the excretion rate used is explained on the following page.

$$CF = \frac{\text{Molar mass of cocaine}}{\text{Molar mass of EME} \times (45/100)} = \frac{303.4}{199.3 \times (45/100)} = 3.4 \qquad \text{Equation 1.2}$$

The calculated amount, mg/day, is then normalized for the number of people served by a particular WWTP (expressed in mg/day per 1000 people). A worked example from the analysis of a real waste water sample is provided in sections 5.31 & 5.3.2 (pages 168-170)

For drugs where metabolite standards are not commercially available, especially cathinones and piperazines, excretion rates of the parent drug can be used if known

(Castiglioni, et al., 2013; Reid, et al., 2014a). In the case of unavailability of excretion rates of the parent drugs or metabolites, quantification of levels in waste water can be made without linking to consumption (Chen, et al., 2014).

Although outside the scope of this research, the area of pharmacogenomics (the study of how genetic make-up affects an individual's response to drugs) is worth mentioning here since it affects the percentage drug excreted as a metabolite between individuals and ethnic groups (Edenberg, 2007; Baik, et al., 2011; Wang, et al., 2011). Therefore, average values of human urinary excretion rates have been used in this thesis and various published literature in sewage epidemiology to calculate drug consumption and do not account for variations between individuals and ethnic groups (Lai, et al., 2013a). Other factors that affect the urinary excretion rate in individuals include the sex, age, co-administration of other drugs, route of administration, amount of dose and pH of urine (Oyler, et al., 2002; van Nuijs, et al., 2011b; Kasprzyk-Hordern, & Baker, 2012; Castiglioni, et al., 2013; Lai, et al., 2013a). In this regard, different percentages of human urinary excretion rates have been reported for the same drug in sewage epidemiological calculations (van Nuijs, et al., 2011a). For instance, the excretion rate for cocaine is reported as 1.45 % by Baker (2013) while Moffat (2011b) reports it as 9 % while that for BZE ranges from 6.5 to 55 % (Castiglioni, et al., 2013). For calculations in this research, urinary excretion rates based on published articles in sewage epidemiology and as reported in Moffat (2011b) were used (Table 5.3, page 169).

In order for the target drugs to be detected, they require to be effectively extracted from the relevant sample matrix and analysed. The following sections cover the analytical process used in this research from sample collection through to instrumental analysis. Extraction techniques based on SPE and LLE, gas chromatography, as well as the chemical derivatization process are also discussed.

1.5 SAMPLE COLLECTION, PRE-TREATMENT AND ANALYSIS

A number of comprehensive review articles have been written on sample collection, preservation, storage, preparation and analysis in relation to sewage epidemiology (Postigo, et al., 2008a; Boles & Wells, 2010; van Nuijs, et al., 2011a; Baker & Kasprzyk-

Hordern, 2011b; Wille, et al., 2012; Pal, et al., 2013). Key aspects relevant to this research are discussed below.

1.5.1 Water Sources and Sampling

The variety of water sources used by researchers in this field include, surface water (e.g. rivers, streams), groundwater and waste water influent and effluent (Jurado, et al., 2012; Pal, et al., 2013; Racamonde, et al., 2013). The choice has been governed by the aim of the research (e.g. comparative studies, detection and/or quantification), the expected concentration of the target analyte, and accessibility to the water source. In this research, untreated waste water was used for quantification as this have been noted to have higher concentrations of PhACs than treated waste water or surface water (section 1.3.4.2) (Chen, et al., 2014; Gilart, et al., 2014).

Sampling methods used in sewage epidemiological studies have been varied and include grab, composite and passive sampling. Although useful information can still be obtained from grab samples, they don't provide time-weighted average (TWA) concentrations over an extended sampling period (Vrana, et al., 2010). Composite samples, on the other hand, provide TWA concentrations since discrete aliquots of samples are collected at specific time intervals over a certain period e.g. 12-99 h and pooled into one sample at the end of the collection period (van Nuijs, et al., 2009c; Boles & Wells, 2010 & 2014). Composite samples are therefore considered a better representation of possible analytes passing through a WWTP over a specified timeframe. Passive sampling is an alternative to composite sampling as it also provides TWA concentrations over a specific and continuous time period ranging from a few hours up to 60 days (Mills, et al., 2007; Yargeau, et al., 2014). Reported passive samplers used for measuring pharmaceutical compounds in waste water include polar organic chemical integrative samplers (POCIS) and Chemcatcher® passive samplers (Bartelt-Hunt, et al., 2009; Greenwood, et al., 2009; Wille, et al., 2012; Yargeau, et al., 2014). Both consist of a sorbent contained between two microporous membranes which allow free-flowing water with dissolved compounds to pass through to the receiving phase where they are trapped. Passive samplers can sample much larger volumes of water (0.05 - 0.35 L/day) and the receiving phase can be tailored for the target analytes thereby increasing the ability to detect compounds present even at utlratrace levels (Mills, et al., 2007).

Sampling has either been conducted using amber glass bottles or plastic containers (Hernandez, et al., 2011; Castiglioni, et al., 2013; Racamonde, et al., 2013). The latter, based on polyethylene terephthalate (PET), polypropylene (PP) and high-density polyethylene (HDPE), were used during this research. Sample volumes quoted in literature range from 500 mL to 2500 mL and volumes extracted ranged from 5 mL to 1500 mL (van Nuijs, et al., 2011a; Racamonde, et al., 2013).

Therefore, the sampling technique chosen depends on the aim of the research, the water source to be sampled, the nature and expected concentration of the target analytes as well as the analytical technique to be used.

1.5.2 Sample Pre-treatment and Storage

Once the sample has been collected, it needs to be transported to the laboratory (if off-site) for pre-treatment and storage. Therefore, preserving the sample during transportation and storage is important in ensuring the reliability of the data obtained. According to published literature, samples were sometimes transported to the laboratory in cooled dark containers and immediately extracted or stored in the dark at 4 - 5 °C and extracted within 7 days to prevent degradation of analytes (Lacina, et al., 2013). Alternatively, samples were stored at -20 °C for extraction at a later date (Postigo, et al., 2008b; Lai, et al., 2013a). In a further step to preserve analytes and minimise decomposition within the matrix due to microbiological activity, some research groups acidified the samples to pH 2-3 at the sampling site or in the laboratory (Vazquez-Roig, et al., 2013) or dosed them with sodium azide (Gerrity, et al., 2011), sodium thiosulphate (Boleda, et al., 2011) or mercury chloride (Senta, et al., 2014) before cold storage. Whatever the sample preservation method used, the aim should be to get the samples as quickly as possible to the laboratory for cold storage or extraction. Cold transport may be necessary to prevent decomposition of the analytes especially if the sampling site is located far from the laboratory or if collection and analysis occur in different countries (Fick, et al., 2009; Baker, et al., 2012; Bayen, et al., 2013).

Before the sample is extracted or stored, it is taken through a filtration step. Sample filtration is normally conducted immediately after arrival at the laboratory and before storage or immediately before extraction, after storage. Different types of filters with

various pore sizes (0.2 - 0.7 µm) have been used in sewage epidemiology. These include nylon membrane, nitrocellulose, and glass microfiber filters (González-Mariño, et al., 2010; Pedrouzo, et al., 2011; Chen, et al., 2014). The aim with filtration is to remove as much of the particulate matter as possible which can clog up the solid phase extraction (SPE) cartridges, gas chromatography (GC) and liquid chromatography (LC) columns as well as injection needles and liners (for GC) if not effectively removed. Throughout this research waste water samples were vacuum filtered through a disposable 1000 mL capacity polystyrene stericup funnel and receiver system with a 0.22 µm GP Millipore Express® Plus membrane (Millipore, UK). As far as the author is aware, this type of filter has not been used before in waste water analysis but it is very convenient and effective at filtering sample volumes ranging from 150 to 1000 mL. Due to the high surface area of the filter (40 cm^2), it did not easily clog up during filtration and the SPE cartridges on which the filtrate was loaded also did not clog up. Tap and deionized water samples were not filtered before extraction due to the high clarity of the samples and a lack of visible suspended solids.

Once the sample is filtered, it is taken through an extraction process with the aim of separating the target analytes from the sample matrix. Commonly used extraction methods are discussed in the following section.

1.5.3 Sample Extraction

Prior to instrumental analysis, target compounds require extraction from the relevant matrix. Commonly applied extraction techniques for emerging contaminants in aqueous environmental samples mainly utilize SPE and to a much less extent LLE (Pedrouzo, et al., 2011; Ademollo, et al., 2012; Rabii, et al., 2014). In particular, automated on-line SPE and solid-phase microextraction (SPME) are increasing in popularity due to their time-saving advantages (Mills & Walker, 2000; Racamonde, et al., 2013; Östman, et al., 2014). LLE is more commonly used in clinical and toxicological settings (Liu, et al., 2010; Couchman & Morgan, 2011; Peters, 2011) and has rarely been used in the extraction of PPCPs from waste water (Mol, et al., 2000; Jimenez, 2013; Loos, et al., 2013; Robles-Molina, et al., 2014). Since LLE is a solvent-based extraction process, any improvements in the recovery of analytes from a sample matrix mainly rely on the solvent(s) selected (Couchman & Morgan, 2011). On the other hand, SPE relies on the use of extraction sorbents with

varying properties and selectivities for analytes and hence optimisation of the extraction process not only relies on the solvents used but also on the sorbents selected (section 3.4.2).

Alternatives to SPE & LLE that are increasingly being used include SPME & liquid-phase microextraction (LPME) (Farre, et al., 2012; Spietelun, et al., 2013). SPME & LPME are techniques whereby sample extraction and concentration are simultaneously conducted (Willie, et al., 2012). When coupled with GC-MS or LC-MS, SPME & LPME techniques are quick, cost effective, result in low LODs and LOQs and adhere to the priniciples of 'green chemistry' (section 1.5.3.2) by requiring low to no volumes of solvents (Racamonde, et al., 2013; Spietelun, et al., 2013 & 2014). Sample volumes used range from 0.5 mL to 100 mL and on-line derivatization methods can be incorporated into the method to further cut down on sample preparation time (Mills & Walker, 2000; Hyotylainen, 2009; Racamonde, et al., 2013). Other advantages of SPME and LPME include reduction in matrix effects compared with SPE and LLE.

In this research, LLE was used in comparative studies with SPE during the preliminary method development stages but subsequent extraction was based on SPE. Therefore, more emphasis is given to the SPE process.

1.5.3.1 Solid Phase Extraction (SPE)

SPE is a sample preparation technique used to separate and concentrate target analytes from various sample matrices. The desired analytes are systematically retained onto a solid-phase sorbent and subsequently extracted with a suitable solvent (Thurman & Mills, 1998). This enables analytes to be isolated from one solution (the sample matrix) and re-dissolved in a different solvent (elution solvent). Most sample matrices comprise a mixture of target analytes and undesired and possibly interfering compounds. Therefore, the more the target analytes are selectively separated from the other matrix components, the cleaner the extract will be with a reduction in interfering compounds (Couchman & Morgan, 2011). The entire SPE process is typically achieved with the use of small volumes of suitable solvent during the conditioning, rinsing and elution steps. Typical sample volumes extracted during waste water analysis of drugs of abuse range from 50 mL to over 1500 mL (van Nuijs, et al., 2011a; Vazquez-Roig, et al., 2013). It stands to reason

that the larger the initial sample volume used, the higher the chances of detecting and quantifying compounds which normally occur in trace amounts (ng/L or pg/L) (Racamonde, et al., 2013; Wilson, et al., 2014). However, as SPE is not exclusively selective, this may also concentrate suspended solids and interferents within the matrix which could result in clogging of the column and signal suppression or enhancement (Hyotylainen, 2009; Baker & Kasprzyk-Hordern, 2011a; Gilart, et al., 2014). The target analytes are then eluted into 4 - 10 mL of a suitable solvent and concentrated further for analysis (Thurman & Mills, 1998).

Sorbents used in SPE processes occur in many different formats such as disks, cartridges and syringe barrels (Thurman & Mills, 1998; Poole, 2003). The sorbent format used for a particular extraction mainly depends on the nature of the sample matrix, the volume of sample, the expected concentration of the target analytes, and the analytical technique used to separate and identify the components (Hennion, 1999; Poole, 2003; Chromacademy). Syringe barrels were used in this research. The majority of single use syringe barrels are made from polypropylene and are packed with sorbent material with different chemical properties. The main sorbent packing materials are either silica-based or polymer-based and have reversed phase, normal phase, ion exchange and mixed-mode (Thurman & Mills, 1998). Polymeric Oasis® mixed-mode reverse phase and ion exchange sorbents supplied by Waters, UK, were used in this research and are described in the following section.

1.5.3.1.1 Oasis® Mixed Mode Sorbents

Oasis MCX and HLB sorbents have been the most widely reported in sewage epidemiology for multianalyte extraction of acidic, basic and neutral pharmaceutical compounds (Boles & Wells, 2010; Baker & Kasprzyk-Hordern, 2011b; van Nuijs, et al., 2011a; Wille, et al., 2012; Burgard, et al., 2013; Gilart, et al., 2014; Lopes, et al., 2014). Depending on the extraction protocol and pharmaceuticals under analysis, on comparison with other sorbent types, either the Oasis MCX or HLB sorbent were found to be more suitable (Gheorghe, et al., 2008; Gracia-Lor, et al., 2010; Vazquez-Roig, et al., 2013). MCX has strong cationic exchange properties suitable for bases while HLB is a universal reverse phase sorbent that can retain polar acids and bases and neutral analytes.

The Oasis® SPE sorbents used in this research are depicted in Figure 1.2. The backbone of all Oasis products is based on the reverse phase HLB (hydrophilic-lipophilic balanced).

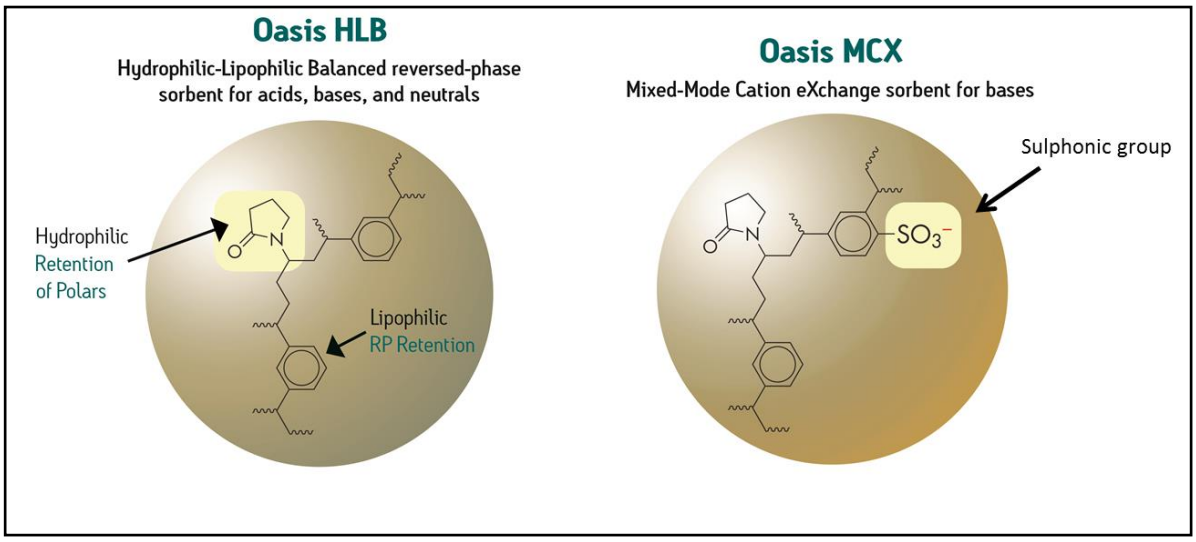


Figure 1.2: Chemical properties of Oasis HLB and MCX SPE sorbents used during analysis (used with permission from Waters, UK).

Although it can be used on its own for its reverse phase properties, the HLB sorbent also forms the backbone of all the other Oasis mixed mode sorbents. It comprises a lipophilic divinylbenzene and hydrophilic *N*-vinylpyrrolidone copolymer and was first introduced in 1996 as a polymeric sorbent that could be wetted, yet still retain its hydrophobic reversed-phase properties. The HLB sorbent is suitable for the recovery of acidic, basic and neutral compounds. The Oasis MCX (mixed-mode strong cation exchange) is as a result of the sulphonation of the Oasis HLB sorbent and is most suitable for basic compounds with a pK_a range of 2-10 (Waters, 2006b). Due to the presence of the divinylbenzene and hydrophilic *N*-vinylpyrrolidone copolymer, it can also retain acidic and neutral drugs.

As an alternative to silica-based products, the divinylbenzene and hydrophilic *N*-vinylpyrrolidone copolymer offers advantages such as water wettability, polar retention, stability across pH 1-14, absence of unreacted silanol interactions with analytes which can lead to irreversible bonding (Sigma-Aldrich, 1998), and does not affect recoveries even if it dries out (Waters, 2006a). The last point is important especially with manual simultaneous extractions which could lead to some sorbents without sample/solvent for short periods of time. In addition, Oasis mixed-mode SPE sorbents do not require the

conditioning step and could thereby save time on the extraction process. These advantages have led to numerous references in literature, especially with regard to analysis of various types of water samples for PhACs, including illicit drugs (Kostopoulous & Nikolaou, 2008; van Nuijis, et al., 2009; Gerrity, et al., 2011).

Retention of analytes by mixed-mode SPE is by a number of interactions including electrostatic interactions, hydrogen bonding, dipole-dipole, van der Waals forces and ion exchange. These complement each other with regards to retention of analytes with varying polarities and chemistries, leading to more effective sample clean-up (Telepchak, et al., 2004; Waters, 2006a). The decision to use the Oasis SPE products for this research was mainly due to two factors. Firstly, the majority of studies relevant to the research of this thesis have utilized Oasis cartridges for the extraction of pharmaceutical compounds from various water samples (Pal, et al., 2013; Gilart, et al., 2014). Secondly, the ability of a single type of mixed-mode SPE sorbent to retain acids, bases and neutral compounds without the need for using different sorbent types for each group of analytes was regarded as an advantage for the multianalyte method under development (Waters, 2006 a&b). The majority of the target analytes during this research were basic with only morphine being amphoteric. Therefore SPE sorbents used for preliminary studies were MCX and HLB. All were of 3 mL capacity with 30 µm particle size packed to a mass of 60 mg.

SPE protocols for extracting various types of compounds (acidic, basic, amphoteric and neutral), including recommendations for selecting the most suitable sorbent material and solvents depending on the nature of the target analytes, are readily available in the literature or from the supplier (Telepchak, et al., 2004; Levine, 2006; Waters, 2006a&b). Therefore once the target analytes have been decided on it may be necessary to compare various types of syringe barrels containing packing material with different chemical properties to determine the most suitable protocol for the analytical method under development (sections 3.4.2.1). All preliminary SPE investigations and the final protocol were applied through an SPE vacuum manifold using a manual set-up.

Whilst SPE is an effective technique for sample extraction, concentration and clean-up, an alternative (or complimentary) technique is the more traditional LLE which is discussed

further in section 1.5.3.2. Prior to the development of SPE, LLE was more commonly used in extracting analytes from one liquid phase into another (Hennion, 1999). While there has been an increase in the use of SPE since its development, LLE is still used in many laboratories (Levine, 2006; Hendriks, et al., 2007; Raikos, et al., 2009; Namera, et al., 2011; Jiménez, et al., 2013; Farajzadeh, et al., 2014).

1.5.3.2 Liquid-Liquid Extraction (LLE)

In LLE, target analytes are isolated from complex matrices (e.g. urine, blood, waste water) through their ability to partition between two immiscible solvents. The polarity of the solvents used in respect to the polarity of the target analytes, the pH of the sample with respect to the pK_a of the target analytes and the volume of extraction solvent and number of repeat extractions all play a role in improving the recovery of the target analytes from the matrix (Levine, 2006). The effectiveness of a given solvent in extracting a particular analyte is chiefly determined by how miscible with water the solvent is and its polarity, i.e. dielectric constant, as well as its hydrogen bonding ability (Flanagan, et al., 2007).

Almost all common drug substances have some degree of polarity and hence will be extracted into a polar solvent (Levine, 2006). Some common solvents used in SPE and LLE and their properties are listed in Table 1.6.

Table 1.6: Common solvents and their extraction-related properties.

Solvent	Boiling point[a] °C	Density (g/cm^3)[a] at 20°C	Dielectric constant, ε[b] at (15-30°C)	Hydrogen Bonds[b]	
				H^+ Donor	H^+ Acceptor
Acetone	56.1	0.78	21.0	No	Yes
Acetonitrile	81.7	0.79	36.6	No	Yes
Chloroform	61.2	1.48[c]	4.8	Yes	No
Ethanol	78.3	0.79	25.3	Yes	Yes
Ethyl acetate	77.1	0.90	6.1	No	Yes
n-Hexane	68.7	0.66[c]	1.9	No	No
Methanol	64.6	0.79	33.0	Yes	Yes
2-Propanol	82.3	0.78[c]	20.2	Yes	Yes
Water	100	0.99[c]	80.1	Yes	Yes

[a]Lide & Haynes, 2009; [b]Levine, 2006; [c]25°C

The polarity of a solvent is expressed as its dielectric constant. The higher the dielectric constant, the higher the polarity and vice versa. In most instances a mixture of two miscible solvents are used to optimise the extraction ability of analytes from aqueous solutions. The solvents complement each other with regards to hydrogen bonding ability (Stimpfl, 2011). The ability of an analyte to form hydrogen bonds with a given solvent affects it solubility and subsequent extraction into the solvent (Levine, 2006). Other factors to consider for the solvent are low toxicity, volatility and flammability and it should be able to extract the analytes from the matrix without the interferents as well as not react with the analytes (Couchman & Morgan, 2011). Examples of solvents used in the extraction of PPCPs from various water samples include acetone, ethyl acetate, n-hexane and toluene (Mols, et al., 2000; Jimenez, 2013; Loos, et al., 2013; Robles-Molina, et al., 2014). It is worth mentioning the concept of 'green chemistry' which has, as one of its aims, the reduction of the use of toxic and/or hazardous solvents or their substitution with more health and environmentally friendly alternatives as well as eliminating the toxic waste generated (Sheldon, 2005).

Examples of solvents that have become undesirable for use due to health and environmental concerns include chloroform, carbon tetrachloride and pentane. These can be substituted with more environmentally friendly solvents such as dichloromethane, ethyl acetate, and heptane, respectively (Doble & Kruthiventi, 2007; Alfonsi, et al., 2008). After selection of a solvent, the pH of the aqueous sample matrix requires adjustment in order to convert the target drugs to unionized forms, which enable them to be readily extracted into the polar solvent. This is at least 2 pH units above the pK_a for basic drugs and 2 pH units below the pK_a for acidic drugs (Appendix III). Ideally, it is more effective to extract acidic, basic and neutral drugs in separate fractions but this adds multiple steps to the extraction process thereby prolonging the method. Multianalyte extraction protocols therefore set out to find optimum conditions for the simultaneous extraction of acidic, basic and neutral drugs in as few steps as possible. This presents a challenge due to the analytes containing different physicochemical properties (e.g. pK_a and polarity) (Levine, 2006; Kasprzyk-Hordern, et al., 2007). As mentioned in section 1.5.3.1.1, the majority of drugs investigated in this research were basic with pK_a values ranging from 7.5 to 9.9, with diazepam having a pK_a of 3.3. As with SPE, LLE is also not exclusively selective for the

target analytes and hence can also lead to co-extraction of other unwanted matrix components (Gilart, et al., 2014).

1.5.3.3 Comparison between SPE and LLE

Some of the key factors which influence the choice on whether to use SPE or LLE include the chemical properties of the analytes, the complexity of the sample matrix and subsequent analytical technique to be used. Each sample extraction technique has advantages and disadvantages which need to be considered based on the aim of the analysis. These are listed in Table 1.7 below.

Both SPE and LLE, although helping to clean up the sample and concentrate the analytes, add extra time to the analytical process and may lead to loss of some of the analytes as recoveries are rarely 100 % for all compounds (Frenich, et al., 2009). However, sample clean-up also protects instrumental components from excessive clogging or getting dirty (columns, ionisation source, injection liners for GC-MS, injection needles) thereby reducing maintenance costs. Both SPE & LLE are also applicable for a wide range of compounds. Overall, SPE has a higher enrichment factor thereby leading to increased selectivity and sensitivity (Farajzadeh, et al., 2014; Wilson, et al., 2014). This is even more important with matrices where analytes are present in trace amounts such as waste water and surface water. SPE has, therefore, increasingly found widespread use in the preparation of environmental samples for emerging organic contaminants (van Nuijs, et al., 2010; Pal, et al., 2013; Gilart, et al., 2014; Ribeiro, et al., 2014b; Wilson, et al., 2014).

Table 1.7: Advantages and drawbacks of SPE and LLE.

Extraction Method	Advantages	Drawbacks
SPE	• Relatively high and reproducible absolute recoveries • High sample throughput and less labour intensive • Ease of use and automation – also compatible with on-line extraction and derivatization • Use of low volumes of organic solvents • Higher enrichment factor and more effective sample clean up than LLE	• Expensive to set-up and run in the long-term • Method development has many steps to optimise • Long sample preparation times especially for multiple analysis using manual set-up • Inconsistent control of flow-rate for manual set-ups • Uncertainty with batch-to-batch reproducibility of sorbents
LLE	• Relatively easy and cost-effective to set up • Simple extraction process • Affordable even for laboratories with limited budgets	• Automated or on-line set ups are not easily done • Not easy to do multiple simultaneous extractions • Emulsion formation • Higher potential for loss of the analytes during extraction • High, reproducible recoveries are more difficult to attain • Environmentally unfriendly - use of large volumes of toxic organic solvents • Expensive to dispose of solvents according to environmental regulations. • Longer evaporation time to remove excess solvent

Thurman & Mills, 1998; Telepchak et al., 2004; Levine, 2006; Alfonsi, et al., 2008; Flanagan, et al., 2007; Couchman & Morgan, 2011; Farajzadeh, et al., 2014; Furey, et al., 2013

How effectively a drug is extracted from its matrix depends on a number of factors, including its chemical structure and pK_a. Most drugs will ionise in solution based on their pK_a and the pH of the solution in which they are dissolved (Levine, 2006; Stimpfl, 2011). Therefore, the pH of the sample matrix relative to the pK_a of the analytes plays an important role in the effective reproducible and quantitative isolation of analytes from the sample matrix. However this poses a challenge when developing a single multianalyte method containing analytes with varying pK_a values (sections 3.4.1 and 3.4.2). Table 1.8 lists the known pK_a values of the analytes investigated in this research.

Table 1.8: pK_a values of target drugs.

DRUG	pK_a	DRUG	pK_a
AMP	9.9	**6-MAM**	9.6[a]
AMIT	9.4	**MAMP**	9.9
BUTY	-	**MBDB**	10.5[b]
BZP	9.6	**MBZP**	-
CAT	-	**MCAT**	7.1[b]
COC	8.7	**MDMA**	8.8
3-CPP	8.6	**MEPH**	8.6[c]
DIAZ	3.3	**2-MEOPP**	-
EME	-	**4-MEOPP**	9.0
4-FMA	-	**METH**	8.9
3-FMC	-	**MOR**	8.0 & 9.0
2-FPP	-	**4-MPP**	-
4-FPP	-	**PIP**	9.7[d]
HEROIN	7.6	**3-TFMPP**	8.7
KET	7.5	**4-TFMPP**	-

[a]Boleda, et al., 2011; [b]Baker & Kasprzyk-Hordern, 2013; [c]Santali, et al., 2011; [d]Khalili, 2009

Most had one pK_a value ranging from 7.1 (methcathinone) to 9.9 (amphetamine and methamphetamine). The exceptions were diazepam (pK_a 3.3) and morphine which is amphoteric (pK_a 8.0 and 9.0). Amphoteric compounds have the ability to act as either an acid or a base and hence will have different recoveries at various pH values (Levine, 2006; Stimpfl, 2011). At the time of writing of this thesis, pK_a values of a number of cathinones and piperazines were not readily available. However, based on the pK_a of methcathinone

and benzylpiperazine (9.6), their pK_a values can be estimated to be in the range 7 - 10. Unless otherwise stated, pK_a values are from Moffat (2011b).

Therefore by taking advantage of the physico-chemical properties of the analytes (e.g. pK_a) a suitable sample preparation procedure can be developed. For SPE and LLE, this has a number of benefits such as, adding selectivity to the process, optimising recovery from the sample matrix and ensuring reproducible sample consistency (Couchman & Morgan, 2011).

A further aspect of sample preparation, especially when GC-MS is used, is derivatization. This is discussed in the next section.

1.6 CHEMICAL DERIVATIZATION

The main aims of chemical derivatization are to reduce the polarity of a compound as well as increase its volatility and thermal stability (Braithwaite & Smith, 1999; Telepchak, et al., 2004). The latter is essential due to the often high temperatures (e.g. 300 °C) used during GC-MS analysis. The overall outcome of derivatization should be the reduction in peak tailing and improved peak shape, detectability and selectivity (Telepchak, et al., 2004). Other advantages of derivatization include: changing the retention times which can lead to improved resolution; generating more abundant ions with a higher atomic mass and a more distinct mass spectrum resulting in less interference from other compounds in the matrix (Halket, 1993; Levine, 2006).

1.6.1 To Derivatize or Not?

Not all compounds require derivatization in order to be detected and reliably quantified and the decision of whether to derivatize or not needs to be established during the initial stages of method development. The nature and chemical properties of the target analytes, the constitution of the sample matrix and the presence of interfering substances that could affect the analytical results as well as the methodological approach need to be taken into consideration when deciding whether or not to include a derivatization step (Blau & Halket, 1993). Analysis by GC-MS almost always includes a derivatization step, especially with polar compounds, while this is not always necessary for high performance liquid chromatography (HPLC) or LC-MS. However, as derivatization leads to increased

sensitivity and improvement in bioanalytical quantitation, it has also been used for some studies involving LC-MS (Holcapek, et al., 2012).

Most pharmaceutical compounds are organic in nature and hence will have some degree of polarity due to their functional groups such as alcohols, phenols, carboxylic acids, amines and amides (Blau & Halket, 1993). Therefore, a knowledge of the chemical structure of a molecule allows for an informed decision on whether a compound can and should be derivatized as well as which derivatizing reagent would be most suitable (Flanagan, et al., 2007). Figure 1.3 depicts the chemical structures of the target analytes and their varied functional groups. The functional groups vary even within drugs of the same class and give rise to varying: acid dissociation constant (K_a) values, stabilities in different solvents and the sample matrix, susceptibilities to derivatization, extractabilities from a sample matrix, interactions with the stationary phase in the GC column and mass spectral fragmentation patterns. All these factors present a challenge when developing one analytical method that will simultaneously extract and quantify the target drugs. In most instances the most suitable method in terms of recovery, precision and limit of detection (LOD) may still favour certain classes of drugs over others. These aspects are discussed in more detail in Chapters 3 and 4.

The functional groups relevant for derivatization are the amine (-NH and -NH_2) and hydroxyl (-OH) groups as depicted in Figure 1.3. Derivatization replaces the labile hydrogen atom with another group that does not contain free hydrogen atoms thereby reducing the tendency of the molecule to bond with the stationary phase and improving the chromatographic properties (Knapp, 1979). Since GC-MS was the instrumental method to be used in this research, a decision was made during preliminary studies to include derivatization in the method development, as this improved the chromatographic and mass spectral properties of the target drugs (section 3.2.1). This is also corroborated by literature references in clinical and toxicological analyses where similar drugs were derivatized in order to achieve better chromatographic properties when using GC-MS (Segura, et al., 1998; Saito, et al., 2007).

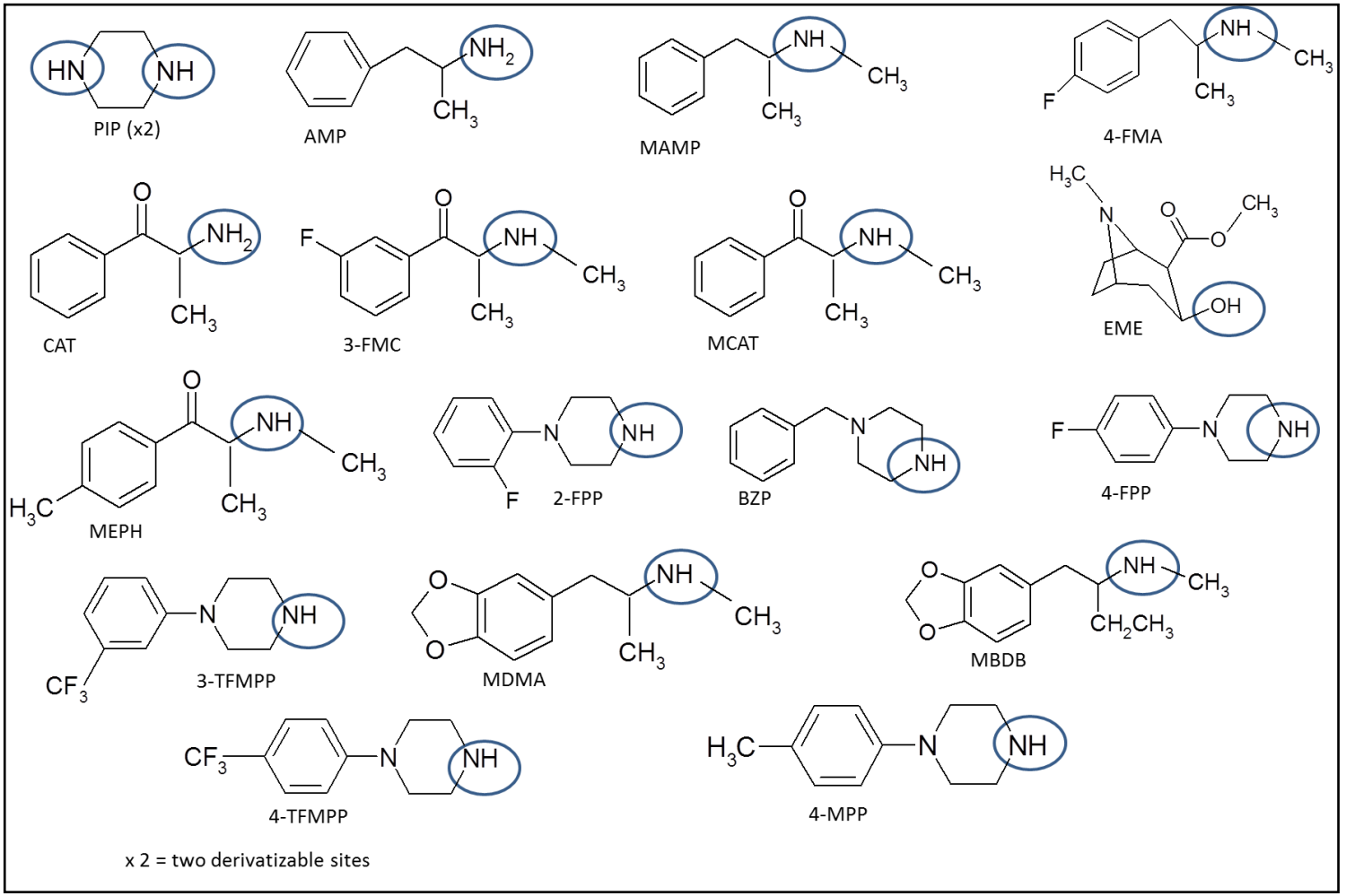


Figure 1.3: Chemical structures of the target analytes and functional groups relevant for derivatization (circled).

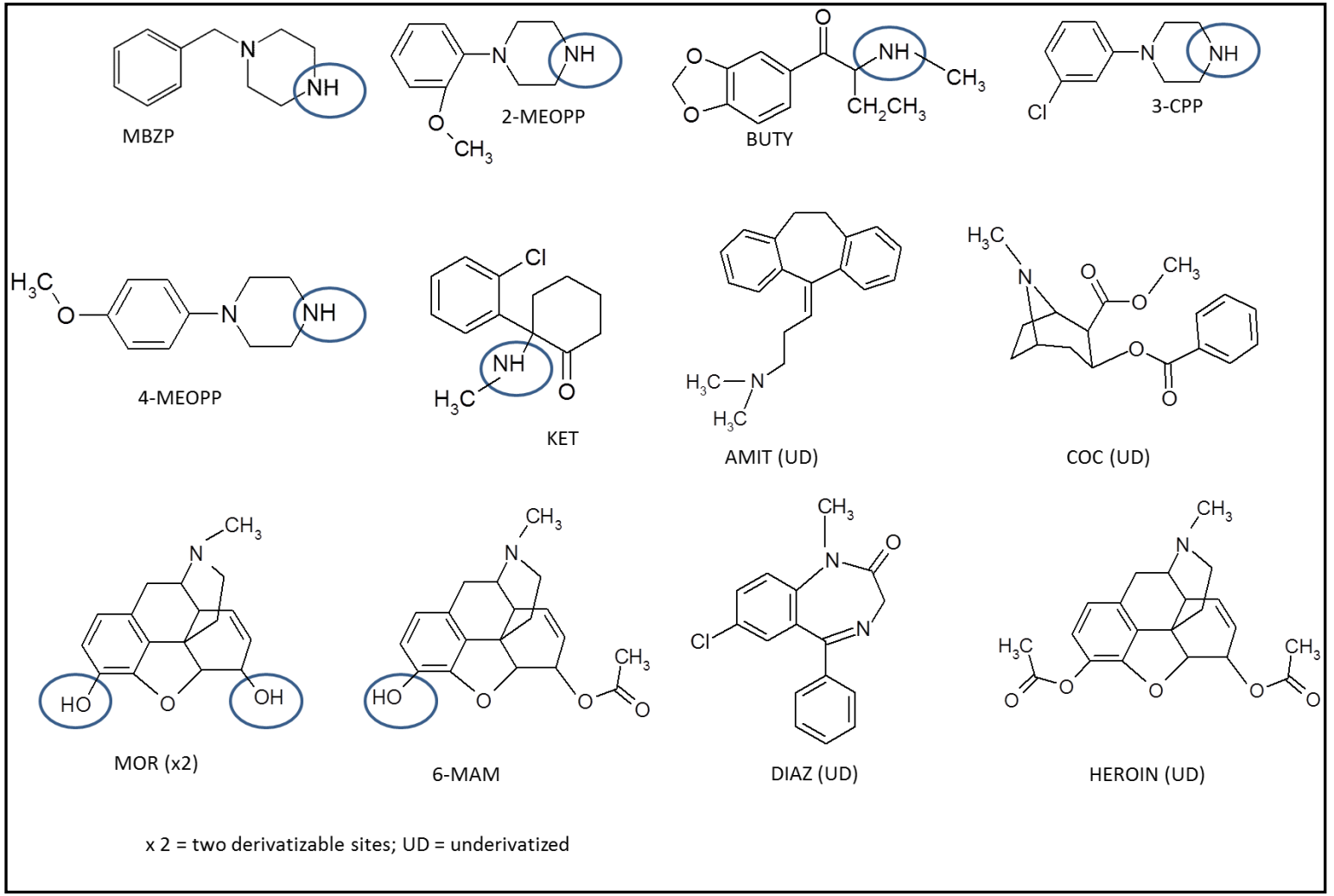


Figure 1.3 *cont'd:* Chemical structures of the target analytes and functional groups relevant for derivatization (circled).

In this research, target drugs without a labile hydrogen, and hence were unaffected by the derivatization process, were amitriptyline, cocaine, diazepam and heroin. All other drugs had at least one derivatizable site, with only morphine and piperazine having two (Fig. 1.3).

Two main categories of derivatizing reagents used in this research, silylation and acylation, are described in the next section. These were selected based on their reported use in derivatizing various types of compounds, including drugs of abuse (Halket, 1993; Telepchak, et al., 2004; Farajzadeh, et al., 2014).

1.6.2 Silylation

During silylation, the labile hydrogen is substituted with an alkylsilyl group (Braithwaite & Smith, 1999). Popular silylation reagents include *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), which replace the labile hydrogen with a trimethylsilyl (TMS) group i.e. $(CH_3)_3Si$. This is depicted in Figure 1.4, using benzylpiperazine as an example.

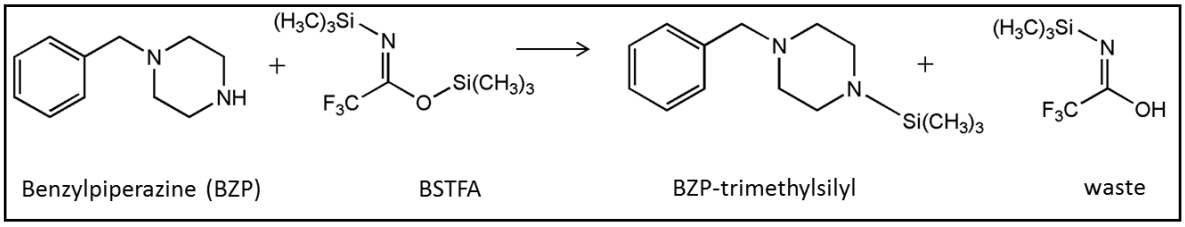


Figure 1.4: Silylation reaction for benzylpiperazine.

According to Telepchak (2004), almost 85 % of derivatization reactions using GC use some form of silylating agent due to its compatibility with most functional groups and is widely accepted to be quite versatile. This is supported by Evershed (1993) and Farajzadeh (2014) who report silylation for enhancing chromatographic and mass spectrometric performance of compounds. Since most silylating agents are moisture sensitive, catalysts such as trimethylchlorosilane (TMCS) are normally added to the silylating agent to not only enhance the substitution reaction (especially for sterically hindered reaction sites) but to also improve its hydrolytic stability (Telepchak, et al., 2004). Therefore for effective and reproducible derivatization the sample should be completely moisture-free. Any water in the sample can cause the decomposition of both the reagent and

derivatives. Hence, it is also considered good practice to use an excess of derivatization reagent in order to react with any possible water in the sample. The by-products formed are inert and highly volatile and not likely to interfere with the analyte peaks (Thermo Fisher Scientific, 2008).

An advantage of silylation over other derivatizing reactions is that it requires no further evaporation and reconstitution steps as it can be directly injected onto the GC column. Both BSTFA and MSTFA were investigated in this research (Table 2.5).

1.6.3 Acylation

During acylation, the labile hydrogen is replaced with an acyl group to give an ester or amide (Segura, et al., 1998; Telepchak, et al., 2004). Acylating reagents fall under three main categories: acid anhydrides, acid halides and reactive acyl derivatives such as acylated imidazoles (*ibid*). The acid anhydrides, trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) were used in this research (Table 2.5). An example of the acylation reaction with PFPA is represented in Figure 1.5 using 4-fluoromethamphetamine.

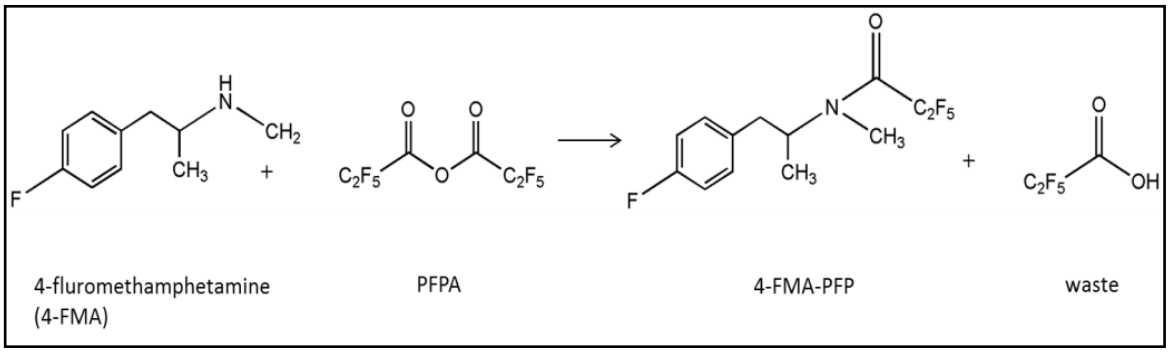


Figure 1.5: Acylation reaction for 4-fluoromethamphetamine.

Acylation is also used on a wide scale for derivatization and is often a worthy alternative to silylation for functional groups such as amines, hydroxyls and phenols (Blau, 1993; Braithwaite & Smith, 1999; Farajzadeh, et al., 2014). The resulting derivatives tend to have even higher and more stable atomic masses for GC-MS than TMS derivatives and are also sensitive to other GC detectors such as an electron capture detector (ECD) (Braithwaite & Smith, 1999; Telepchak, et al., 2004). TFAA, PFPA and HFBA are similar in chemical behaviour and hence any can be effectively used for acylation. The reaction

time and temperature, the retention time (RT), as well as final atomic mass of the derivative may need to be considered when making the final selection (Blau, 1993).

Acylating reagents are highly reactive and can give rise to negative effects during chromatography such as: altering the chemical composition of the stationary phase, corrosion of parts of the GC-MS system and appearance of additional peaks due to re-derivatization of compounds stuck at the head of the column. Therefore, in contrast to silylating reagents, the excess acylating agent and acid by-products need to be removed before injection thereby necessitating an additional step where the reaction mixture is evaporated to dryness and reconstituted in a suitable solvent (Segura, et al., 1998).

Ultimately, the choice of derivatizing reagent would be based on the most suitable for the mixed target drugs under analysis, especially for the NPS, which were the focus of this research, with the knowledge that compromises are inevitable due to the varied chemical properties of the analytes.

In order for the derivatization process to be beneficial to the method under development, it should be simple, relatively quick to conduct and should result in a single, stable derivative. Most derivatizing agents require heat to be effective and to reduce the reaction time (Evershed, 1993; Telepchak, et al., 2004). Therefore parameters such as reaction time and temperature as well as the volume of derivatizing reagent used need to be optimised for the target analytes to ensure reproducible and reliable results (section 3.2.2). Optimisation also ensures that the derivatization reaction has gone to completion as well as determining whether the derivatives are stable (Blau, 1993; Braithwaite & Smith, 1999).

1.6.4 Evaluating the Derivatization Reaction

Determining when a derivatization reaction is complete (i.e. maximum conversion) is just as important as selecting the right derivatizing reagent. It is essential for reliable, quantitative analysis. The ultimate goal with optimising the derivatization process is to detect the most important factor(s) that affects the completeness of the derivatization process for specific drugs. However, only a few approaches in determining the optimal conditions for the derivatization process have been reported. The Box-Behnken approach has been utilised by Gonzalez-Marino (2010) and Racamonde (2013). This multivariate

approach is based on mathematical modelling that assesses a factor and a response to that factor, with the response being the dependant variable. A set of experiments are used to assess the effect of a particular factor on the results e.g. derivatizing agent volume and reaction temperature. Other researchers assumed derivatization was complete based on the presence of one derivative peak with the right mass spectral profile for a particular analyte (Segura, et al., 1998). A different approach also reported in published literature has been to measure the increase in drug peak area (Lacina, et al., 2013) or the relative response factor (Migowska, et al., 2012; Kumirska, et al., 2013) against the reaction temperature and time. The presence of one derivative peak and the most intense instrumental response indicates maximum conversion, and hence the optimal derivatization condition for that drug (Migowska, et al., 2012; Kumirska, et al., 2013; Lacina, et al., 2013).

After derivatization, the prepared samples are then analysed for qualitative and quantitative purposes. In the following section, instrumental techniques, relevant to this research are discussed.

1.7 INSTRUMENTAL ANALYSIS

The interest in the detection of emerging contaminants (including illicit drugs) in environmental samples has coincided with more recent advancements in analytical techniques capable of detecting target analytes at very low concentrations (ng/L) in complex matrices (Fatta, et al., 2007; Willie, et al., 2012). These analytical techniques mainly comprise LC-MS and GC-MS (van Nuijs, et al., 2011a; Pal, et al., 2013). To a lesser extent, HPLC with a diode array or fluorescence detector (Fatta, et al., 2007; Patrolecco, et al., 2013), capillary electrophoresis (Bishop, et al., 2005), GC with electron capture detector (GC-ECD) (Migowska, et al., 2012) have also been used. These same techniques have historically been used to detect illicit drugs in various biological matrices such as hair, urine, sweat, saliva and blood (Rivier, 2003; Boleda, et al., 2007; Bones, et al., 2007; Liu, et al., 2010; Peters, 2011). However, illicit drugs normally occur at much lower concentrations in environmental samples (ng/L) than in biological matrices (µg/mL) thereby requiring instrumental techniques capable of trace-level detection.

1.7.1 Advances in Instrumental Techniques

Over the past decade, improvements in instrumental designs have been made to enable trace-level detection of emerging contaminants in various complex matrices. Reviews by various authors have covered the recent trends in analytical techniques relating to the trace-analysis of emerging contaminants. Hyphenated systems, and in particular those coupled to tandem mass spectrometry (MS/MS), have maintained their popularity and are increasingly being used (Farre, et al., 2012; Pal, et al., 2013). This includes systems capable of achieving high resolution of compounds within a short analysis period such as ultra-high pressure liquid chromatography (UHPLC) (Pedrouzo, et al., 2011). The improvements in analytical separation techniques have coincided with improvements in highly sensitive mass spectrometric detectors capable of achieving very low limits of detection (LOD) and limits of quantification (LOQ) and increasing the range of compounds that can be detected by these methods (Wu & French, 2013).

These include the triple quadrupole (Zuba, 2012), ion trap (Hogenboom, et al., 2009), time of flight (Lacina, et al., 2013) and orbitrap (Wille, et al., 2012) or various hybrid combinations of these. In addition, multidimensional as well as two-dimensional LC and GC techniques are also growing in popularity (Farre, et al., 2012; Lacina, et al., 2013). In multidimensional techniques two columns are used in tandem with the first column used for sample pre-concentration and the second column for analytical separation. In two dimensional techniques the entire sample is separated on two different columns (Farre, et al., 2012). Improvements in instrumentation have also enabled the move towards multianalyte (also referred to as multi-residue and multi-class) methods capable of simultaneously analysing a number of drugs with similar or different physico-chemical properties (Peters, 2011). Multianalyte methods save on analysis time, resources and the number of methods that need to be validated in the laboratory (Peters, 2011). However due to the varied physico-chemical properties of compounds analysed under these multianalyte techniques, compromises in various aspects of method development are inevitable (Wille, et al., 2012). This has, nevertheless, not impeded researchers from utilising multianalyte techniques as the benefits far outweigh any negatives.

Other notable improvements in instrumental design include automated sample extractions (off-line and on-line) and handling, improved software and databases for

interpretation, comparing and reporting of mass spectral data as well as improved instrument reliability (less maintenance) (Boleda, et al., 2007; Wu & French, 2013). All these have led to increased sample throughput which is especially key when results are required within a short period of time.

However, it is worth noting that while improvements in instrumental and extraction techniques have enhanced the detection of trace levels of compounds in complex matrices, this has also pushed the costs of the instruments up thereby making some of these techniques inaccessible to many laboratories globally (Deng, et al., 2004; de Vos, et al., 2013).

1.7.2 Mass Spectrometry

Chromatographic separation techniques such as GC and LC are based on the partitioning of components of a mixture between a stationary and mobile phase (Braithwaite & Smith, 1999). While many different types of detectors can be coupled to gas chromatographs to aid in the reliable identification of the analyte, a mass spectrometer was used in this research due to its superior structural elucidation and confirmation abilities (Braithwaite & Smith, 1999; Frenich, et al., 2009; Dawling, et al., 2013). In a mass spectrometer, the analytes in the gaseous phase are converted into charged molecules which are then separated by their mass-to-charge (*m/z*) ratio and accelerated towards an ion detection source. Different types of ions are produced depending on the ionization source which includes chemical ionisation (CI), electron impact ionisation (EI) and electrospray ionisation (ESI). The resulting mass spectrum, referred to as a total ion chromatogram (TIC), can provide structural information by which even unknown compounds can be identified, thereby making mass spectrometry a powerful technique for identification, confirmation and quantification of analytes (Ardrey, 2003; McNair & Miller, 2009; Dawling, et al., 2013). Therefore, not only does a mass spectrometer increase the selectivity and sensitivity of a method but it also enables accurate identification of target analytes among other matrix components (Repice, et al., 2013).

1.7.3 Comparison between GC-MS and LC-MS

While LC-MS with ESI is the more popular technique used in the analysis of emerging contaminants in wastewater, GC-MS with EI ionisation was used in this research as an

alternative instrumental technique for sewage epidemiological studies.

When considering the analysis of drugs of abuse in waste water over the past three years, the majority of researchers, by far, have used LC-MS/MS (Kumirska, et al., 2013). To the author's knowledge, only three studies have used GC-MS, with derivatization either based on silylation and iso-butyl formate (Racamonde, et al., 2012 & 2013) or no derivatization (Robles-Molina, et al., 2014). In a review by Vazquez-Roig (2013), only two out of eleven studies for determining legal and illegal drugs in water used GC-MS while eight used methods based on LC-MS/MS and one on LC- quadrupole linear ion trap. Table 1.9 lists some of the few studies that have used GC-MS for the analysis of emerging contaminants in waste water over the past three years. Only 3 out of the twelve listed were solely focussed on or included drugs of abuse and none of them simultaneously analysed 29 drugs and metabolites from different classes. Despite the increasing popularity of using LC-MS for the analysis of emerging contaminants in waste water, GC-MS has many advantages which have kept it as the instrument of choice for many types of analyses, and these can be capitalised on for sewage epidemiological studies. Some of the main advantages and disadvantages of GC-MS are listed in Table 1.10.

Table 1.9: References for GC-MS analysis of emerging contaminants in water samples.

Method (Derivatization)	Sample Matrix	Target Analytes	Reference (Country)
GC-MS/MS (Oximation and silylation)	WW, SW	Steroids	Andrasi et al., 2011 (Hungary)
GC-MS/MS (Silylation)	WW, SW	Hormones	Trinh, et al., 2011 (Australia)
GC-MS (Silylation)	WW, SW	PhACs	Migowska, et al., 2012 (Poland)
GC-MS (Hydrazine)	SW	Disinfectant	Oh & Shin, 2012 (South Korea)
GC-MS (Silylation)	WW, SW	Drugs of abuse	Racamonde, et al., 2012 (Spain)
GCxGC-TOF/MS (none)	SW	PPCPs	Lima Gomes, et al., 2013 (Brazil)
GC-MS (Methylation)	SW	PPCPs & industrial	Jimenez, 2013 (Spain)
GC-MS (Silylation)	WW, SW, TW	PhACs	Kumirska, et al., 2013 (Poland)
GCxGC-TOF/MS (Silylation)	WW, SW	PhACs	Lacina, et al., 2013 (Czech Republic)
GC-HRMS (none)	WW	PPCPs & industrial	Loos, et al., 2013 (multiple)
GC-MS (Acylation with IBC)	WW, SW	Drugs of abuse	Racamonde, et al., 2013 (Spain)
GC-MS/MS (none)	SW	Varied EOCs & drugs of abuse	Robles-Molina, et al., 2014 (Spain)

HRMS = high resolution mass spectrometry; TOF = time of flight; IBC = iso-butyl chloroformate; SW = surface water; WW = waste water; TW = tap water

Table 1.10: Advantages and disadvantages of GC-MS.

Advantages	Disadvantages
• Suitable for compounds whose molecules can be in the gaseous or vapour phase below 400°C and are stable at such temperatures • Lower instrumentation costs than LC-MS and hence more widely available in laboratories • Suitable for analysis of volatile compounds • The same gaseous mobile phase and column can be used for different types of analytes • Can alternate between soft (CI) and hard (EI) ionization techniques • Less affected by matrix effects compared with LC-MS • Mass spectra can be shared between instruments and laboratories and compared with reference spectra from databases such as the NIST or in-house libraries • Methods conducted in different laboratories and on different instruments are relatively transferrable • Very sensitive - LODs as low as LC-MS/MS	• Unsuitable for the analysis of polar, hydrophobic, thermo-labile and high molecular weight compounds without laborious sample pre-treatment e.g. derivatization • Derivatization exposes the analysts to potentially harmful and carcinogenic reagents • Mobile phase limited to inert gases, e.g. He, N_2 • Decomposition of thermally labile compounds may interfere with analysis • Long sample run times for mutianalyte methods compared with LC-MS • Frequent maintenance due to injection septum and liners

On the other hand, there have been numerous studies that have used LC-MS for the detection and quantification of drugs of abuse in water samples over the past three years. The majority of studies incorporate drugs of abuse into multianalyte methods assessing various emerging contaminants. Table 1.11 lists some of the sewage epidemiological studies that have used LC-based techniques.

Table 1.11: References for LC-MS analysis of emerging contaminants in water samples.

Method	Sample Matrix	Target Analytes	Reference (Country)
LC-MS/MS	SW, WW	PhACs & drugs of abuse	Baker & Kasprzyk-Hordern, 2013 (UK)
LC-MS/MS	SW	PhACs and EDCs	Bayen, et al., 2013 (Singapore)
LC-MS/MS	WW	Drugs of abuse	Bijlsma, et al., 2013 (Netherlands)
LC-MS/MS	WW	PhACs & drugs of abuse	Burgard, et al., 2013 (USA)
LC-MS/MS	WW	Drugs of abuse	Emke, et al., 2014 (Netherlands)
LC-MS/MS	WW	Drugs of abuse	Kankaanpää. et al., 2014 (Finland)
LC-MS/MS	WW	Drugs of abuse	Lai, et al., 2013a&c (Australia)
LC-MS/MS	WW	Drugs of abuse	Lai, et al., 2013b (Hong Kong)
LC-MS/MS	WW	Drugs of abuse	Nefau, et al., 2013 (France)
HPLC- UV-FL	SW, WW	PhACs	Patrolecco, et al., 2013 (Itay)
LC-MS/MS	WW	Drugs of abuse	Repice, et al., 2013 (Italy)
LC-MS/MS	WW	Drugs of abuse	Vuori, et al., 2014 (Finland)
LC-MS/MS	WW	PhACs & drugs of abuse	Baker, et al., 2014 (UK)
LC-MS/MS	WW	PhACs & drugs of abuse	Gilart, et al., 2014 (Spain)
LC-MS/MS	WW	PhACs & drugs of abuse	Östman, et al., 2014 (Sweden)
LC-MS/MS	WW	PhACs	Verlicchi, et al., 2014 (Italy)

UV - ultraviolet; FL – fluorescence; SW – surface water; WW – waste water

It was easier to find articles published over the past two years that have used LC-MS/MS for detecting drugs of abuse in waste water than it was to find similar published articles that have used GC-MS over the past five years. As listed in Table 1.12, LC-MS also has many advantages which have made it the instrumental method of choice for emerging contaminants in water samples, especially since they are usually polar and non-volatile (Repice, et al., 2013). However, LC-MS also has disadvantages as listed in Table 1.12.

Table 1.12: Advantages and disadvantages of LC-MS.

Advantages	Disadvantages
• Suitable for polar, high molecular weight or thermo-labile compounds without sample pre-treatment	• Labour intensive and complex during the initial phases of method development
• Does not require derivatization and hence reduces sample preparation time	• Much higher cost of the instruments than GC-MS and hence limited availability in laboratories
• Aqueous samples can be directly analysed by LC-MS	• Matrix effects are more pronounced with LC-MS
• Various combinations of mobile phase composition and pH for wide variety of analytes	• Use of high volumes of expensive solvents and additives which can be harmful to the environment and analyst
• Short run-times compared with GC-MS and hence increased sample throughput	• Greater chance of adduct formation due to reaction of target analytes with mobile phase additives
	• Libraries/databases and mass spectra are not readily transferable between laboratories
	• Limited to soft ionization techniques which provides less structural information
	• Methods are not easily transferrable between different laboratories instruments

The choice on whether to use GC-MS or LC-MS depends on many factors including, the thermal stability and polarity of the compounds to be analysed and the sensitivity of the detector to these compounds, availability in the laboratory and budget (Rivier, 2003). The methods complement each other and should not be solely regarded as either/or (Maurer, 2005). Some sewage epidemiological studies have utilised both methods to enhance the results for a wide variety of compounds (Oh & Shin, 2012; Robles-Molina, et al., 2014). Both types of instrument are capable of conducting unmanned, continuous automated assays for days without needing maintenance intervention. In addition, both GC-MS and LC-MS are conducive for developing methods for the simultaneous analysis of different compounds from different classes.

However, the higher costs can limit the applicability of LC-MS and LC-MS/MS in less developed countries or laboratories with limited budgets (Jjemba, 2006; Racamonde, et al., 2013). In addition, a problem commonly associated with LC-MS, is matrix effects (Frenich, et al., 2009). This broadly refers to the suppression or enhancement of the signal from the analyte due to interference from co-extracted matrix components which may or may not co-elute with the analyte (Frenich, et al., 2009; Peters & Remane, 2012; Bayen, et al., 2013; Petrie, et al., 2013). The interference is thought to occur during the ionisation process (Vogeser & Seger, 2010; Peters & Remane, 2012). Matrix effects can lead to false negatives and deflate or inflate the LOD and LOQ values which ultimately result in unreliable analytical accuracy, reproducibility and quantification (Postigo, et al., 2008; Wille, et al., 2012; Racamonde, et al., 2013). Therefore, matrix effects need to be considered during method validation for LC-MS in order to obtain reliable and realistic results (Peters, 2011). Steps taken to try and minimise matrix effects include optimising the sample pre-treatment & clean-up/extraction methods, use of deuterated or stable isotope-labelled internal standards, reducing or diluting the amount of extracted sample matrix, and standard addition calibration, but none have been able to completely eliminate them (Chambers, et al., 2007; González-Mariño, et al., 2010).

In addition, since compounds are normally not derivatized with LC-MS, m/z values of the molecular and fragment ions tend to be low and could negatively affect positive identification of target analytes due to isobaric compounds altering ion ratios (Racamonde, et al., 2013). Isobaric compounds are either structural isomers of the target

analyte that share its elemental formula or structurally unrelated compounds that have the same nominal molecular mass as the target analyte (Vogeser & Seger, 2010).

Although the development and use of LC-MS-ESI for clinical, toxicological and environmental analysis has accelerated in the last two decades, GC-MS-EI remains the most widely applied analytical technique since its development in the 1950s due to its universality, widespread availability and lower cost of analysis (Ragunathan, et al., 1999; Peters, 2011; Migowska, et al., 2012; Favretto, et al., 2013; Kumirska, et al., 2013). It can also be considered to have more reliable quantification especially when isotopically-labelled internal standards are used as compared with LC-MS in which matrix effects and isobarism always have to be taken into account during method validation (Vogeser & Seger, 2010). On the other hand, GC-MS is less affected by matrix effects (Favretto, et al., 2013; Kumirska, et al., 2013). In addition, Heath (2010) reports GC-MS to be more sensitive than LC-MS/MS in the analysis of acidic drugs in more complex matrices and several studies have shown GC-MS to have LODs rivalling or better than LC-MS/MS (Fatta, et al. 2007; Mwenesongole, et al., 2013; Vazquez-Roig, et al., 2013). With regard to measurement uncertainty for the trace analysis of organic compounds in complex matrices, an inter-laboratory study showed GC-MS to be superior to LC-MS/MS (Heath, et al., 2010).

Since very little published information was available on the analysis of waste water for drugs of abuse using GC-MS, this instrumental technique was used in this research to provide further data on its suitability for sewage epidemiological studies, and as a cheaper and sensitive alternative to LC-MS/MS. In addition, as a result of preliminary investigations, derivatization by PFPA was used as an alternative to more commonly applied silylation, due to its universality. To the author's knowledge, GC-MS, with derivatization by PFPA, has not been used for the analysis of drugs of abuse, especially NPS, in waste water.

1.8 VALIDATION OF AN ANALYTICAL METHOD

Method validation determines whether a method is suitable for its intended use by testing different performance characteristics. Various published papers are available which explain in detail what successful method validation entails (Jimenez, et al., 2002;

Peters, et al., 2007; Huber, et al., 2010; Willie, et al., 2011). The extent of method validation, the evaluation criteria selected and the acceptance range of the results all depend on the intended purpose of the analytical method. In this regard, various guidelines are available to assist laboratories in designing their own method validation strategies while maintaining the integrity and reliability of the method, such as the International Conference on Harmonisation (ICH, 2005) and Eurachem (Eurachem, 1998) guidelines. Each laboratory can decide which are the key performance tests and the minor (supportive) ones (Jimenez, et al., 2002).

1.8.1 Key Performance Tests

The key performance tests which were deemed relevant for this research are briefly mentioned below:

Selectivity - the ability of a method to accurately detect and distinguish the target analyte from other known and unknown compounds in the sample matrix.

Linear range - the ability of a method to give test results that are directly proportional to the concentration of analyte in the sample, within a specific range.

Precision (intra-assay and intermediate) - the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

A measure of the precision under the same operating conditions over a short interval of time is referred to as intra-assay precision. The precision measured within the same laboratory but on different days, or by different analysts, or different equipment etc. is referred to as the intermediate precision.

Limit of detection (LOD) - the lowest concentration of the target analyte in the sample that can be reliably differentiated from background noise or a blank.

Limit of quantification (LOQ) - the lowest concentration of the target analyte in the sample that can be reliably quantified with suitable precision and accuracy.

Extraction recovery - measures how efficiently the target analyte is extracted from the sample matrix.

Stability - measures the changes in drug concentration over a pre-determined time interval.

1.8.2 Quantification by Standard Addition

For complex samples where a blank matrix cannot be obtained, such as waste water, the standard addition method of quantification is recommended (Frenich, et al., 2009; Cooper, et al., 2010). In the standard addition method, increasing concentrations of a mixed drug standard are added to aliquots of the same volume of the sample containing possible target analytes. The unknown concentration of the analyte originally present in the sample is calculated by extrapolation. Since the calibraton is conducted within the sample matrix, any co-extracted components are accounted for thereby correcting for any matrix effects (Furey, et al., 2013). The majority of published studies in sewage epidemiology used matrix-matched calibration spiked with drug standards (Baker & Kasprzyk-Hordern, 2013; Gilart, et al., 2014; Kumirska, et al., 2013) or calibration standards in pure water or solvent (Jimenez, 2013; Lai, et al., 2013a; Lopes, et al., 2014; Verlicchi, et al., 2014). Isotopically-labelled internal standards were used in the majority of studies to compensate for any variabilities during sample preparation and analysis (Frenich, et al., 2009). Standard addition has been avoided due to its laborious nature (Furey, et al., 2013). However, matrix matched calibration works best when a blank sample matrix is used. Due to the ever changing nature of the composition of waste water and surface water, it is very difficult to find a blank sample matrix with the same make-up as the sample matrix (Furey, et al., 2013). As a result of this, various matrices have been used as a 'substitute' of the blank sample matrix, such as surface water from uncontaminated sources, tap water, distilled or ultrapure water (Ostman, et al., 2014; Vuori, et al., 2014). Some calibration standards have also been prepared in solvents such as methanol and acetonitrile (Nefau, et al., 2013; Lopes, et al., 2014). These do not, however, take into account any other matrix components that may interfere with the analysis. In addition, the behaviour of drugs in a neat standard is different from when in the complex sample matrix. Therefore, this does not adequately compensate for interfering matrix components even when an internal standard is used. With standard

addition, the same matrix is used which takes into account any interefering matrix components. The method of standard addition therefore adequately compensates for matrix effects and removes any bias due to the use of a substitute matrix because both quantification and calibration are performed on the same sample (Peters, et al., 2007; Frenich, et al., 2013; Rabii, et al., 2014). This leads to more accurate quantification (Furey, et al., 2013). However, based on a comprehensive literature review, standard addition has not been used for the analysis of drugs of abuse in waste water to the author's knowledge. This is surprising considering standard addition is the most effective way of compensating for or eliminating matrix effects that plague analysis by LC-MS, the more commonly used analytical technique for sewage epidemiological studies (Danzer & Currie, 1998).

Therefore, in this research, to compensate for any matrix components that might interfere with qualitative and quantitative aspects of the analysis, to reduce any bias arising from the use of a substitute blank matrix, and to compensate for any variabilities during sample preparation and analysis, both standard addition and the use of isotopically-labelled internal standards were used.

1.9 AIM OF THE RESEARCH

In the light of the above discussions, the aim of this research was to develop and validate an analytical method based on GC-MS with a suitable derivatization reagent, for the simultaneous extraction, detection and quantification of 29 drugs of abuse in waste water samples from Cambridge, UK.

The main objectives were to:

1. Obtain real-time qualitative and quantitative data on the most commonly abused drugs, including NPS, in Cambridge, UK.
2. Use the quantitative findings to estimate drug consumption in Cambridge, UK.
3. Present GC-MS as an equally effective and more sensitive alternative to LC-MS/MS in sewage epidemiological studies.

CHAPTER TWO

EXPERIMENTAL PROCEDURES

This chapter details the experimental protocols and materials used during the development of the method for the detection of drugs of abuse in waste water. The chapter is divided into sub-sections which highlight the various preliminary studies conducted such as chemical derivatization, stability studies, and sample extraction. The final method selected after optimising each segment of the method is mentioned in the relevant sub-section. This is followed by protocols conducted during validation studies.

2.1 DRUG STANDARDS, CHEMICALS AND SOLVENTS

The solvents, chemicals and drug standards used are listed in Tables 2.1, 2.2 and 2.3. All drug standards were of analytical grade (purity ≥ 97 %), except where indicated, and were purchased as solids or as 1 mg/mL or 0.1 mg/mL standard solutions in methanol or acetonitrile. For the solids, 1 mg/mL stock solutions were prepared in methanol or acetonitrile (fresh individual drug stock solutions were made every 6 months). All standards were stored at -20 °C in the dark. Mixed drug standards were made from aliquots of freshly made individual stock standards and used immediately or the solvent evaporated and the dried mixed standard stored at -20 °C until required. These mixed dried standards were later reconstituted in methanol (when used for spiking water samples before extraction) (section 2.4.3) or ethyl acetate (when used for instrumental validation studies) (section 2.5). Un-extracted and extracted drug standards were derivatized and reconstituted in 0.05 to 0.1 mL ethyl acetate before GC-MS analysis. Alkane standards were made at concentrations of 1 mg/mL in pentane and further diluted to mixed working standards of 0.1 mg/mL and 0.01 mg/mL. Calibration of the pH meter was conducted with pH 4.0, 7.0 and 10.0 buffers (Table 2.2). All research work was conducted in laboratories at Anglia Ruskin University, Cambridge, UK, which has a Schedule 1 Home Office 'Licence to be in Possession' provided under the Misuse of Drugs Act 1971.

Table 2.1: Derivatizing reagents and solvents used during research.

Derivatizing Reagents	Solvents
Acetic anhydride[a]	Acetone[h]
N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane (TMCS)[g]	35 % Ammonium hydroxide (NH_4OH)[h]
Heptafluorobutyric anhydride (HFBA) [a]	Chloroform[h]
N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) [a]	37 % Hydrochloric acid (HCl)[h]
Pentafluoropropionic anhydride (PFPA) [a]	Ethanol[h]
Pyridine[a]	Ethyl acetate [h]
	Formic acid [h]
	Methanol [h]
	Pentane [h]
	2-Propanol[h]

Sigma[a], Supelco[g], Fisher[h]

Table 2.2: n-Alkanes and chemicals used during research.

n-Alkanes	Other chemicals
Decane [h] (general grade)	Sodium sulfate[i]
Docosane[f]	pH 4 (phthalate)[h]
Dodecane [h] (lab reagent)	pH 7 (phosphate)[h]
Eicosane [i]	pH 10 (borate)[h]
Hexadecane[h] (general grade)	
Hexacosane[f]	
Octadecane [i]	
Octacosane[f]	
Tetracosane[f]	
Tetradecane [i]	

Alfa Aesar[f], Fisher[h], Acros[i]

Table 2.3: Drug standards used during research.

Amitriptyline hydrochloride[a] (AMIT)	Mephedrone hydrochloride[d] (MEPH)
Amphetamine sulphate[a] (AMP)	Methamphetamine hydrochloride[a] (MAMP)
Amphetamine hydrochloride[a] (AMP)	Methcathinone hydrochloride[a] (MCAT)
Amphetamine-d_6[a] (AMP-d_6), **IS**	2-Methoxyphenylpiperazine[b] (2-MEOPP)
Benzylpiperazine[b] (BZP)	4-Methoxyphenylpiperazine[a] (4-MEOPP)
Butylone hydrochloride[c] (BUTY)	Methylbenzodioxolylbutanamine hydrochloride[e] (MBDB)
Cathinone hydrochloride[a] (CAT)	Methylbenzylpiperazine[a] (MBZP)
3-Chlorophenylpiperazine[a] (3-CPP)	Methylenedioxymethamphetamine[b] (MDMA)
Cocaine hydrochloride[a] (COC)	Methylenedioxymethamphetamine-d_5 [e] (MDMA-d_5), **IS**
Cocaine-d_3[e] (COC-d_3), **IS**	4-Methylphenylpiperazine[a] (4-MPP)
Diazepam[a] (DIAZ)	6-Monoacetylmorphine[a] (6-MAM)
Ecgonine methylester hydrochloride hydrate[a] (EME)	Morphine sulphate pentahydrate[a] (MOR)
4-Fluoromethamphetamine hydrochloride[c] (4-FMA)	Morphine-d_3[e] (MOR-d_3), **IS**
3-Fluoromethcathinone[c] (3-FMC)	Piperazine[b] (PIP)
2-Fluorophenylpiperazine[a] (2-FPP)	Piperazine hydrochloride hexa hydrate[f] (PIP)
4-Fluorophenylpiperazine[a] (4-FPP)	3-Trifluoromethylphenylpiperazine[f] (3-TFMPP)
Heroin[b]	4-Trifluoromethylphenylpiperazine[b] (4-TFMPP)
Ketamine[a] (KET)	

Sigma[a], Fluka[b], National Measurement Institute, Australia[c], Toronto Research Chemilab, Canada[d], Cerilliant[e], Alfa Aesar[f]
IS = internal standard

2.2 SAMPLE COLLECTION AND PREPARATION

During the course of the method development, different water samples were used. Tap water was obtained from Anglia Ruskin laboratories. Deionized water was obtained from an Elga Purelab Option (Veolia, UK). All waste water influent and effluent samples were collected from Anglian Water in Cambridge, UK, in 1 L PET containers. Waste water samples used for preliminary studies (sections 2.4.3.2.2, 2.5.4 and 2.5.5) were grab and 48 h composite influent and grab effluent samples randomly collected between March 2011 and September 2012 while the sample used for quantification by standard addition (section 2.5.6) was a 72 h composite influent sample collected in September 2012. After collection, the waste water samples were immediately transported in the dark to the laboratory, which was only a 10 minute drive away.

Ethical approval for the research project was provided by the Research Ethics Subcommittee at Anglia Ruskin University, Cambridge, UK. Since the waste water samples were collected from a WWTP and could not be linked to a particular individual or group, the project was given a 'low risk'.

Prior to extraction, waste water samples were vacuum filtered through a disposable 1 L capacity stericup funnel and receiver system with a 0.22 µm GP Millipore Express® Plus membrane (Millipore, UK). Once filtered and before extraction or storage, the samples were acidified with 37 % HCl to pH 2.4 to 2.7. Samples were extracted on the day of collection, or either stored in the dark at 5 °C and extracted within 24 h of collection or at -20 °C and extracted within 3 months of collection. Frozen samples were defrosted at 5 °C and at room temperature. Samples were stored in PP and HDPE containers (Fisher Scientific, UK). See also section 1.5.1 for further background on sample collection.

A risk assessment for handling the waste water samples was conducted and approved before research began. All handling of the waste water was conducted according to good laboratory practice for biological samples i.e. use of gloves when collecting samples, transporting samples in sealed containers, handling of samples in designated fume hoods labelled with 'biological hazard' (in the absence of other students), and disinfecting and/or autoclaving of all laboratory equipment and surfaces in contact with the samples before re-use or disposal.

Throughout the method development, evaporation of organic solvents and eluants with volumes ≤ 10 mL was conducted with a MiVAC DNA concentrator (Genevac, UK) at temperatures between 35 and 40 °C. For volumes > 10 mL, evaporation was conducted on a rotary evaporator (Bibby Scientific Ltd, UK).

2.3 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

Instrumental parameters used during this research are listed in Table 2.4.

Table 2.4: Instrumental parameters for GC-MS.

	GAS CHROMATOGRAPH	
	Preliminary Studies	Method Validation
Injector Temperature	250 °C & 260 °C	250 °C
Injection Volume	1 & 2 µl	1 µl
Injection Mode	Split	Splitless
Split Ratio	20:1, 10:1, 5:1	Not utilised
Injection liners	4 mm quartz liner for split injection with and without deactivated silanised glass wool	2 mm quartz liner for splitless injection plugged with deactivated silanised glass wool
Oven Temperature Programme	30 °C, held for 10 min, increased to 280 °C at 10 °C/min, held for 3 min 100 °C, held for 2 min, increased to 300 °C at 10 °C/min, held for 5 min 80 °C, held for 2 min, increased to 280 °C at 15 °C/min, held for 5 min 100 °C, held for 1 min, increased to 280 °C at 8 °C/min, held for 3 min	50 °C, held for 2 min, increased to 100 °C at 30 °C/min then increased to 280 °C at 8 °C/min and held for 5 min
Instrument Timed Event	Not utilised	Split valve opened at 1 min at a flow rate of 30 mL/min and again at 3 min at a flow rate of 5 mL/min
Run Time	20.33 - 38 min	31.17 min
Solvent Delay	3.5 min	3.5 min
Transfer Line	260 °C & 280 °C	280 °C
	MASS SPECTROMETER	
	Preliminary Studies	Method Validation
Ionisation Mode	EI^+	EI^+
Electron Energy	70 eV	70 eV
Source Temperature	230 °C	230 °C
Detector Voltage	350 – 450V	600V
Mass Analyzer	SIM (when used): dwell time, 20-60 ms per ion; 8 RT windows. Scan: m/z 40–620 amu	SIM: dwell time, 20-60 ms per ion; 14 RT windows; 3-12 ions per window; interscan delay of 5 ms. Scan: m/z 40 – 620 amu

Instrumental analysis was conducted on a Perkin Elmer Clarus 500 GC-MS. Two different types of columns were used during the method development, a Phenomenex ZB-1

column (30 m x 0.25 µm x 0.25 mm i.d.) used during early preliminary studies and a Supelco Equity™-5 capillary column (30 m x 0.25 µm x 0.25 mm i.d.) used during later preliminary studies and method validation. The stationary phase on the ZB-1 column was 100 % dimethylpolysiloxane while for the Equity™-5 column it was 5 % diphenylpolysiloxane/95 % dimethylpolysiloxane. The carrier gas was helium (BOC, 99.95 %) at a flow rate of 1 mL/min. The mass analyser was a single quadrupole. All data was collected, analysed and processed using TurboMass™ 5.4 GC-MS software. Further instrumental settings are listed in Table 2.4.

2.4 PRELIMINARY INVESTIGATIONS

The following sections provide experimental procedures undertaken during preliminary investigations as discussed in Chapter 3.

2.4.1 Chemical Derivatization

2.4.1.1 Comparison of Derivatizing Reagents

The various derivatizing reagents assessed during comparative investigations are listed in Table 2.5, together with the experimental parameters.

Table 2.5: Derivatizing reagents and conditions used.

Derivatizing Reagent	Conditions
[BSTFA:TMCS (99:1v/v)]:ethyl actetate (2:1v/v)	Part A 70 °C for 60 minutes
MSTFA:ethyl actetate (2:1v/v)	
Acetic anhydride:pyridine (3:2v/v)	
HFBA	
PFPA:ethyl actetate (2:1v/v)	
[BSTFA:TMCS (99:1v/v)]	Part B 70 °C for 60 minutes
PFPA:ethyl actetate (2:1v/v)	

In Table 2.5 Part A, a mixed drug standard containing aliquots of individual drugs taken from stock solutions was placed in six vials and evaporated to dryness. 0.2 mL of the derivatizing reagent was added into five of the vials and after the appropriate reaction

time, the derivatizing reagent was evaporated to dryness and the sample reconstituted in 0.2 mL ethyl acetate resulting in a final drug concentration of 100 µg/mL. One vial was not derivatized but was evaporated and also reconstituted in 0.2 mL ethyl acetate. The drugs assessed under this study are listed in Table 3.1.

In Table 2.5 Part B, a mixed drug standard containing aliquots of individual drugs taken from stock solutions was placed in two vials and evaporated to dryness. 0.2 mL of the derivatizing reagent was added into the vials and after the appropriate reaction time. PFPA was evaporated to dryness and the sample reconstituted in 0.2 mL ethyl acetate resulting in a final drug concentration of 100 µg/mL. BSTFA was not evaporated and the sample was analysed as is. The drugs assessed under this study are listed in Figures 3.3 and 3.4.

2.4.1.2 Optimisation of PFPA Derivatization Reactions

After comparative derivatization studies, PFPA: ethyl acetate (2:1v/v) was selected for further optimisation studies (section 3.2.2). Table 2.6 lists the variables assessed under this section. Internal standards added to the mixed drug standards prior to derivatization were COC-d_3 at 25 µg/mL, MDMA-d_5 and AMP-d_6 at 20 µg/mL and MOR-d_3 at 10 µg/mL.

Table 2.6: Variables assessed during PFPA optimisation of a mixed drug standard.

Derivatization Temp. (°C)	Derivatization Time (min)	Volume of PFPA:ethyl acetate, 2:1v/v (mL)	Concentration of Mixed Drug Std. (µg/mL)
80	15, 30, 45	0.1	12.82 – 23.38
90	15, 30, 45	0.1	12.82 – 23.38

Drugs included in this study are shown in Figures 3.9, 3.10 and 3.11.

2.4.1.3 Derivatization Reactions for a Mixed Opiate Standard

Separate derivatization studies were conducted on a mixture of morphine and 6-MAM (section 3.2.3). Table 2.7 lists the variables assessed under this section. COC-d_3 was used as the internal standard at a concentration of 16.67 µg/mL.

Table 2.7: Variables assessed during PFPA optimisation of a mixed opiate standard.

Derivatization Temp. (°C)	Derivatization Time (min)	Volume of PFPA:ethyl acetate, 2:1v/v (mL)	Concentration of Mixed Opiate Std. (µg/mL)
90	15, 30, 45, 60	0.1	16.46 (MOR) 16.67 (6-MAM)

2.4.1.4 Individual Analysis of MOR, 6-MAM and Heroin

0.1 mL each of 100 µg/mL MOR, 6-MAM and heroin was derivatized at 90 °C for 30 min with 0.1 mL of PFPA: ethyl acetate (2:1v/v). After evaporation of the derivatizing reagent, the drugs were individually reconstituted in 0.1 mL ethyl acetate.

2.4.2 Stability Studies

As part of the method development, different stability studies were conducted on the unextracted mixed drug standards. Table 2.8 lists the stability studies undertaken and experimental conditions used.

Table 2.8: Methods and conditions for mixed drug standard stability studies.

Stability Test	Temp. (°C)	Test Solvent	Analysis time point	Drugs, Internal Standards and Concentrations
Autosampler	Room	Ethyl acetate	27 hours	The 26 drugs included in this study are listed in Table 5.4. Individual drugs ranged in concentration from 16.4 to 32.5 µg/mL (except 2-FPP at 41 µg/mL and MOR at 6.1 µg/mL). Internal standards added at 1 µg/mL for MDMA-d_5 and MOR-d_3 and 10 µg/mL for COC-d_3.
Derivatized Storage	5 and -20	Ethyl acetate	0,2,4 (weeks)	The 26 drugs included in this study are listed in Table 5.5. Individual drugs ranged in concentration from 15.4 to 69.6 µg/mL. Internal standards MDMA-d_5, COC-d_3, and MOR-d_3 added at 41.7 µg/mL. Equal aliquots were analyzed at the relevant time period.
Underivatized storage (then derivatized on day of analysis)	5 and -20	Methanol	0,1,2,4 (weeks)	The 26 drugs included in this study are listed in Table 5.6. Individual drugs ranged in concentration from 26.7 to 46.8 µg/mL (except 2-FPP at 66.2 µg/mL and MOR at 9.9 µg/mL). Internal standards added at 1.7 µg/mL for MDMA-d_5 and MOR-d_3 and 16.7 µg/mL for COC-d_3. Equal aliquots were analyzed at the relevant time period.

Autosampler and derivatized storage stability was conducted on a PFPA-derivatized mixed standard reconstituted in ethyl acetate, while underivatized storage stability was conducted on a mixed drug standard in methanol. For autosampler stability, the mixed drug standard was divided into 23 vials and analysed over a 27 h period, with a solvent blank injected between each sample injection. For each storage stability trial, the same initial mixed drug standard was divided into two separate vials and stored at the different temperatures. Three aliquots from each temperature were analysed at the relevant time points.

The analysis time point indicates the time intervals at which the storage stability samples were analysed with, '0' denoting the initial time point.

2.4.3 Analyte Extraction Methods

LLE and SPE techniques were both explored during preliminary studies in order to determine the most suitable extraction method for the target analytes.

2.4.3.1 Liquid-liquid Extraction (LLE)

Different extraction solvents and varying pH values were assessed for LLE to determine which would be the most suitable for the target analytes.

2.4.3.1.1 Selection of Extraction Solvent

The two different LLE extraction protocols investigated during preliminary studies were adapted from Tsutsumi (2005) and Raikos (2009). These were based on chloroform:isopropyl alcohol (3:1, v/v) and chloroform:ethyl acetate:ethanol (3:1:1, v/v), respectively. A 2 mL aliquot of deionised water was spiked with a mixed drug standard containing 26 drugs and 3 internal standards MDMA-d_5, COC-d_3 and MOR-d_3 (with individual drugs ranging in concentration from 0.6 to 2.8 µg/mL). The pH was adjusted to 10.5 with 35 % NH_4OH. A 5 mL aliquot of extraction solvent (3 fractions) was used and the mixture was vortexed for 1 min then centrifuged at 3000 x gr for 5 min. The organic layer was evaporated to dryness after which it was derivatized and reconstituted in 0.1 mL ethyl acetate. In order to enable the calculation of recovery, a similar unextracted mixed drug standard was evaporated, derivatized and reconstituted in ethyl acetate. Drugs included in this study are listed in Table 3.7.

2.4.3.1.2 Optimisation of pH

Extraction recovery using chloroform:ethylacetate:ethanol (3:1:1, v/v) was conducted on 150 mL deionized water spiked with a mixed drug standard containing 26 drugs with individual drugs ranging in concentration from 2.5 to 4.7 µg/mL (except 2-FPP at 6.6 µg/mL). The internal standards MDMA-d_5 and MOR-d_3 were added at concentrations of 0.17 µg/mL while COC-d_3 was added at a concentration of 1.7 µg/mL. pH values of 5.0, 7.0 and 10.5 were investigated. The pH was adjusted with 35 % NH_4OH or 37 % HCl. A 250 mL separating funnel and 30 mL of extraction solvent (3 fractions) were used. The mixture was agitated for at least 10 min and sodium sulfate was added to the organic layer to ensure removal of any residual moisture. The organic layer was then filtered and the sulfate particulates rinsed with the extraction solvent. Lastly, the organic layer was evaporated to dryness, derivatized and reconstituted in 0.1 mL ethyl acetate. Drugs included in this study are listed in Table 3.8.

2.4.3.2 Solid Phase Extraction (SPE)

SPE preliminary experiments concerned the selection of a suitable sorbent, sample pH and elution solvents for the drug analytes under investigation. A Visiprep™ Vacuum Manifold with 24 ports (Supelco, UK) was used for all extractions. Oasis®SPE cartridges (Waters, UK) were used for all extractions.

2.4.3.2.1 Comparison of the SPE Sorbents, Oasis® MCX and HLB

Oasis MCX and HLB sorbents were investigated at different pH values, as shown in Table 2.9, using methods adapted from Waters (2006a), Bones (2007) and González-Mariño (2010). 150 mL of deionised water was spiked with a mixed drug standard containing 26 drugs with individual drugs ranging in concentration from 2.5 to 4.7 µg/mL (except 2-FPP at 6.6 µg/mL). The internal standards MDMA-d_5 and MOR-d_3 were added at concentrations of 0.17 µg/mL while COC-d_3 was added at a concentration of 1.7 µg/mL. pH adjustment was made with 35 % NH_4OH or 37 % HCl. Method blanks using unspiked deionised water samples and unextracted standards for recovery calculations were analysed simultaneously. The drugs included in this study are listed in Table 3.9.

Table 2.9: Protocols for solid phase extraction with Oasis MCX and HLB sorbents.

SPE CARTRIDGE	Oasis MCX[4] (60 mg, 3 mL)	Oasis HLB[5] (60 mg, 3 mL)
SAMPLE pH	2.0, 5.4 and 10	2.8, 7.4 & 8.5
CONDITIONING SOLVENT	Methanol, 2 mL	Ethyl acetate, 2.5 mL
	DH_2O, 2 mL	Acetone, 2.5 mL
	DH_2O (pH 2), 2 mL	DH_2O, 2.5 mL
SAMPLE LOADING FLOWRATE (mL/min)	5-10	5-10
RINSE	DH_2O (pH 2), 1 mL	5 % (v/v) Methanol, 2 mL
	DH_2O, 1 mL	
DRYING TIME (min)	10	10
ELUTION SOLVENT	Methanol, 2 mL	Ethyl acetate, 2 mL
	5 % (v/v) NH_4OH in methanol, 2 mL	Acetone, 4 mL

[4]Tarcomnicu, et al., 2011;[5]González-Mariño, et al., 2010; DH_2O = Deionised Water

2.4.3.2.2 Comparison of Elution Solvents for MCX at pH 2.0

Using the MCX protocol in Table 2.9, two elution solvents were compared with each other; 5 % (v/v) NH_4OH in methanol (Tarcomnicu, et al., 2011) and 5 % (v/v) NH_4OH in acetone:ethyl acetate (1:1v/v) (Bones, et al., 2007).

A mixed drug standard (individual drugs ranged in concentration from 0.81 to 1.3 µg/mL, except 3-FMC at 2.3 µg/mL) was spiked into 50 mL of waste water (pH 2.5) and extracted for each of the solvents. Internal standards AMP-d_6, MDMA-d_5 and COC-d_3, all at 1 µg/mL, were added to the drug mix before extraction. Eluants were evaporated, derivatized and analysed.

The drugs included in this study are shown in Figure 3.25. The final SPE protocol used for validation studies and application to a waste water sample is listed in Table 2.10.

Table 2.10: Final protocol for solid phase extraction with Oasis MCX.

SPE CARTRIDGE	Oasis MCX (60mg, 3mL)
SAMPLE pH	2.4 - 2.8
CONDITIONING SOLVENT	Methanol, 2 mL DH_2O, 2 mL DH_2O (pH 2.5), 2 mL
SAMPLE LOADING FLOWRATE (mL/min)	5-8
RINSE	DH_2O (pH 2.5), 1.5 mL
DRYING TIME	20 min under high vacuum
ELUTION	a) Methanol, 4 mL (800 µl x 5) b) 5 % (v/v) NH_4OH in acetone:ethyl acetate (1:1 v/v), 4 mL (800 µl x 5)
EVAPORATION	30-40 min at 40 °C in MiVAC

DH_2O = Deionised Water

2.5 METHOD VALIDATION STUDIES

Method validation parameters, as discussed in section 1.8.1, were assessed by the use of a derivatized mixed working solution reconstituted in ethyl acetate as well as waste water spiked with known concentrations of analytes. The derivatization and SPE protocols optimised during preliminary studies were used (Table 2.10 and section 3.2.4).

2.5.1 Instrumental Linear Range

The instrumental linear range was determined by dilution of a derivatized mixed drug standard prepared in ethyl acetate at concentrations ranging from 2.0×10^{-4} to 1.4 µg/mL. Triplicate analyses were conducted for each concentration point. Drugs included in this study are listed in Table 4.1. Internals standards were added at 0.07 µg/mL for AMP-d_6, MDMA-d_5, and COC-d_3.

2.5.2 Precision (Intra-assay and Intermediate)

Intra-assay precision of the instrument was determined over an 18 h period under the same instrumental conditions. Three concentrations of mixed drug standards (0.005, 0.1 and 1 µg/mL) were assessed.

Intra-assay precision of the analytical method was assessed over a 6 h period under the same instrumental conditions using a mixed drug standard spiked in 100 mL of treated waste water. Individual drugs ranged in concentration from 0.81 to 1.3 µg/mL, except 3-FMC at 2.3 µg/mL. This study was incorporated as part of the extraction and recovery study (section 2.5.4).

Intermediate precision of the instrument was verified at three concentrations (0.005, 0.1 and 1 µg/mL) on three separate days. Drugs included in this study are listed in Tables 4.2, 4.3 and 4.4.

2.5.3 Instrumental Detection and Quantification Limits

Instrument quantification and detection limits were determined by dilution of a derivatized mixed drug standard in ethyl acetate and measuring the detector response until a signal to noise ratio (S/N) of 10:1 (for LOQ) and 3:1 (for LOD) was attained. This was accomplished through a function available in the Perkin Elmer Clarus 500 GC-MS TurboMass™ 5.4 software. Drugs included in this study are listed in Table 4.5.

2.5.4 SPE Extraction and Recovery Using Optimised Instrumental Method

Matrix-based recovery of the drugs from treated waste water was conducted using the SPE protocol in Table 2.10. 100 mL aliquots of treated waste water were spiked with a mixed drug standard before extraction (individual drugs ranged in concentration from 0.8 to 1.3 µg/mL, except 3-FMC at 2.3 µg/mL). AMP-d_6 (1 µg/mL), MDMA-d_5 (1 µg/mL), & COC-d_3 (1.5 µg/mL) were added as internal standards. Separate 100 mL aliquots of waste water were spiked after elution for recovery calculations. Unextracted standards were also analysed as positive controls. A further 100 mL aliquot of waste water was extracted and not spiked with any drugs pre- or post-extraction and was used as a background control sample.

The eluants were evaporated to dryness, derivatized and stored at -20 °C. On the day of analysis, they were reconstituted in 0.1 mL ethyl acetate. Drugs included in this study are listed in Table 4.7.

2.5.5 Matrix-based Stability

The stability of the underivatized drugs in the sample matrix was investigated by spiking 500 mL of untreated waste water (pH 2.5) with a mixed drug standard and dividing it into 50 mL portions. Individual drugs ranged in concentration from 0.8 to 1.3 µg/mL, except 3-FMC at 2.3 µg/mL. Internal standards AMP-d_6, MDMA-d_5 and COC-d_3 were added at 1 µg/mL. The portions were stored at 5 °C then extracted, derivatized and analysed at time points 0, 3 days and 7 days. Drugs included in this study are listed in Table 4.8.

2.5.6 Standard Addition

A six point standard addition curve of drugs and internal standards (0.0003, 0.0022, 0.0188, 0.2500, 1.0, 3.750 µg/mL) was prepared in untreated waste water before extraction and used for the quantification. The drug standards were spiked into 25 mL of 72 h composite waste water diluted 1:1 with deionised water to give a total volume of 50 mL (section 2.2). Internal standards AMP-d_6, MDMA-d_5 and COC-d_3 were added at 0.06 µg/mL. Duplicate extractions of unspiked deionized water (method blank) and the same volume of waste water as the calibrators but spiked only with internal standards (matrix control) were also conducted. Drugs included in this study are as previously listed in Table 4.8.

2.6 SYSTEM SUITABILITY TESTS (SST) AND QUALITY CONTROL (QC)

Column performance and shifts in RT before, during and after a sample batch were assessed by the use of a GC column check standard (Phenomenex), a mixture of 10 n-alkane standards (C8-C28, as shown in Table 2.2) as well as QC samples (0.1 µg/mL). The QC samples either contained a mixture of 3 of the target drugs plus an internal standard (piperazine, MDMA, MDMA-d_5 and diazepam) or were positive controls containing all target drugs. The alkane and QC standards were included at the beginning, middle and end of the batch (for long batches) and the beginning and end of a batch (for short batch runs). Solvent blanks (i.e. ethyl acetate) were also run in-between sample

injections to check for carryover and any other anomalies such as contamination from GC septa or vial lids.

The GC-MS instrument was subjected to regular tuning, mass calibration and maintenance (change of filament, liner, septum, column and needle as well as baking the column and cleaning the source) as necessary and comprehensive maintenance once a year.

CHAPTER THREE

RESULTS AND DISCUSSION – PRELIMINARY STUDIES

This chapter discusses the results obtained from preliminary studies undertaken prior to the method validation studies. Preliminary studies involved selecting and optimising the derivatizing reagent, the sample extraction method and stability studies to guide sample pre-treatment, preparation, storage and analysis.

3.1 INTRODUCTION

As one of the key aims of this research involved the development of a method to simultaneously analyse a number of drugs, all preliminary studies were conducted on mixed drug standards. However, mass spectra and ion ratios of individual derivatized and underivatized drugs were initially obtained in scan mode for comparison with mass spectra and ion ratios of the drugs in respective mixed standards. In addition, mass spectra were compared with published literature or the National Institute of Standards and Technology (NIST) mass spectral search program [Version 2.0(2)]. To compensate for variability across the entire analytical process, sample preparation was done in duplicate or triplicate and internal standards were added to the mixed drug standards before various assays. A background to the selection of internal standards used during this research can be found in Appendix IV.

During method development, there are various factors that need to be considered in order to improve the sensitivity and selectivity of the method as well as ensure reliable and reproducible results. Those related to sample preparation are discussed in sections 1.5 to 1.6 and 3.2 to 3.4, and those related to instrumental analysis are discussed in section 3.5.

3.2 CHEMICAL DERIVATIZATION

Based on the polar nature of the target drugs under investigation, an informed decision was made to derivatize the drugs in order to determine whether chromatographic and mass spectral parameters would be improved (section 1.6.1). Due to the different functional groups of the drugs (amine, hydroxyl and ester), the

most commonly used derivatizing reagents based on silylation and acylation were investigated.

3.2.1 Comparison of Derivatizing Reagents

Following the procedures as detailed in Table 2.5, Part A (section 2.4.1.1), five derivatizing reagents were investigated in order to determine the most suitable for the mixed drugs under investigation. Figures 3.1 and 3.2 show the TICs of drugs derivatized with PFPA and BSTFA (selected to represent acylation and silylation, respectively). A mixed standard was also analysed for comparative purposes and this is shown in Figure 3.6 (page 88). During this initial investigation, ten drugs representing the different classes of drugs and RTs that would form part of the validated multianalyte method were assessed.

The criteria used for the selection of most suitable derivatizing reagent was the presence of one derivative peak per original drug in the mixed standard, representing complete derivatization of the initial drug as confirmed by a comparison of mass spectra of the underivatized drugs with mass spectra of the expected derivatives (section 1.6.4), and the distinctiveness of mass spectra generated with higher *m/z* ratios (Halket, 1993). Any extra peaks were evaluated as they could arise from artefacts, adducts or from underivatized drug. Likewise, if fewer peaks were detected then the derivatization process was evaluated as it may not have been suitable for the drugs under analysis. In addition, when targeted diagnostic ions from underivatized drugs were detected, this indicated that the conversion process was incomplete (Mol, et al., 2000).

COC and DIAZ do not get derivatized (section 1.6.1) and hence their RT and mass spectra remain the same in all chromatograms. Aside from the presence of peaks corresponding to underivatized EME (EME-UD) for both acylating and silylating reagents, the TICs for drugs derivatized by the acylating reagents had one derivative peak per original drug with no other additional peaks (Figure 3.1).

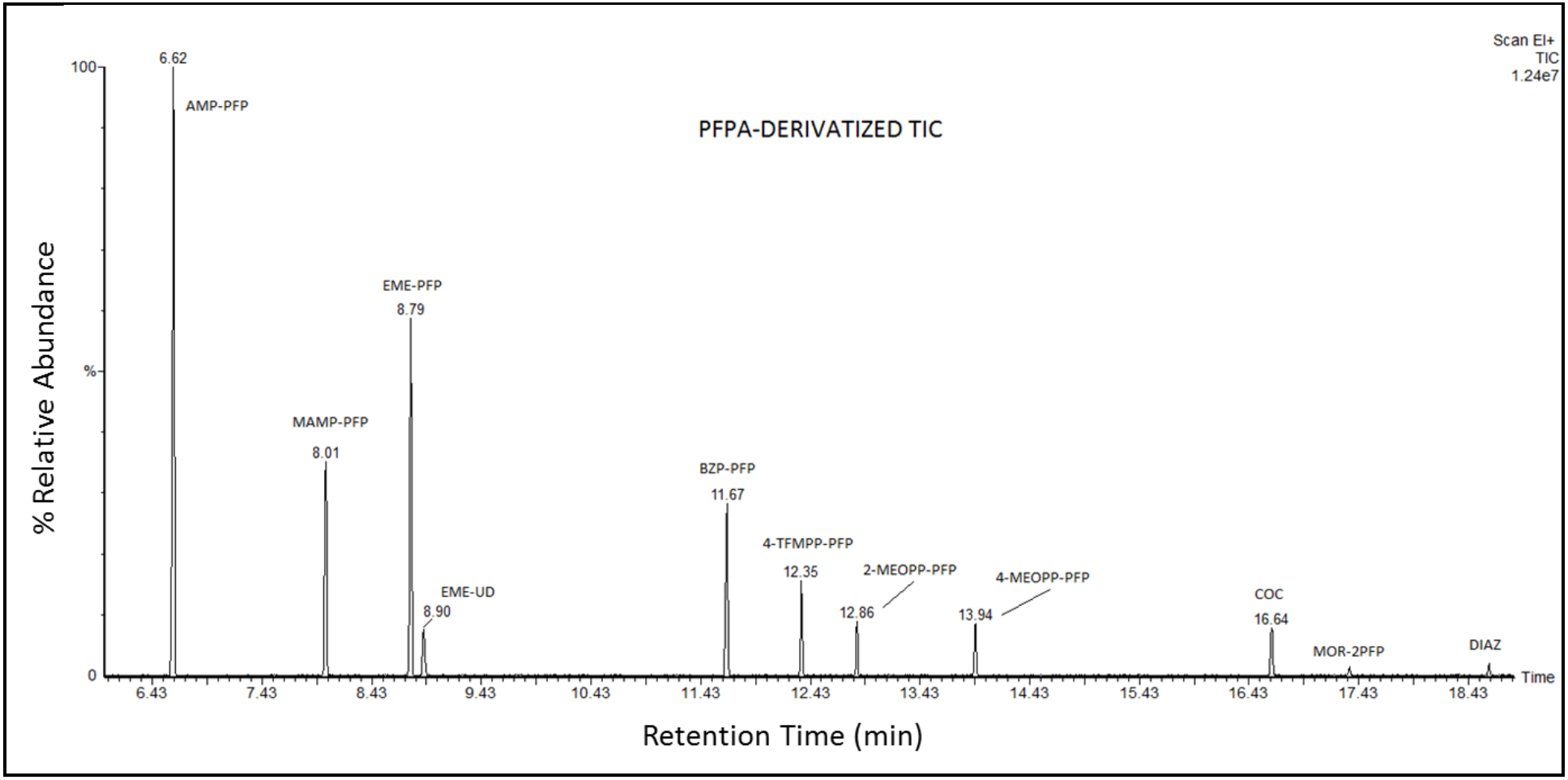


Figure 3.1: Total ion chromatogram of 10 PFPA-derivatized drugs reconstituted in ethyl acetate (UD = underivatized).

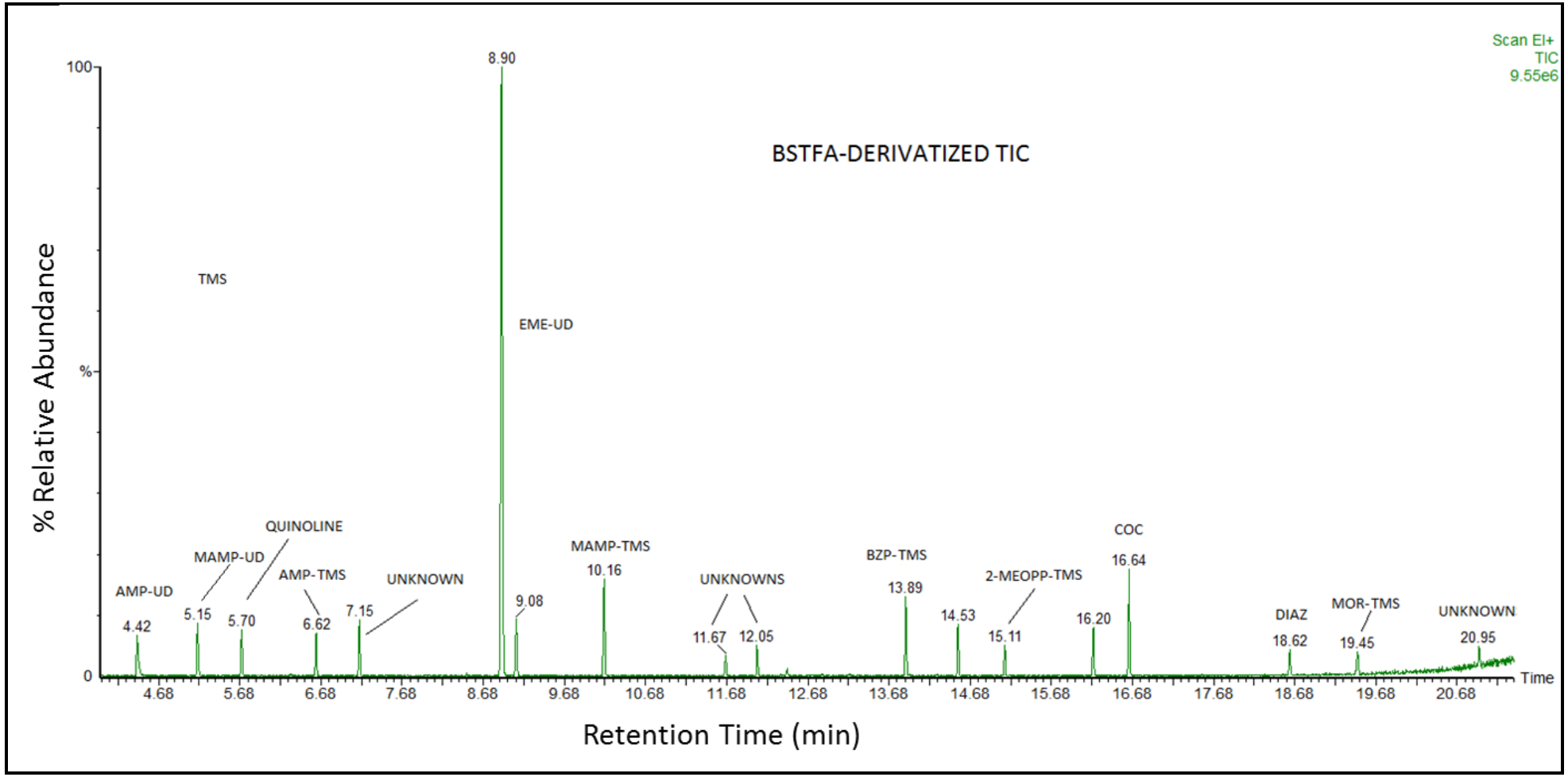


Figure 3.2: Total ion chromatogram of 10 BSTFA-derivatized drugs reconstituted in ethyl acetate (UD = underivatized).

On the other hand, drugs derivatized by silylation, especially BSTFA, had several extra unknown peaks in their TICs as well as peaks corresponding to underivatized AMP, MAMP and EME. For instance, the TIC for BSTFA had 19 peaks when only 11 drugs were in the original mixed standard (quinoline was initially added then discounted as an internal standard but is mentioned here as it contributed to the number of peaks observed).

While evaporation of the derivatizing reagent and reconstitution of the derivatives in a suitable organic solvent prior to instrumental analysis is essential for acylating reagents, it is not always necessary for silylating reagents (sections 1.6.2 & 1.6.3). Silylating reagents can be analysed directly after the derivatization reaction or can be diluted with a suitable solvents such as ethyl acetate, prior to analysis or during the derivatization reaction (Blau & Darbre, 1993; Evershed, 1993; Migowska, et al., 2012; Kumirska, et al., 2013). However, since all excess derivatizing reagents (including the silylation reagents) were evaporated and the derivatives reconstituted in ethyl acetate, a decision was made to conduct additional derivatization reactions for PFPA and BSTFA, without dilution with ethyl acetate or evaporation of the derivatizing reagent for the latter (Table 2.5, Part B in section 2.4.1.1). This was to eliminate any potential contribution of ethyl acetate to the presence of the underivatized products observed in the BSTFA TIC (Figure 3.1). Analysis for both derivatization reagents was conducted on a mixed drug standard containing additional drugs (i.e. 27) to also factor in the effect of a higher number of drugs as may be found in a waste water influent sample. Some of the analytes, such as quinoline, methadone, benzoylecgonine and cannabis, were subsequently discounted as target analytes in order to focus on the NPS. The drugs included in the additional preliminary derivatization reactions are listed in Figures 3.3 and 3.4.

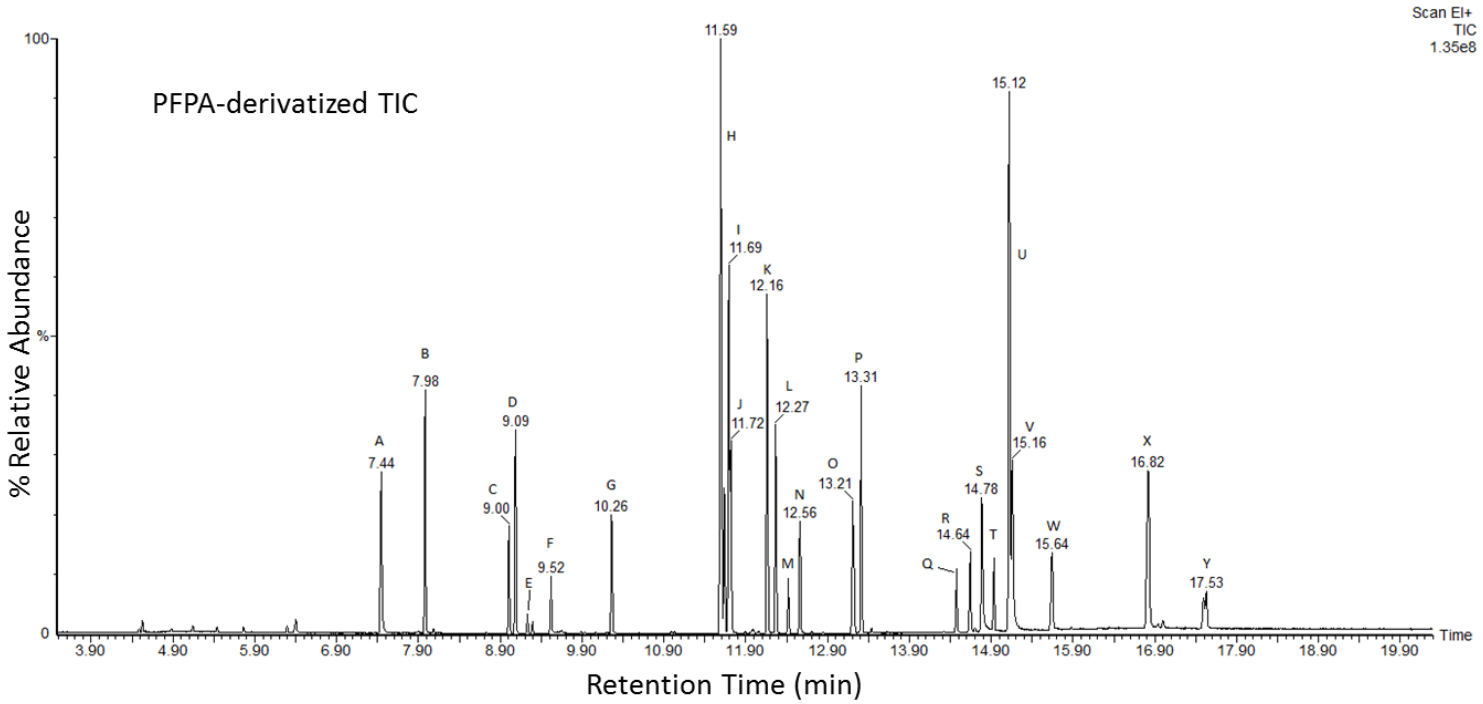


Figure 3.3: Total ion chromatogram of 27 PFPA-derivatized drugs and 5 internal standards reconstituted in ethyl acetate .
(A) Quinoline, (B) Amphetamine,-PFP (C) Methamphetamine-PFP, (D) 4-Fluoromethamphetamine-PFP, (E) 3-Fluoromethcathinone-PFP, (F) Ecgonine methyl ester-PFP, (G) Mephedrone-PFP, (H) 1-Benzylpiperazine-PFP & 4-(Fluorophenyl)piperazine-PFP, (I) 1-(3-Trifluoromethylphenyl) piperazine-PFP, (J) 3,4-Methylenedioxymethamphetamine-d_5-PFP & 3,4-Methylenedioxymethamphetamine-PFP, (K) Methylbenzodioxolylbutanamine-PFP, & 1-(4-Trifluoromethylphenyl) piperazine-PFP, (L) 1-(4-Methylphenyl)piperazine-PFP, (M) 1-(2-Methoxyphenyl) piperazine-PFP, (N) Butylone-PFP, (O) 1-(3-Chlorophenyl) piperazine -PFP & 1-(4-Methoxyphenyl) piperazine,-PFP (P) Ketamine-PFP, (Q) 11-Nor-9-carboxy- Δ9-tetrahydrocannabinol-PFP (R) Δ9-Tetrahydrocannabinol & Δ9-Tetrahydrocannabinol -d_3-PFP, (S) Methadone, (T) Cannabinol-PFP, (U) Amitriptyline, (V) Cocaine, & Cocaine-d_3 (W) Morphine -PFP & Morphine-d_3-PFP, (X) Diazepam & Diazepam-d_5, (Y) Underivatised 6-Monoacetylmorphine and Cannabinol.

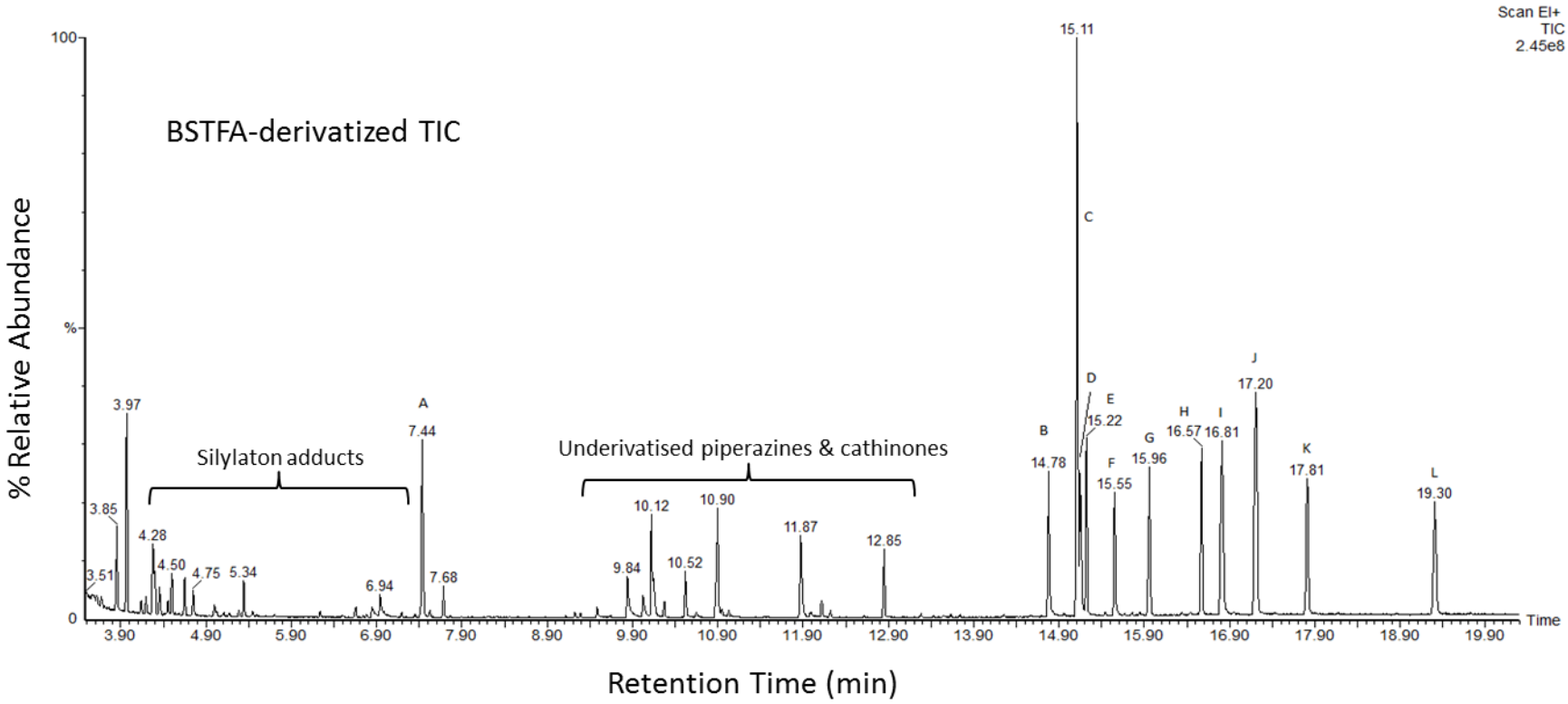


Figure 3.4: Total ion chromatogram of 27 BSTFA-derivatized drugs and 5 internal standards not reconstituted in ethyl acetate
(A) Quinoline, (B) Methadone, (C) Amitriptyline, (D) Cocaine, & Cocaine-d_3, (E) Cannabidiol-TMS, (F) Benzoylecgonine-TMS, (G) Δ9-Tetrahydrocannabinol-TMS & Δ9-Tetrahydrocannabinol -d_3-TMS, (H) Cannabinol-TMS, (I) Diazepam & Diazepam-d_5, (J) Morphine -TMS & Morphine-d_3-TMS, (K) 6-Monoacetylmorphine-TMS, (L) 11-Nor-9-carboxy- Δ9-tetrahydrocannabinol-TMS.

Figures 3.3 and 3.4 depict the chromatograms from the additional PFPA and BSTFA derivatization reaction, respectively. While fully derivatized MOR and 6-MAM peaks were observed for BSTFA as compared to PFPA, which had incompletely derivatized MOR and 6-MAM peaks, the TIC for BSTFA still had a number of extra unknown peaks compared with PFPA. In addition, phenylethylamines, piperazines and cathinones, the key target analytes, were not derivatized with BSTFA but were completely derivatized with PFPA. The heroin peak was not observed in either chromatogram.

Therefore, based on the number of derivative peaks observed and the higher number of completed derivatives for the key target analytes, the acylating reagents performed better than the silylating reagents, and were further assessed for suitability. The silylating reagents were eliminated from the method development. With multianalyte procedures, compromises are inevitable and therefore the derivatization reagent that gave better results for the emerging drugs of abuse (i.e. NPS) over the classic drugs of abuse was selected.

However, BSTFA appeared to perform better for opioids and cannabinoids and this will be taken into consideration for future analyses that specifically target these compounds.

To confirm whether PFPA was the most suitable of the acylating reagents, the RT and peak areas for the PFPA-, HFBA-, acetic anhydride:pyridine (AcAn:Pyr)-derivatives and underivatized drugs are listed in Table 3.1. PFPA-derivatives had higher peak areas than both HFBA- and AcAn:Pyr-derivatives for all the drugs. However, COC and DIAZ had higher peak areas in the underivatized chromatogram than for the acylated products. As both COC and DIAZ do not have derivatizable groups, perhaps the derivatization process causes some degradation of these products e.g. hydrolysis in the presence of ethyl acetate and anhydride (PFPA). AcAn:Pyr did not have a peak corresponding to EME-UD indicating complete derivatization of EME in contrast to PFPA and HFBA in which EME-UD could still be detected. MOR was not detected in the AcAn:Pyr and underivatized chromatogram indicating that derivatization with the appropriate reagent was particularly essential for its detection. Therefore, based on a combination of peak area and number of drugs fully derivatized, PFPA was the most

effective derivatizing reagent and could have been selected as the derivatizing reagent at this stage.

Table 3.1: Retention time and peak area of acylated drug derivatives and underivatized drugs.

DRUG	RT (min) & PEAK AREA	PFPA	HFBA	AcAn:Pyr	UNDERIVATIZED
AMP	RT	6.62	7.16	9.09	4.41
	Peak Area	266378	97049	47275	7992
MAMP	RT	8.01	8.48	10.17	5.21
	Peak Area	93944	28774	22659	36344
EME	RT	8.79	9.26	10.52	NA
	Peak Area	132914	38201	54279	NA
EME-UD	RT	8.90	8.90	ND	8.90
	Peak Area	23966	1771	ND	117238
BZP	RT	11.67	12.06	13.89	9.05
	Peak Area	74871	19912	8454	7049
4-TFMPP	RT	12.35	12.72	14.53	10.14
	Peak Area	34404	3888	3461	10306
2-MeOPP	RT	12.86	13.19	15.11	10.67
	Peak Area	20996	3750	2425	1863
4-MeOPP	RT	13.94	14.27	16.20	12.00
	Peak Area	21877	4782	3294	1477
COC-UD	RT	16.64	16.64	16.64	16.64
	Peak Area	23393	2974	1572	29434
MOR	RT	17.35	17.49	ND	ND
	Peak Area	3705	120	ND	ND
DIAZ-UD	RT	18.62	18.62	18.62	18.62
	Peak Area	4835	697	717	8277

AcAn:Pyr = Acetic anhydride: pyridine; UD = underivatized; ; NA = not applicable; ND = not detected

However, in addition to peak area it was necessary to also evaluate the selectivity of the *m/z* ions contributing to the peak areas for the acylating reagents. Figure 3.5 shows the mass spectra for acylation products for 4-MEOPP.

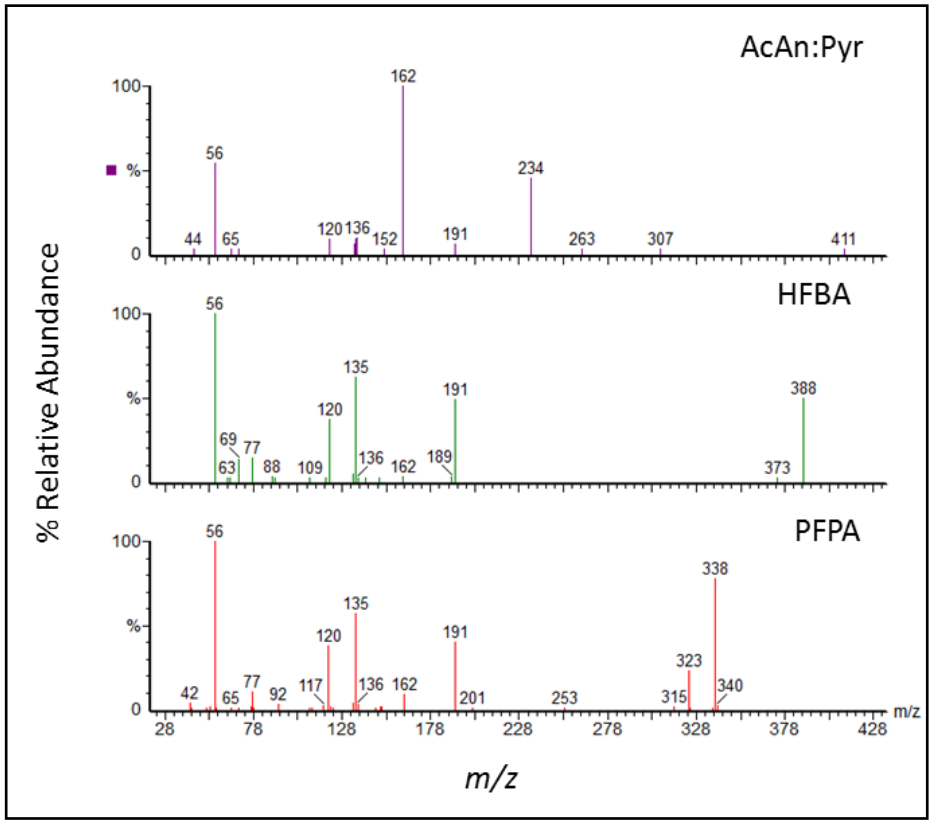


Figure 3.5: Mass spectra of AcAn:Pyr-, HFBA- and PFPA-derivatives of 4-MEOPP.

The quantifier ion for 4-MEOPP when AcAn:Pyr is used as a derivatizing reagent is *m/z* 162 while that for HFBA and PFPA are *m/z* 338 and *m/z* 388, respectively. Therefore, the mass spectra for PFPA- and HFBA-derivatives resulted in ions with higher *m/z* values than for AcAn:Pyr. This was seen to be an advantage especially since waste water contains a multitude of unknown compounds and the aim was to try and distinguish the target drugs from other matrix components, after extraction and derivatization, to aid with positive identification.

Overall, the fragmentation patterns for PFPA and HFBA were very similar differing only in the last two *m/z* ions. In the example of 4-MEOPP, PFPA- and HFBA-derivatives share the 56, 120, 135 and 191 *m/z* ions but differ in the molecular ion *m/z* i.e. 338 and 388, respectively. As a result, PFPA was selected as the derivatizing reagent based on a combination of highest peak areas (intensity) and mass spectra

that were distinctive enough. Other observations made that helped guide the selection of PFPA for derivatization were that PFPA-products had the lowest RTs (Table 5.1), which help reduce the analysis time, and evaporation of the derivatizing reagent took half the time of that for the AcAn:Pyr mixture.

To further confirm the informed decision to proceed with derivatization as opposed to not derivatizing at all, the TIC and mass spectra of PFPA-derivatized and underivatized drugs was compared (Figure 3.6).

Aside from the higher intensity of the PFPA-derivatized peaks (Table 3.1), the method resulted in peak separation for all 11 analytes (including quinoline). In addition, the RT increased as a result of a higher molar mass and hence slightly longer interaction of the drug with the stationary phase.

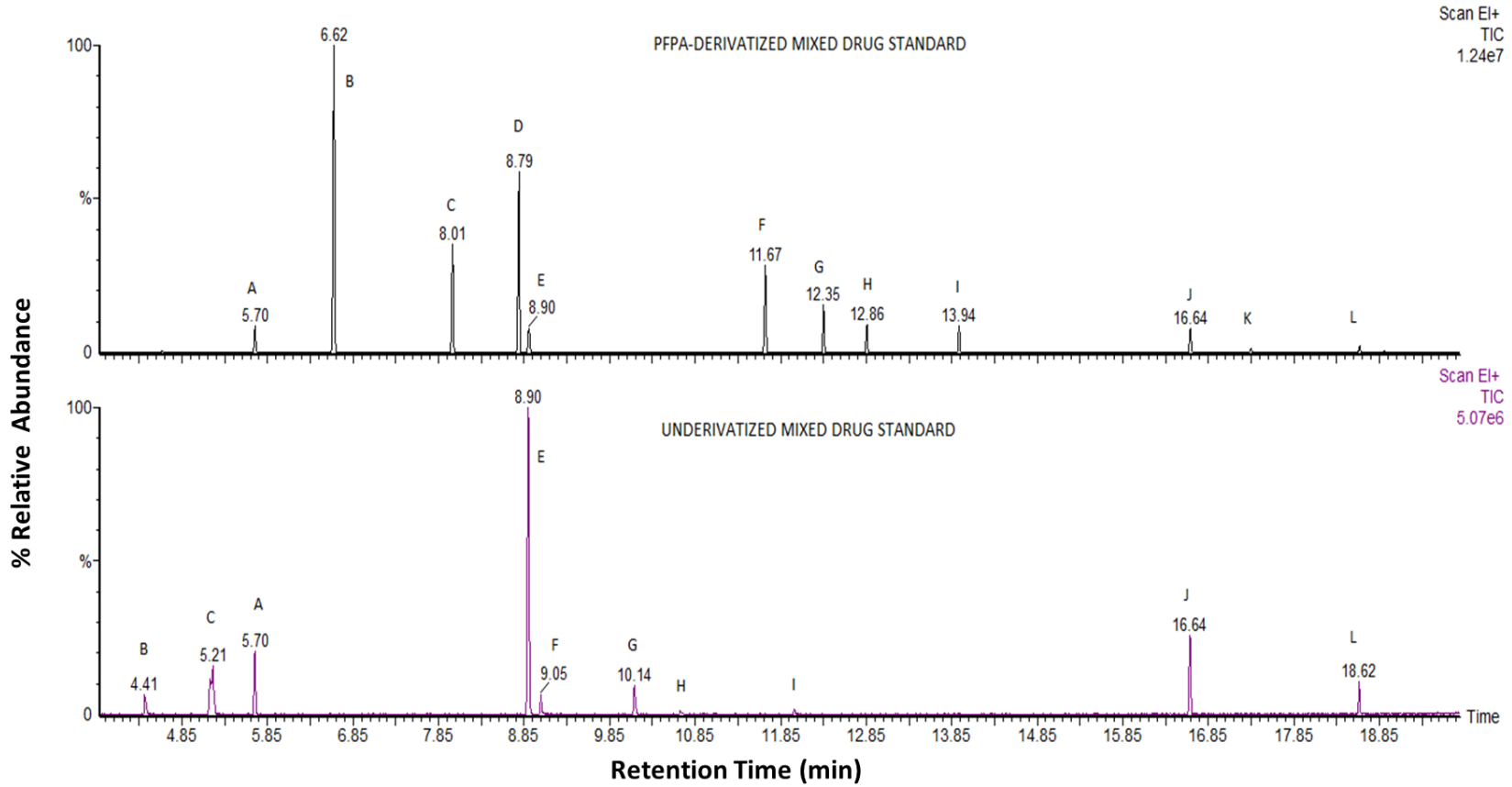


Figure 3.6: Total ion chromatogram of PFPA-derivatized and underivatized mixed drug standard.
(A) Quinoline, (B) Amphetamine, (C) Methamphetamine, (D) Ecgonine methyl ester, (E) Ecgonine methyl ester -underivatized, (F) Benzylpiperazine, (G) 4-trifluoromethylphenylpiperazine, (H) 2-methoxyphenylpiperazine, (I) 4-methoxyphenylpiperazine, (J) Cocaine, (K) Morphine (L) Diazepam.

An example of the derivatization reaction for BZP showing a higher molar mass of the derivative is depicted in Figure 3.7. The molar masses of derivatives for the remaining target analytes can be found in Appendix VI a-f.

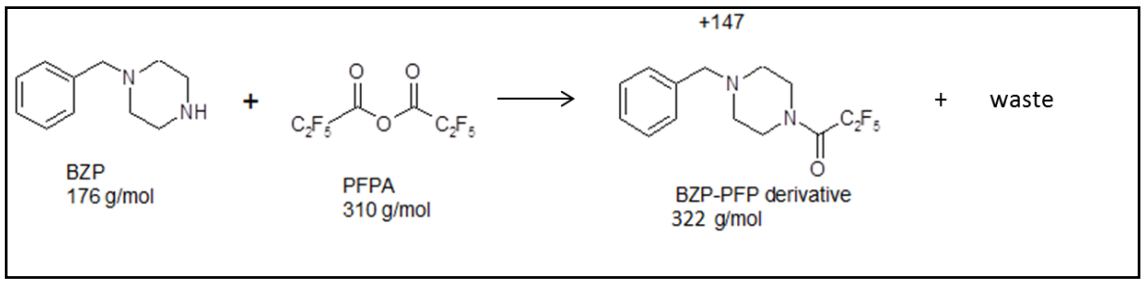


Figure 3.7: PFPA derivatization reaction for BZP showing increased molar mass of derivative.

In addition to higher peak areas, derivatization led to improved peak shape and mass spectra as opposed to underivatized drugs. This is depicted in Figure 3.8, using 4-TFMPP as an example.

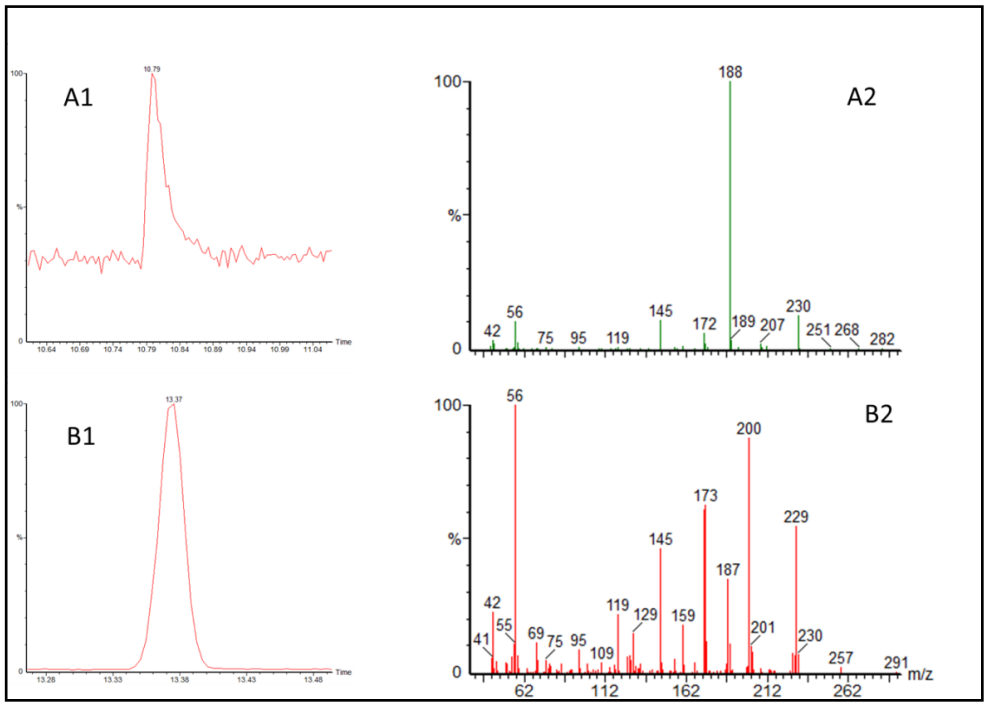


Figure 3.8: Total ion chromatogram and mass spectra for underivatized (A1 and A2) and derivatized (B1 and B2) 4-TFMPP showing improved peak shape and mass spectrum for the latter.

In summary, PFPA was selected as the derivatizing reagent based on a combination of highest peak areas (intensity), one derivative per drug added, mass spectra that were distinctive enough to enable selectivity in complex matrices, better peak shape (versus underivatized) and lowest RT.

After selection of PFPA as the derivatization reagent for the method under development, the next step was to optimise the reaction temperature and time for the target drugs. The results are discussed in section 3.2.2.

3.2.2 Optimisation of PFPA Derivatization Reactions

Since earlier PFPA derivatization studies conducted at 70 °C for 60 min had not resulted in the complete derivatization of EME (Table 3.1 and Figure 3.1), a decision was taken to perform further derivatization studies at higher reaction temperatures of 80 and 90 °C. The aim of selecting higher temperatures was, therefore, an attempt to improve the degree of conversion as well as reduce the derivatizing time.

Following the procedures as detailed in section 2.4.1.2, Figures 3.9, 3.10 and 3.11 depicts the results of drugs and internal standards derivatized at 80 and 90 °C. The derivatization reaction was regarded as complete when there was no change in peak area with increasing time at each temperature (Lacina, et al., 2013). In addition, the number of peaks in the chromatogram and the presence of relevant diagnostic ions for the derivatives were used to assess the completion of the derivatization reaction (section 1.6.4).

In the chromatogram for the drugs and internal standards derivatized at 80 °C (Figure 3.9), the majority of peak areas continued to increase with time indicating that perhaps the derivatization reaction was still progressing even after 45 min. The only exceptions were 4-MPP and 4-MEOPP which were fully derivatized by 15 min. When derivatized at 90 °C (Figure 3.10), most drugs and internal standards, including EME, had been fully derivatized by 30 min indicating complete derivatization. Figure 3.11 shows the expanded graphs for 3-FMC. The opioids (MOR, 6-MAM and heroin) were not detected at 80 and 90 °C during this derivatization trial, possibly due to low

instrumental response, and hence were analysed separately to better understand their interactions (section 3.2.3).

Since internal standards would subsequently be used in the calculation of the peak area ratio (PAR), the internal standard itself needs to be completely derivatized under the conditions of testing in order to obtain reliably quantifiable results. The internal standards, AMP-d_6, MDMA-d_5, COC-d_3, and MOR-d_3 were added to the mixed drug standard and evaluated simultaneously as detailed in section 2.4.1.2. The graphs from the derivatization of the internal standards are included in Figures 3.9 and 3.10. At 80 °C, the peak areas for AMP-d_6, MDMA-d_5, and COC-d_3 also increased with derivatization time even up to 45 min (COC-d_3 was included in the study even though it does not get derivatized). At 90 °C the maximum peak area was achieved by the 30 min reaction time for AMP-d_6, MDMA-d_5, and COC-d_3. Although MOR-d_3 was also added, it was not detected as per other opioids (section 3.2.2). Therefore, these results indicate that the internal standards, AMP-d_6 and MDMA-d_5 were fully derivatized at 90 °C after a 30 min reaction time (the same time as the drugs in the mixed standard) and hence were suitable for use in calculating the PAR. The use of the PAR of the target analytes, with respect to their assigned internal standard, is standard practice and has been used in various publications to assess different validation parameters and for quantification (Al-Asmari & Anderson, 2007; Saito, et al., 2007; Capriotti, et al., 2013; Furey, et al., 2013; Belsey, et al., 2014; Carmona, et al., 2014; Kankaanpää, et al., 2014; Negreira, et al., 2014)

The use of high reaction temperature was a concern as the stability of PFPA and the drugs at that temperature had not been evaluated by the author. However, based on the work of other researchers who derivatized similar drugs at 90 °C and 100 °C using PFPA (Wang, et al., 2006; Damm, et al., 2009) as well as the evaluation of the reaction products (one single derivative, high peak area) during this research, the PFPA derivatives were considered to be stable at the reaction conditions.

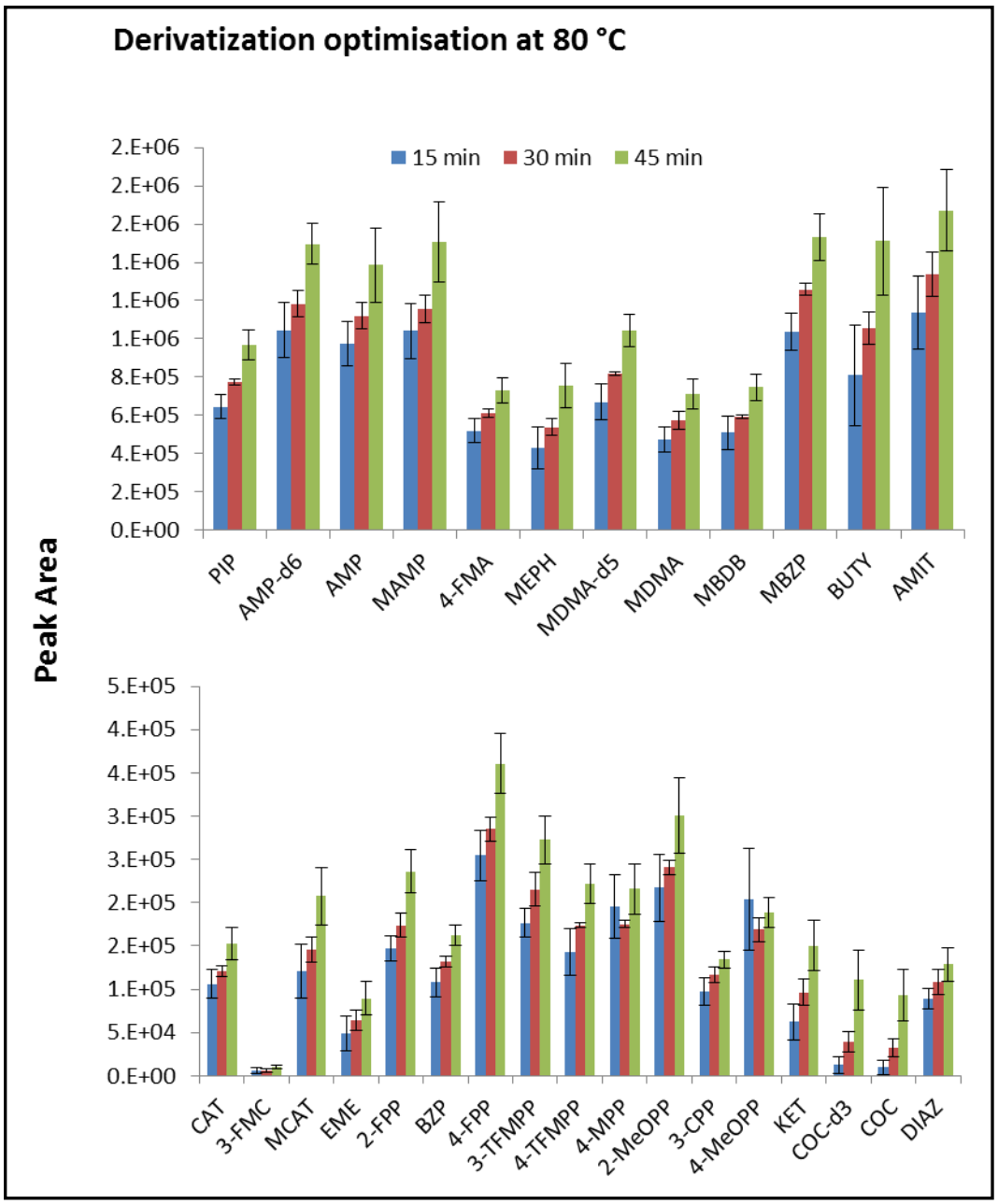


Figure 3.9: Graphs showing derivatization optimisation results for various drugs at 80 °C. Error bars represent standard deviation at n = 3.

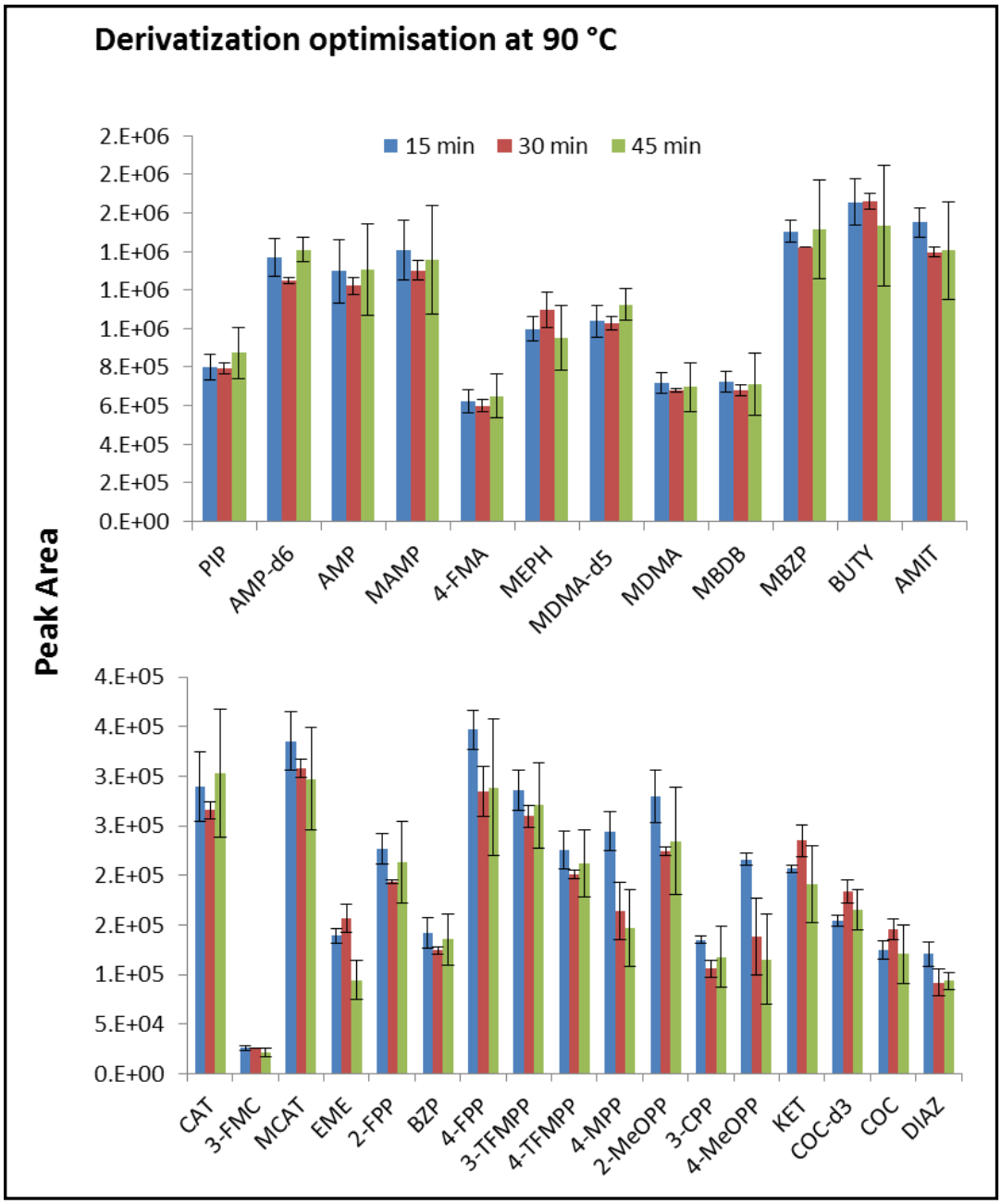


Figure 3.10: Graphs showing derivatization optimisation results for various drugs at 90 °C. Error bars represent standard deviation at n = 3.

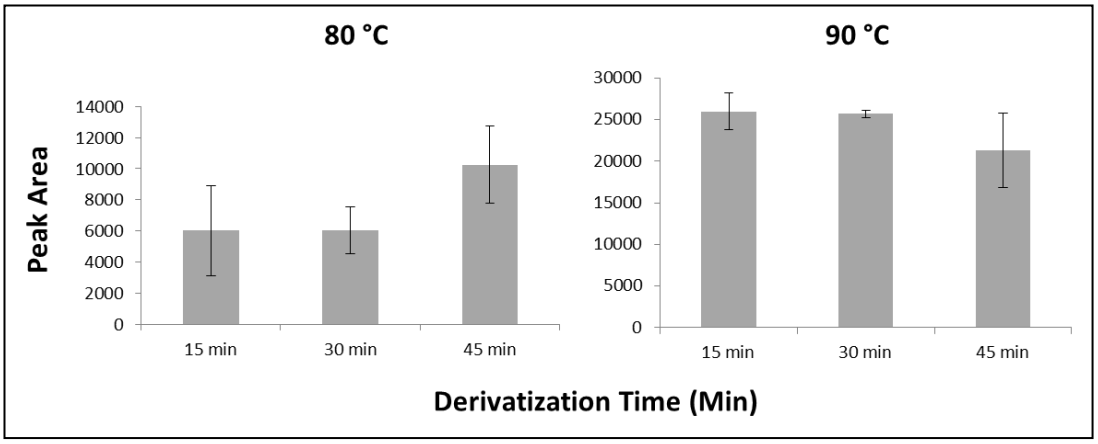


Figure 3.11: Graphs showing derivatization optimisation results for 3-FMC. Error bars represent standard deviation at n = 3.

Therefore, in this research, optimisation of derivatization was conducted by varying the reaction temperature and time and finding the optimal combination of these two factors (Kumirska, et al., 2013; Lacina, et al., 2013). Based on the results from the detected drugs in the mixed standard (the exceptions were MOR, 6-MAM and heroin), the derivatization conditions of 90 °C for 30 min was selected as the most suitable for the method under development.

3.2.3 Derivatization Reactions for a Mixed Opiate Standard

Since all opioids were not detected in the derivatization optimisation studies for the mixed drug standard (section 3.2.2), a decision was made to further investigate the derivatization reactions for opioids (MOR, 6-MAM and heroin) separately as these were essential target drugs and metabolites for quantification in waste water.

Following the procedures as detailed in section 2.4.1.3, the RT and diagnostic ions of the opiate drugs and derivatives are listed in Table 3.2. In this first part of the opiates derivatization trial, only MOR and 6-MAM were included in the mixture. Heroin was excluded so as to remove its possible contribution to both MOR and 6-MAM through hydrolysis (Guillot, et al., 1997).

Table 3.2: Retention time and diagnostic ions of opiate drugs and derivatives.

DRUG/DERIVATIVE	RT (min)	DIAGNOSTIC IONS (*m/z*)
MOR-2PFP	20.36	<u>414</u>, 577
MOR-PFP	20.66	<u>268</u>, 431
6-MAM-PFP	21.62	<u>414</u>, 473
6-MAM	23.32	<u>268</u>, 327

Quantifier ion underlined

Four peaks were observed in the chromatogram at different RTs (Table 3.2). Mass spectra (SIM) of the peaks corresponded with diagnostic ions for MOR-2PFP, MOR-PFP, 6-MAM-PFP and 6-MAM even after a 60 min reaction time.

MOR has two derivatizable sites (Fig 1.3). When both sites are derivatized, this is denoted as -2PFP and when only one site is derivatized, this is denoted as –PFP. The mass spectra and proposed fragmentation pattern for both MOR-2PFP and MOR-PFP are depicted in Figure 3.12.

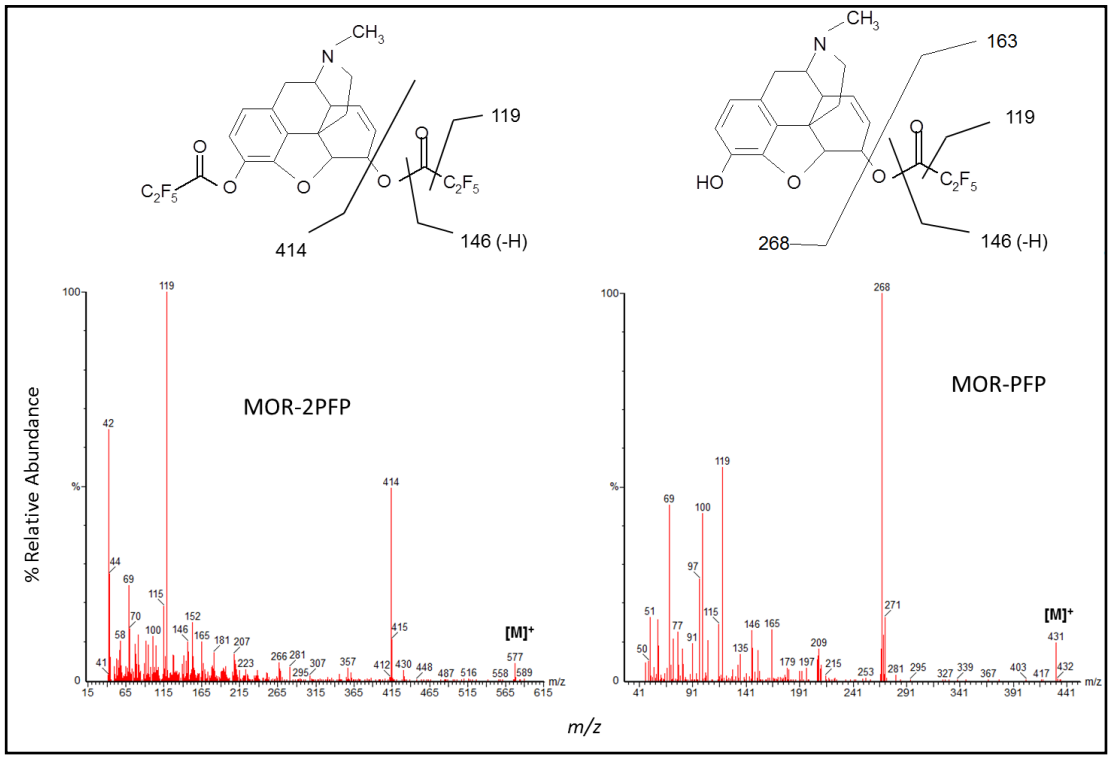


Figure 3.12: Mass spectra and fragmentation patterns for MOR-2PFP and MOR-PFP.

In addition, the PAR for 6-MAM-PFP, 6-MAM, MOR-2PFP and MOR-PFP was plotted against the reaction time and the results are depicted in Figure 3.13. The PAR was calculated by dividing the peak area of the drug with the peak area of the internal standard. In this instance, COC-d_3 was used as the internal standard for the calculation of PAR since MOR-d_3 was also not detected in earlier studies (section 3.22).

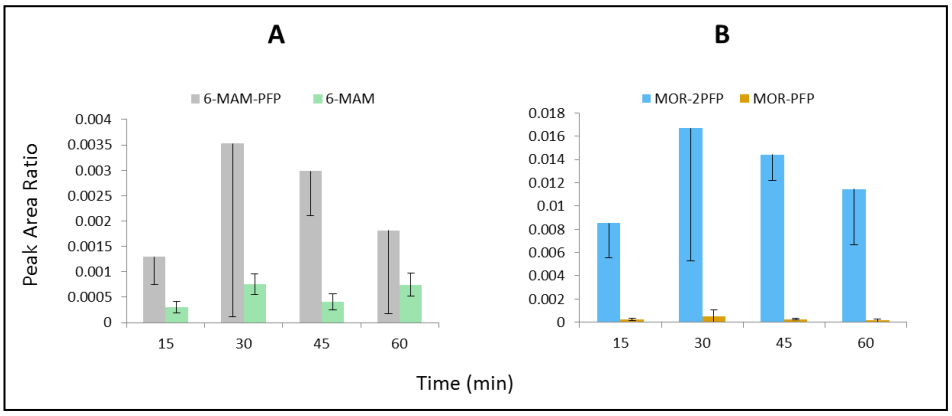


Figure 3.13: Graphs of PAR versus reaction time for (A) 6-MAM-PFP and 6-MAM and (B) MOR-2PFP and MOR-PFP. Error bars represent standard deviation at n=3.

All four compounds reach their maximum PAR at 30 min after which the PAR decreases, possibly due to degradation. Greater standard deviations were observed for fully derivatized morphine (MOR-2PFP) and 6-monoacetylmorphine (6-MAM-PFP), especially at 30 min possibly due to degradation reactions being more pronounced at the 30 min time point leading to greater fluctuations in detector response. However, higher PARs occurred for the fully derivatized analogues (6-MAM-PFP and MOR-2PFP) than for the underivatized (6-MAM) or partially derivatized (MOR-PFP) analogues throughout the time frame of analysis. This would indicate that at any one time during the reaction, the derivatized analogues were present at much higher concentration (indicated by the PAR) than the underivatized analogues and therefore the reaction favoured the fully derivatized product. This has been represented in Figures 3.14 and 3.16-B.

Two possible explanations are suggested by the author for the observance of both underivatized and derivatized 6-monoacetylmorphine (6-MAM and 6-MAM-PFP, respectively). These are depicted in Figures 3.14 and 3.15.

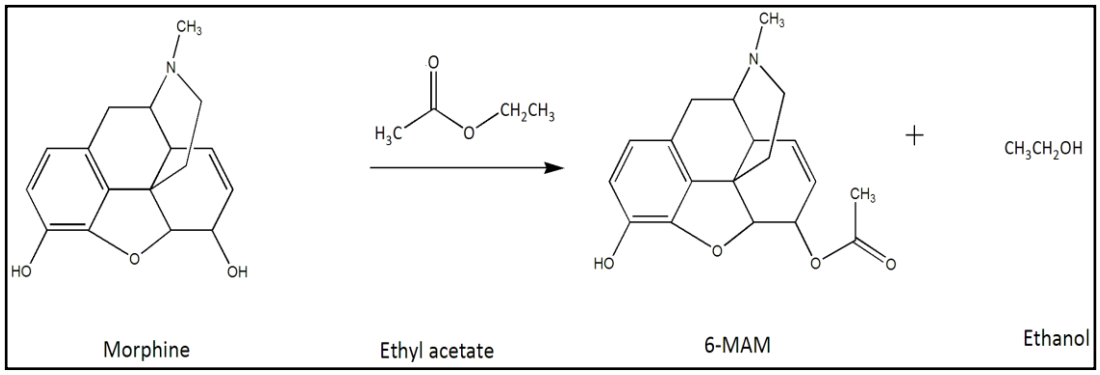


Figure 3.14: Proposed partial acetylation of MOR to 6-MAM in the presence of ethyl acetate.

In Figure 3.14, the MOR present in the mixture is partially acetylated to 6-MAM with ethanol as a side-product. As no heroin was detected, it is unlikely that further acetylation occurred to form heroin (unless it was present below the LOD).

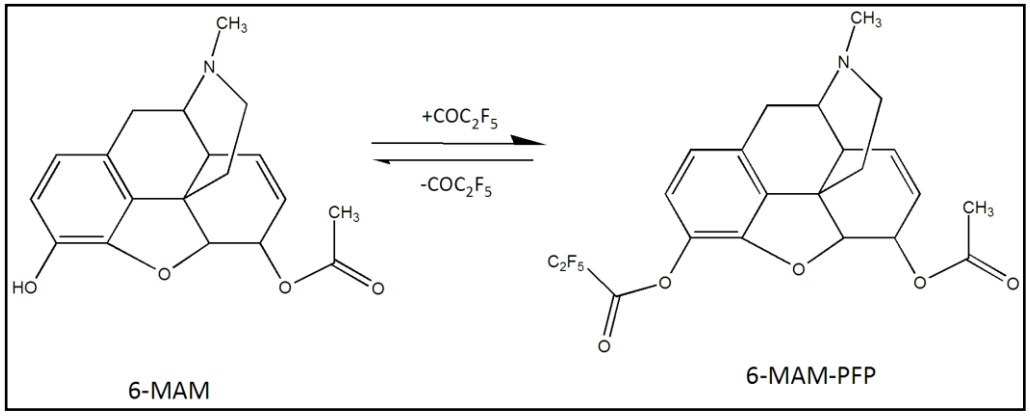


Figure 3.15: Proposed reversible reaction between underivatized 6-MAM and the derivatized product (6-MAM-PFP).

In Figure 3.15, the reaction between 6-MAM and the acylating agent is reversible, leading to a mixture containing both derivatized and underivatized products.

With regard to morphine, the presence of both partially derivatized (MOR-PFP) and fully derivatized (MOR-2PFP) products suggest the following: (1) partial derivatization of MOR; (2) de-acetylation of MOR-2PFP to MOR-PFP. These reactions are depicted in Figure 3.16 as A and B, respectively.

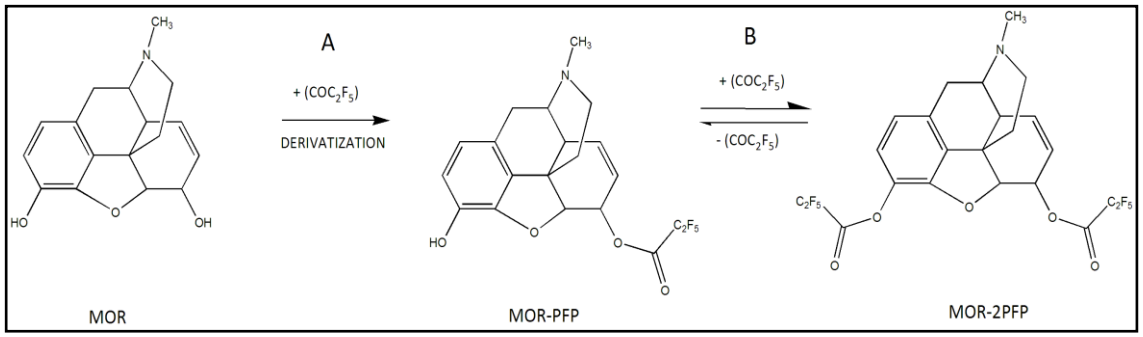


Figure 3.16: Proposed partial (MOR-PFP) and full (MOR-2PFP) derivatization of morphine.

Since no underivatized MOR was detected, the most likely explanation for the presence of MOR-PFP is therefore cleavage of one -PFP moiety from MOR-2PFP in a reversible reaction (B in Figure 3.16).

3.2.3.1 Individual Analysis of MOR, 6-MAM and Heroin

To understand whether the observations mentioned above were once-off or regular occurrences, the opioids, MOR, 6-MAM and heroin, were analysed individually at much higher concentrations (100 µg/mL) according to the procedure in section 2.4.1.4. The chromatograms were assessed for all peaks as observed in Table 3.2 plus the heroin diagnostic ions *m/z* 327 and 369. Similar results as discussed above (Section 3.2.3) were observed. For the morphine standard, MOR-2PFP was found to occur together with MOR-PFP confirming the reaction in Figure 3.16-B. Heroin does not have derivatizable functional groups (Figure 3.6) but it was found to occur with underivatized 6-monoacetylmorphine (6-MAM). This indicates that heroin had most likely undergone hydrolysis to 6-MAM (Figure 3.17).

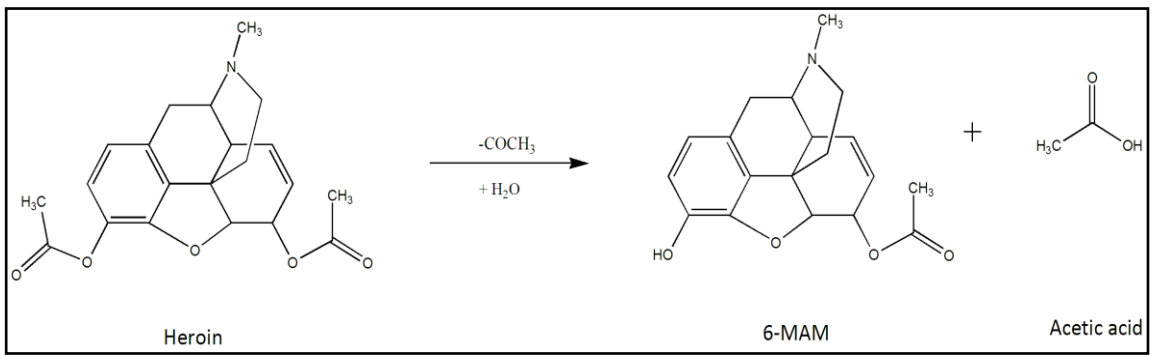


Figure 3.17: Hydrolysis of heroin to 6-MAM.

With regard to 6-MAM, both 6-MAM-PFP and 6-MAM were detected confirming earlier suggestions of the cleavage of the -PFP moiety (Figure 3.15). Therefore, underivatized 6-MAM occurred when both heroin and 6-MAM-PFP were analysed separately indicating that hydrolysis to 6-MAM was the favoured reaction (Huizer & Poortman, 1989).

The opioids investigated have hydroxyl and/or ester functional groups, which are prone to transacetylation, hydrolysis or esterification when dissolved in methanol or ethyl acetate and injected into the GC-MS, depending on which other drugs are present in the mixture. Heroin has long been known to hydrolyse to 6-MAM or MOR (Fig 3.17) during GC-MS analysis due to various factors such as the solvent used, derivatization reagent used, injection process, use of glass wool in the injection liners and the presence of oxygen or water in the carrier gas (Huizer & Poortman, 1989; Guillot, et al., 1997; Cole, 2003; Flanagan, et al., 2007). On the other hand MOR is known to undergo esterification to 6-MAM or heroin (Fig 3.14) (Flanagan, et al., 2007). Although published hydrolysis and esterification reactions have been reported for underivatized opioids especially when methanol is used as a solvent, observations from this research indicate that these reactions also occur even when MOR and 6-MAM are derivatized with PFPA in the presence of ethyl acetate. Both methanol and ethyl acetate are commonly used as reconstitution solvents for GC-MS analysis after evaporation of the acylation reagent (Baptista, et al., 2002; Damm, et al., 2009; Migowska, et al., 2012). The reactions depicted in Figures 3.14 to 3.16 are theoretical proposals based on observations made during this research and the exact reaction mechanism is unknown at the time of writing of this thesis. However, suggestions include the influence of PFPA, use of glass wool in injection liners and ethyl acetate (Huizer & Poortman, 1989).

Therefore, what was initially thought as incomplete derivatization of MOR, and 6-MAM could very well be due to transacetylation, esterification and hydrolysis reactions. These reactions can result in fluctuations in the concentration of opioids and lead to under or over-reporting of concentrations depending on which reaction is occurring at the time of analysis. Some suggestions of avoiding transacetylation are by using silylating agents for derivatization (Huizer & Poortman, 1989) or using

on-column injection or programmable temperature injection to avoid degradation during injection (Guillot, et al., 1997). These unwanted reactions observed for the opioids were discovered later on during the method development and may account for some of the observations made during stability and recovery studies as discussed in sections 3.3 and 3.4.2.1, respectively. As noted earlier on in this section, although the PARs for the derivatized products were much higher than for the underivatized products (Figure 3.14), the impact of these reactions on the total amount of MOR detected would need to be taken into consideration. Further work would take account of these reactions and the suggested ways of reducing or preventing them.

3.2.4 Final Derivatization Conditions

In light of the results obtained in sections 3.2.2 and 3.2.3 above, the optimal derivatization conditions for the drugs under analysis and their respective internal standards were: 0.1 mL PFPA: ethyl acetate (2:1 v/v) at 90 °C for 30 min. The final derivatization conditions were the most ideal for the mixed drug standard but not necessarily the best for some individual drugs. For instance, most drugs could have been derivatized at 70 °C or 80 °C for 15-30 min (Dickson, et al., 2010a) but the presence of EME, MOR and 6-MAM which were not fully derivatized (or as later found out were undergoing degradation reactions) meant a higher temperature of 90 °C was used.

3.2.5 Diagnostic Ions and Mass Spectra

Figure 3.18 depicts the TIC of the PFPA-derivatized drugs and their internal standards. The careful evaluation of chromatograms for extra peaks was continually made throughout the method development to ensure the optimised method was suitable even when different concentrations and combinations of drugs were used. As indicated in Figure 3.18, extra peaks could be due to underivatized drugs, column bleed, artefacts or adducts and hence they needed to be categorized.

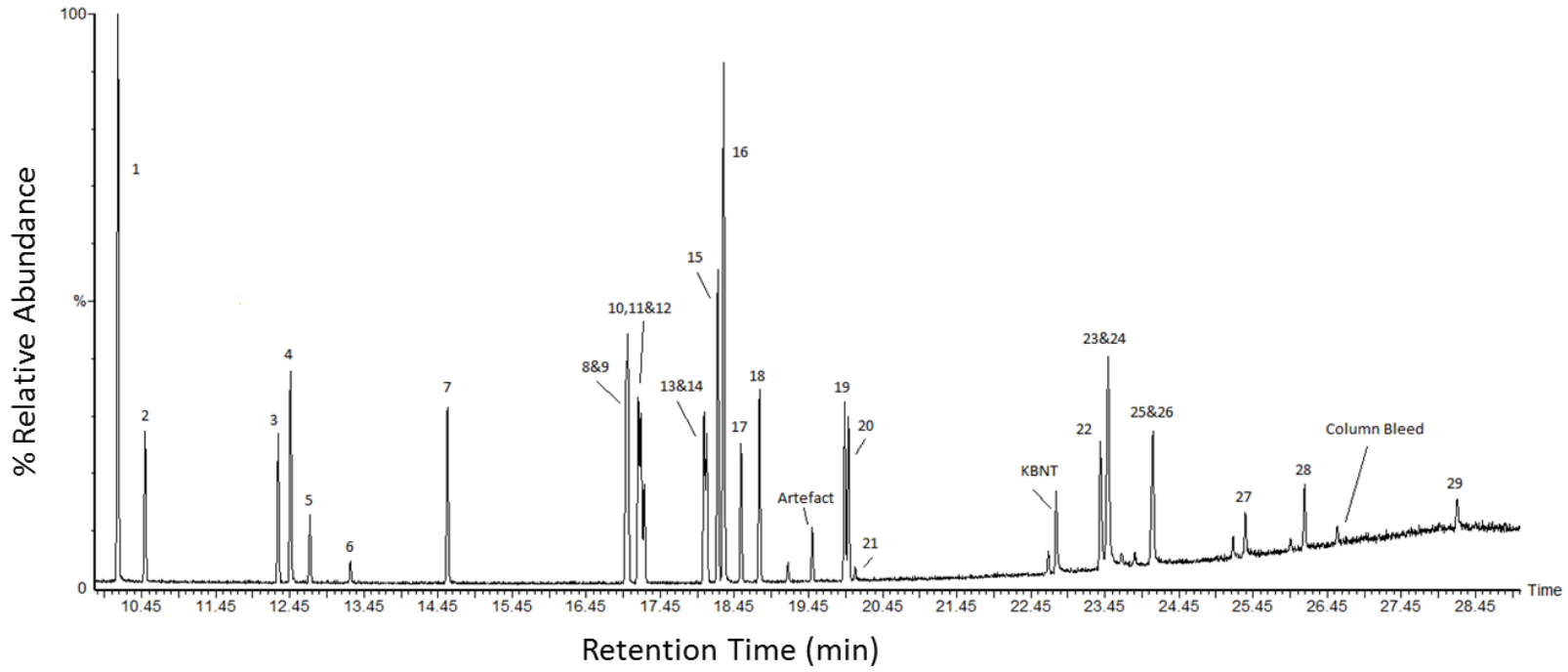


Figure 3.18: Total ion chromatogram of a derivatized mixed drug standard with internal standards.
(1) Piperazine , (2) Amphetamine, (3) Methamphetamine, (4) 4-fluoromethamphetamine, (5) 3-fluoromethcathinone, (6) Ecgonine methyl ester, (7) Mephedrone, (8) Benzylpiperazine, (9) 4-fluorophenylpiperazine, (10) 3-trifluoromethylphenylpiperazine, (11) 3,4-methylenedioxymethamphetamine-d_5, (12) 3,4-methylenedioxymethamphetamine, (13) Methylbenzodioxolylbutanamine, (14) 4-trifluoromethylphenylpiperazine, (15) 4-methylphenylpiperazine, (16) Methylbenzylpiperazine, (17) 2-methoxyphenylpiperazine, (18) Butylone, (19) 3-chlorophenylpiperazine, (20) 4-methoxyphenylpiperazine, (21) Ketamine, (22) Amitriptyline, (23) Cocaine, (24) Cocaine-d_3, (25) Morphine, (26) Morphine-d_3, (27) 6-monoacetylmorphine, (28) Diazepam, (29) Heroin. KBNT = known but not target drug.

Table 3.3 lists the RT, retention index (RI), internal standards and diagnostic ions for the PFPA-derivatized drugs which were used during preliminary studies, method validation (Chapter 6) and standard addition (Chapter 7).

Table 3.3: Retention time, retention index, internal standard and diagnostic ions for derivatized drugs.

DRUG STANDARD	Diagnostic Ions (m/z)	RT (min)	RI	Internal Standard	References for Diagnostic Ions
PIP	202, 231, 259	9.76	1241	MDMA-d_5[1]	N/Av
AMP-d_6	93, 123, 194	10.06	1252	N/Av	N/Av
AMP	91, 118, 190	10.11	1254	MDMA-d_5[1]	Saito, et al., 2007
MAMP	118, 119, 160, 204	11.73	1408	MDMA-d_5[1]	Saito, et al., 2007
4-FMA	119,136, 160, 204	11.89	1413	MDMA-d_5[1]	N/Av
CAT	77, 105, 119	11.96	1415	MDMA-d_5[1]	N/Av
3-FMC	119,123, 160, 204	12.14	1421	MDMA-d_5[1]	N/Av
MCAT	77, 105, 160, 204	12.40	1431	MDMA-d_5[1]	N/Av
EME	82, 94, 182	12.64	1438	COC-d_3	Saito, et al., 2007
MEPH	119, 160, 204	13.92	1482	MDMA-d_5[1]	Dickson, et al, 2010b
2-FPP	150, 179, 326	15.51	1638	MDMA-d_5	N/Av
BZP	91, 146, 175	16.27	1665	MDMA-d_5	Dickson, et al, 2010a
4-FPP	150, 179, 326	16.29	1666	MDMA-d_5	N/Av
3-TFMPP	200, 229, 376	16.41	1671	MDMA-d_5	Maher, et al., 2009
MDMA-d_5	136, 163, 208	16.46	1672	NA	Saito, et al., 2007
MDMA	160, 162, 204	16.51	1674	MDMA-d_5	Saito, et al., 2007
MBDB	160, 176, 218	17.31	1804	MDMA-d_5	Thigpen, et al., 2007
4-TFMPP	200, 229, 376	17.34	1805	MDMA-d_5	Maher, et al., 2009
4-MPP	146, 175, 322	17.49	1811	MDMA-d_5	N/Av
MBZP	105, 231, 336	17.56	1813	MDMA-d_5	N/Av
2-MEOPP	162, 191, 338	17.79	1823	MDMA-d_5	N/Av
BUTY	149, 160, 218	18.04	1833	MDMA-d_5	N/Av
3-CPP	166, 195, 342	19.18	1878	MDMA-d_5	Dickson, et al, 2010a
4-MEOPP	162, 191, 338	19.23	1880	MDMA-d_5	N/Av
KET	160, 312, 320	19.32	1883	MDMA-d_5	N/Av
AMIT	58, 115, 202	22.60	2226	MDMA-d_5	N/Av
COC-d_3	85, 105, 185	22.68	2230	NA	N/Av
COC	82, 105, 182	22.69	2231	COC-d_3	NIST
MOR-d_3	119, 149, 417	23.28	2258	COC-d_3	N/Av
MOR	119, 146, 414	23.30	2260	COC-d_3	Saito, et al., 2007
6-MAM	204, 414, 473	24.53	2419	COC-d_3	Saito, et al., 2007
DIAZ	256, 283, 285	25.32	2459	MDMA-d_5	NIST
HEROIN	204, 268, 327, 369	27.43	2660	COC-d_3	NIST

Quantifier ion is underlined; N/Av = Not available; NA = not applicable; [1]replaced with AMP-d_6 during method validation

The RI was calculated based on Kovat's retention index formula for linear temperature programing as shown in Appendix V. The reproducibility of the method is shown by comparison with earlier chromatographic data in which the RI differs by < 0.40 % even though different GC-MS parameters were used (see Mwenesongole, et al., 2012, in

Appendix I b, Table 4, page 160). Further identification and confirmation of target drugs within spiked and unspiked samples was based on a RT or RI within ± 0.2 min or 1 % of the reference standard (WADA, 2003) and at least one ion ratio within ± 20% of the reference standard (EC, 2002; Cooper, et al., 2010; van de Steene, et al., 2010; Baker & Kasprzyk-Hordern, 2011a; Trinh, et al., 2011; Migowska, et al., 2012).

The corresponding mass spectra and proposed fragmentation patterns for all drugs analysed can be found in Appendix VI a-f, some examples of which are provided in Figures 3.19 and 3.20. Although some researchers monitored only 2 diagnostic ions (Migowska, et al., 2012; Kumirska, et al., 2013), in this thesis at least 3 diagnostic ions (one quantifier and at least two confirmation ions) were monitored to improve the reliability in identifying and confirming the detected drugs (WADA, 2003). Using 3 diagnostic ions would also enable a minimum of 1 ion ratio for identification according to recommended guidelines (EC, 2002). The diagnostic ions were selected based on a combination of the most abundant ions and those with a high relative molar mass as per their mass spectral patterns. The ion with the highest relative abundance was used for quantification (Tarcomnicu, et al., 2011; Racamonde, et al., 2013). References for some of the spectra and/or diagnostic ions for PFPA-derivatized drugs can be found in literature as indicated in Table 3.3. However, reference spectra and/or diagnostic ions could not be found for over half of the drugs especially for the NPS (i.e. piperazines and cathinones). Therefore, as far as the author is aware, this is the first time PFPA spectra and diagnostic ions have been reported for drugs such as CAT, 3-FMC, MCAT, 2 and 4-FPP, 4-MPP, MBZP, 2-and 4-MEOPP, and BUTY. The diagnostic ions for these and other drugs have recently been published (Mwenesongole, et al., 2012 & 2013). Since the molecular structure of the derivatized and underivatized NPS were known (Fig 3.7), the mass fragmentation pattern could be predicted based on various theoretical rules related to the interpretation of mass spectra (Watson & Sparkman, 2007).

3.2.5.1 Peak Separation and Isomers

As shown in Table 3.3 and Figure 3.18, all peaks were separated from each other apart from partial co-elution between BZP and 4-FPP and MBDB and 4-TFMPP. The internal standards (AMP-d_6, MDMA-d_5, COC-d_3 and MOR-d_3) also co-eluted with their

respective undeuterated analogues. However, the extracted or SIM diagnostic ions for the co-eluting compounds were different and hence could be distinguished from each other (Table 3.3). The mass spectra for MBDB and 4-TFMPP, together with their proposed fragmentation patterns, are depicted in Figure 3.19. Although they co-elute, their diagnostic ions are sufficiently different to maintain selectivity and enable independent quantification since a different quantifier (not present in the other drug) is used for each drug.

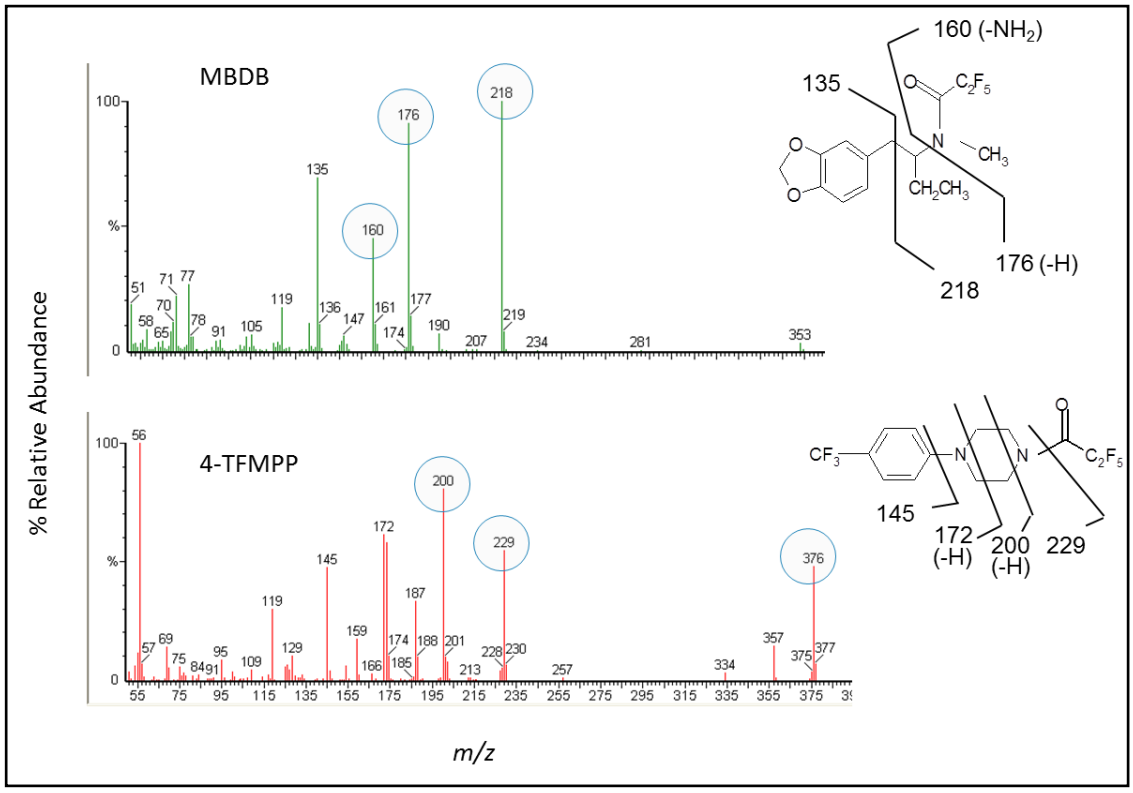


Figure 3.19: Mass spectra and proposed fragmentation patterns for MBDB and 4-TFMPP showing different diagnostic ions.

In addition, there were four sets of constitutional isomers included in the method i.e. 2-FPP/4-FPP, 3-TFMPP/4-TFMPP, 2-MEOPP/ 4-MEOPP and BZP/4-MPP. These constitutional isomers have similar mass spectral patterns and hence quantification would be difficult if they occurred at the same RT since they would share the same m/z ions. However, all these isomeric pairings occurred at different RT and could therefore be independently quantified (Table 3.3). The similarity in the mass spectral patterns for 2-MEOPP and 4-MEOPP is depicted in Figure 3.20. Although they share the same quantifier ion *m/z* 338, the mass spectrum for 4-MEOPP has a higher abundance of the *m/z* 323 ion (triangulated) which would result in different ion ratios

when used in calculations and hence can be used for identification purposes if for some reason the actual isomer included in the drug mix was unknown (e.g. in the case of an unmarked sample or labelling error).

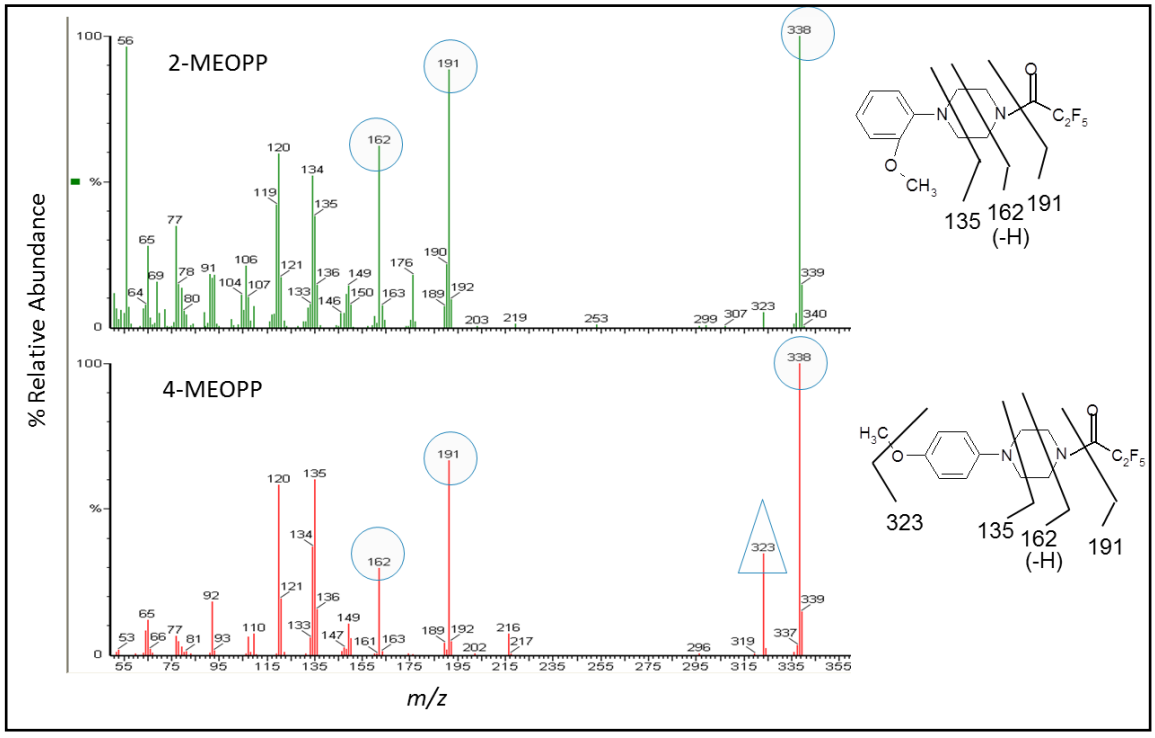


Figure 3.20: Mass spectra and proposed fragmentation patterns for the constitutional isomers 2-MEOPP and 4-MEOPP.

Therefore, despite the presence of co-eluting compounds and isomers, the developed method was selective enough to enable reliable quantification as evidenced by the results from the application of the method (Chapter 5).

3.2.6 PFPA Derivatives – Novel in Sewage Epidemiology

This is the first time, as far as the author is aware, that derivatization with PFPA has been reported for sewage epidemiological studies on illicit drugs. The majority of research in the field of sewage epidemiology has been conducted with LC-MS and therefore derivatization has not been necessary. The few researchers that have used GC-MS have used alkylation (Verenitech, et al., 2006), silylation (Mari, et al., 2009; Sebok, et al., 2009; González-Mariño, et al., 2010; Lacina, et al., 2013) and iso-butylchloroformate (Racamonde, et al., 2013). In preliminary trials, Gonzalez-Marino (2010) had investigated various silylating reagents and found MSTFA to be the most suitable for the target analytes. However, they acknowledged that MSTFA derivatives

had low m/z values for amphetamines and a single product ion in MS/MS results which could lead to false positives or negatives when identifying analytes from a complex matrix. The higher m/z values produced by derivatization with PFPA as well as more distinct mass spectral patterns (more ions with abundances greater than 50 %) can therefore lead to more reliable positive identification of analytes in complex matrices. In order to increase the sensitivity of the method and thereby improve the detection of the target analytes within a complex matrix such as untreated waste water, splitless injection and SIM using the selected diagnostic ions in retention time windows was incorporated into the method validation (section 3.5.1).

3.3 STABILITY STUDIES

Determining the appropriate analysis and storage conditions that prevent the degradation of the drugs throughout the analytical process is essential for reliable quantitative analysis. Stability studies were conducted on derivatized and underivatized mixed drug standards according to the procedures as detailed in section 2.4.2 and Table 2.8.

3.3.1 27 Hour Autosampler Stability of a Derivatized Mixed Standard

Autosampler stability study was conducted on a derivatized mixed drug standard in order to determine the stability of the analytes during instrumental analysis. The stability was evaluated by regression analysis on a plot of PAR against the injection time. Instability was indicated by a negative slope, significantly different from zero ($p \leq 0.05$) (Saar, et al., 2010). Statistical analysis was done with Microsoft Excel 2010© and GraphPad Prism 4.03©.

Figure 3.21 shows the plots from the analysis of EME and MAMP as examples. Plots of all drugs can be found in Appendix VII a-g. Based on the results, slopes from 23 of the drugs, including MAMP were not significantly different from zero ($p \leq 0.05$) indicating stability during the 27 h analysis period. These include the newer drugs of abuse BZP, BUTY, 4-FMA, 3-FMC, 2-FPP, 4-FPP, MEPH, 3-TFMPP, 4-TFMPP, 4-MPP, KET, 2-MEOPP, 4-MEOPP, and MBZP. However, EME (shown in Figure 3.21), 6-MAM and heroin had slopes significantly different from zero indicating instability. This is indicated by the higher correlation coefficient for EME (0.5431), 6-MAM (0.4042) and

heroin (0.4094) compared with the rest of the drugs as shown in Table 3.4, which lists the slopes and correlation coefficient (R^2) from the linear regression equation.

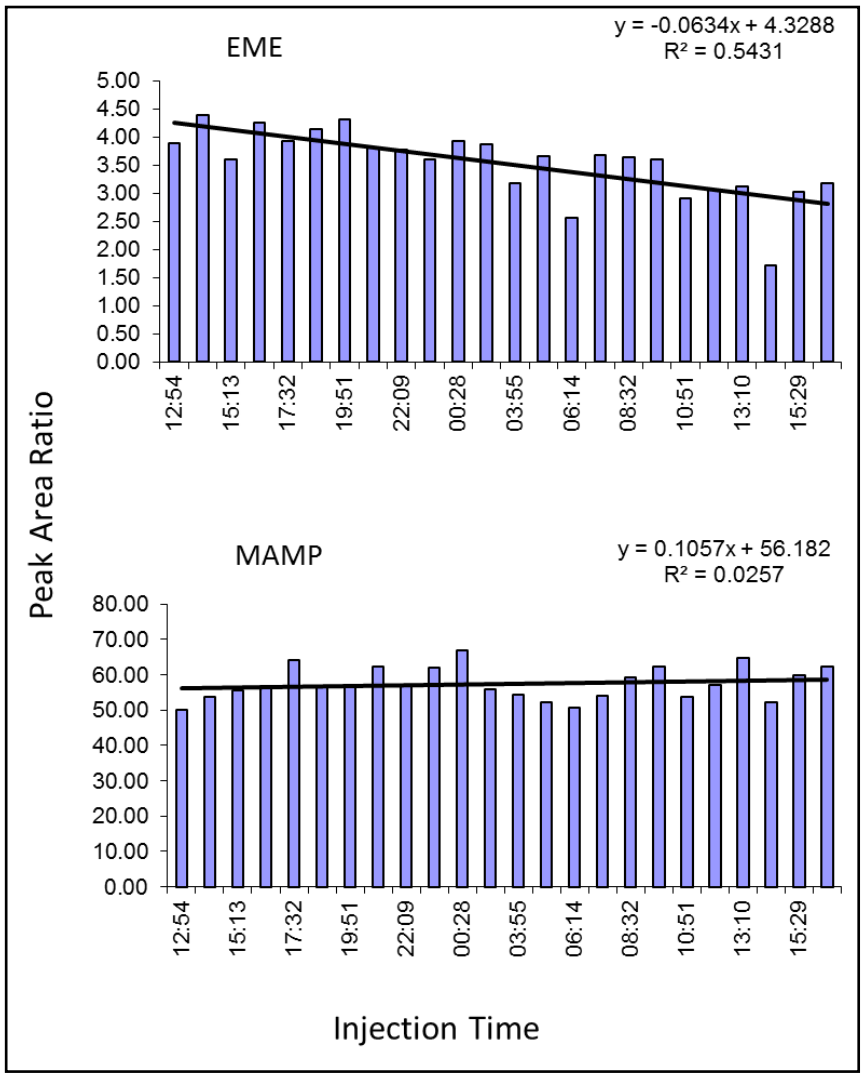


Figure 3.21: Graphs of PAR versus injection time for EME and MAMP, including R^2 and regression equation, n=24.

Aside from EME, it is interesting to note that the drugs which were found to be unstable are the same drugs which were observed to undergo degradation reactions during the PFPA optimisation studies (section 3.2.3). Therefore, the degradation reactions played a role in the autosampler instability of 6-MAM and heroin. EME is known to undergo hydrolysis to ecgonine and this could be the reason for the observed instability (Castiglioni, et al., 2008; Gheorghe, et al., 2008).

Table 3.4: Correlation coefficients (R^2), slopes and *p*-values for autosampler stability.

DRUG	R^2	Slope ± Stdev	*p*-value
PIP	0.0148	0.0533 ± 0.09266	0.5708
AMP	0.0515	0.1229 ± 0.1125	0.2863
MAMP	0.0258	0.1057 ± 0.1387	0.4534
4-FMA	0.0565	0.0729 ± 0.0636	0.2639
3-FMC	0.0158	0.0213 ± 0.0358	0.5582
EME	0.5431	-0.0634 ± 0.0124	<0.0001[a]
MEPH	0.0373	0.1334 ± 0.1444	0.3654
2-FPP	0.0393	0.0326 ± 0.0344	0.3516
BZP	0.0060	0.0043 ± 0.0119	0.7212
4-FPP	0.0451	0.0297 ± 0.0292	0.3201
3-TFMPP	0.1039	0.0369 ± 0.0231	0.1241
MDMA	0.0597	0.0627 ± 0.0530	0.2503
4-TFMPP	0.0276	0.0177 ± 0.0244	0.4375
4-MPP	0.0147	0.0122 ± 0.0195	0.5686
MBZP	0.0746	0.1901 ± 0.1427	0.1963
2-MeOPP	0.0184	0.0134 ± 0.0208	0.5307
BUTY	0.1192	0.2714 ± 0.1573	0.0986
3-CPP	0.1129	0.0161 ± 0.0096	0.1075
4-MeOPP	0.0312	0.0199 ± 0.0236	0.4112
KET	0.0216	0.0144 ± 0.0206	0.4940
AMIT	0.0327	-0.1102 ± 0.1278	0.3986
COC	0.1060	0.0073 ± 0.0046	0.1216
MOR	0.0295	0.0008 ± 0.0011	0.4539
6-MAM	0.4042	-0.0038 ± 0.0010	0.0009[a]
DIAZ	0.0511	-0.0282 ± 0.0259	0.2903
HER	0.4094	-0.0032 ± 0.0008	0.0012[a]

[a]slope significantly different from zero

Since the internal standards MDMA-d_5, COC-d_3 and MOR-d_3 were used in calculating the PAR, they were added to the mixed drug standard prior to derivatization and their PAR assessed against each other as shown in Figure 3.22.

For example, the PAR for MDMA-d_5 was calculated using COC-d_3 and MOR-d_3 and the results plotted against the injection time. MDMA-d_5 and COC-d_3 had fewer fluctuations in PAR when used against each other but MOR-d_3 caused greater fluctuations in PAR wherever it was used. Therefore, MOR-d_3 was eliminated from use as an internal standard for MOR, 6-MAM and heroin due to its instability and the PAR for these drugs was re-calculated using COC-d_3. As well as its proximity, in terms of

RT, to the opioids, COC-d_3 was seen as a suitable replacement because it also had similar functional groups (ester and tertiary amine groups) (see Figure 1.3 and Table 3.3).

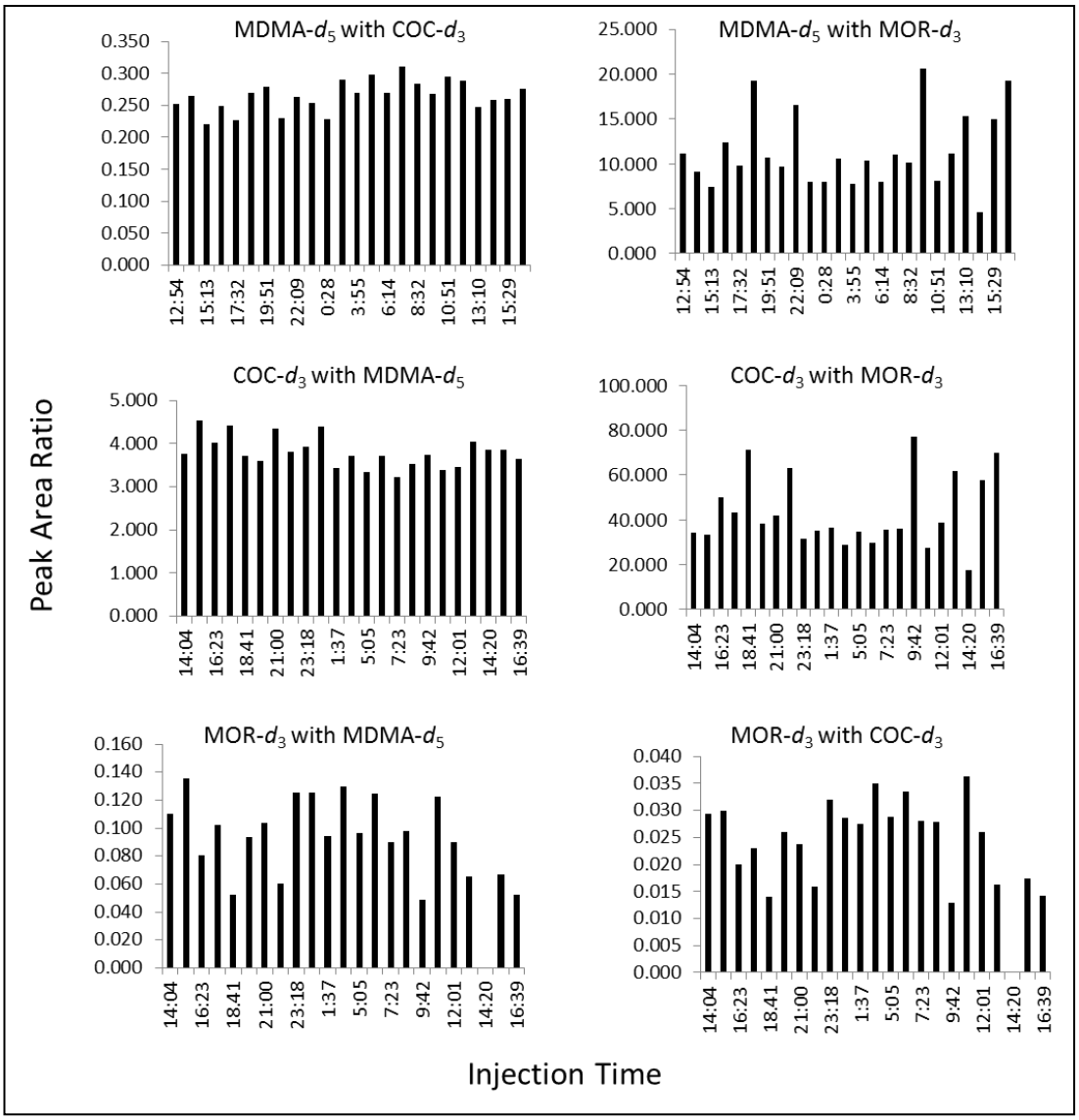


Figure 3.22: Graphs of PAR versus injection time for MDMA-d_5, COC-d_3 and MOR-d_3.

Based on these results, for overnight runs on the GC-MS instrument, sample vials were kept refrigerated and put on the autosampler to ensure that they would not stand for more than 20 h before injection.

3.3.2 4 Week Storage Stability of a Derivatized Mixed Standard

In case of unforeseen circumstances such as instrument breakdown, sometimes derivatized samples may need to be stored for several days or weeks. Therefore, longer term stability of PFPA derivatives was also determined over a 4 week period

(Table 2.8). The change in PAR from time point zero was used to evaluate the stability. Data was analysed and interpreted as: a loss of < 15 % is considered stable (denoted as 1), a loss of ≥ 15 - ≤ 30 % is considered moderately stable (denoted as 2) and a loss of > 30 % is considered unstable (denoted as 3) (Saar, et al., 2012). Statistical analysis was conducted with Microsoft Excel 2010©. The results are summarized in Table 3.5.

Table 3.5: PAR change (%) for 4-week storage stability of a derivatized mixed standard.

DRUG	2 weeks				4 weeks			
	5 °C		-20 °C		5 °C		-20 °C	
	% PAR[a]	Stability	% PAR[a]	Stability	% PAR[a]	Stability	% PAR[a]	Stability
PIP	-3	1	1	1	-13	1	-10	1
AMP	2	1	-1	1	-7	1	-5	1
MAMP	-2	1	-3	1	-13	1	-7	1
4-FMA	-6	1	-4	1	-10	1	-7	1
3-FMC	0	1	2	1	-4	1	-7	1
EME	86	3	28	2	98	3	51	3
MEPH	6	1	8	1	19	2	19	2
BZP	-13	1	-7	1	-50	3	-60	3
4-FPP	-7	1	-1	1	-36	3	-45	3
3-TFMPP	1	1	2	1	-3	1	-6	1
MDMA	1	1	0	1	0	1	-6	1
MBDB	-4	1	-1	1	-5	1	-4	1
4-TFMPP	-8	1	-8	1	-13	1	-16	1
4-MPP	-6	1	-3	1	-30	3	-39	3
MBZP	4	1	2	1	20	2	20	2
2-MeOPP	-12	1	-8	1	-41	3	-48	3
BUTY	3	1	3	1	11	1	8	1
3-CPP	-10	1	-5	1	-35	3	-41	3
4-MeOPP	-12	1	-9	1	-34	3	-47	3
KET	-3	1	-3	1	-8	1	-10	1
AMIT	-14	1	-14	1	6	1	5	1
COC	6	1	5	1	11	1	14	1
MOR	14	1	10	1	23	2	-3	1
6-MAM	22	2	-6	1	40	3	-28	2
DIAZ	-32	3	-29	2	-51	3	-64	3
HEROIN	-18	2	-7	1	-43	3	-45	3

1 = stable; 2 = moderately stable; 3 = unstable (Saar, et al., 2012); a = 100 – ((PAR day n/PAR day 0)x100)

According to the tabulated results, all investigated drugs were stable to moderately stable for up to 2 weeks at -20 °C while only EME and DIAZ showed instability after 2 weeks at 5 °C. After 4 weeks of storage, EME, BZP, 4-FPP, 4-MPP, 2- and 4-MEOPP,

3-CPP, DIAZ and heroin showed instability at both temperatures (including 6-MAM at 5 °C). The overall percentage change in PAR at both temperatures ranged from 1 – 14 % for the majority of drugs indicating stability. However, EME showed the most dramatic change in PAR at 86 % after 2 weeks and by 98 % at 4 weeks when stored at 5 °C. When stored at -20 °C, the change in PAR was 28 % and 51 % at 2 and 4 weeks, respectively. This can be attributed to hydrolysis reactions as stated in section 3.3.1.

On the other hand, MAMP, which showed stability throughout the study period at both temperatures, had overall percentage changes in PAR ranging from 2 to 13 %. Figure 3.23 depicts plots of the PAR against analysis time point for EME and MAMP.

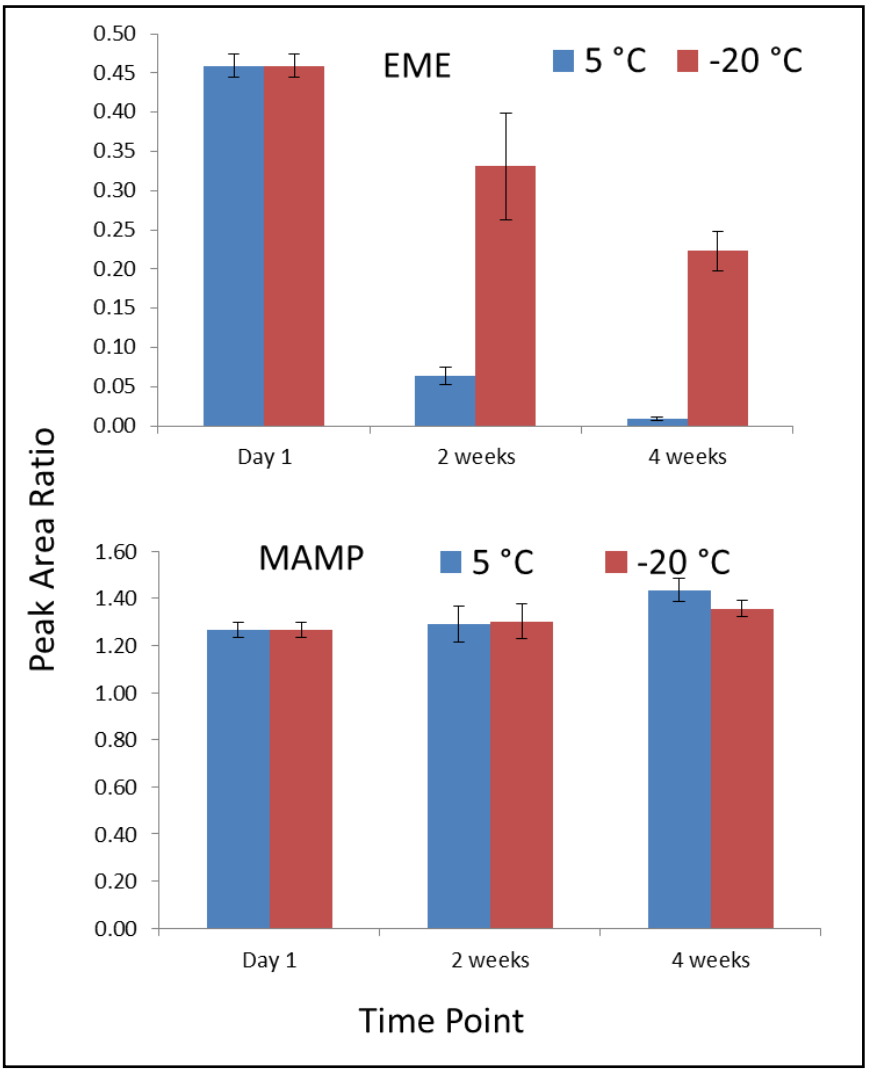


Figure 3.23: PAR versus analysis time point for EME and MAMP. Error bars represent standard deviation at n = 3.

The other unstable drugs mentioned above showed percentage PAR changes ranging from 30 - 51 % for storage at 5 °C and 39 - 64 % or storage at -20 °C.

Therefore, the majority of PFPA-derivatized drugs, dissolved in ethyl acetate and investigated in this research, can be stored up to 4 weeks without any significant degradation at both 5 and -20 °C. However, the degradation observed for EME and DIAZ at 5 °C was enough to ensure that drugs were stored at -20 °C throughout the method development. Since more drugs showed instability after 4 weeks storage at both temperatures, it is not advisable to store the investigated drugs for longer than 2 weeks in ethyl acetate. In practice, during this research, derivatized samples in ethyl acetate were never stored for more than 1 week and hence the two week cut-off was more than adequate. In comparison with published literature for different derivatizing reagents, Gonzalez-Marino (2010) reports silylated derivatives of similar drug classes to be stable for up to a week at -20 °C while Kosjek (2012) reports acylated benzodiazepines to be stable for up to 2 weeks. However, both research groups do not mention the acceptance criteria for stability and in the latter example even the storage conditions are not mentioned.

3.3.3 4 Week Storage Stability of an Underivatized Mixed Standard

In order to determine how long mixed drug standards could be stored before being used, a 4 week stability study of the underivatized mixed drug standards stored in methanol was conducted (Table 4.8). The samples were only derivatized after removal from storage immediately prior to analysis at time points zero and 4 weeks. Evaluation of stability was as stated in section 3.3.2. The results are summarized in Table 3.6.

All drugs were stable to moderately stable when stored at -20 °C for 4 weeks except for 3-FMC. At 5 °C, all drugs were stable to moderately stable except 3-FMC, MEPH and heroin. In fact, 3-FMC was not detected at 5 °C.

Table 3.6: PAR change (%) for 4-week storage stability of an underivatized mixed standard.

DRUG	5 °C		-20 °C	
	% PAR[a]	Stability	% PAR[a]	Stability
PIP	16	2	10	1
AMP	20	2	12	1
MAMP	19	2	17	2
4-FMA	20	2	17	2
3-FMC	ND	ND	58	3
EME	5	1	18	2
MEPH	53	3	23	2
2-FPP	13	1	8	1
BZP	15	1	17	2
4-FPP	6	1	-1	1
3-TFMPP	12	1	8	1
MDMA	6	1	-2	1
4-TFMPP	2	1	-5	1
4-MPP	10	1	5	1
MBZP	10	1	8	1
2-MeOPP	6	1	-3	1
BUTY	14	1	7	1
3-CPP	13	1	5	1
4-MeOPP	21	2	23	2
KET	8	1	4	1
AMIT	10	1	18	2
COC	15	1	12	1
MOR	-6	1	18	2
6-MAM	-20	1	-1	1
DIAZ	-9	1	-18	2
HEROIN	43	3	1	1

1 = stable; 2 = moderately stable; 3 = unstable (Saar, et al., 2012); ND = not detected;
a = 100 – ((PAR day n/PAR day 0)x100)

A notable difference in stability was observed for heroin at the two storage temperatures. Figure 3.22 compares the stability of heroin and 3-TFMPP. Heroin had degraded by 43 % of its original PAR when stored at 5 °C and by only 1 % when stored at -20 °C. On the other hand, after 4 weeks of storage 3-TFMPP had degraded by 12 % at 5 °C and 8 % at -20 °C. Therefore, for heroin, storage at -20 °C or lower is imperative. In addition, EME, which was only moderately stable when stored as the PFPA-derivative in ethyl acetate at -20 °C for 2 weeks, was stable when stored

underivatized in methanol. Therefore EME possibly also undergoes degradation reactions in ethyl acetate similar to the opioids.

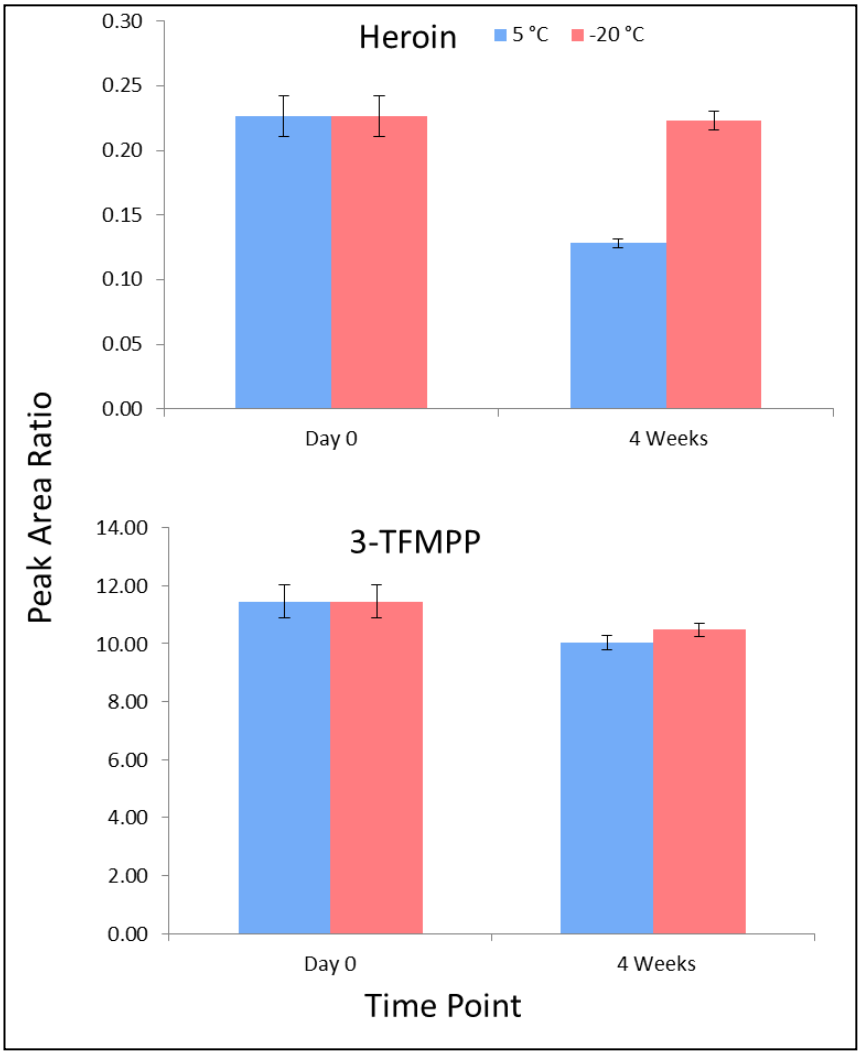


Figure 3.24: PAR versus analysis time point for heroin and 3-TFMPP. Error bars represent standard deviation at n = 3.

Overall, when stored at -20 °C, 25 of the 26 drugs had shown a change in PAR of 1- 23 % after 4 weeks which falls in the stable to moderately stable range. A similar trend was observed for drugs stored at 5 °C with PAR losses ranging from 2 - 21 %, with the exception of MEPH (53 %) and heroin (43 %).

The main aim with this study was to determine the stability of the target drugs after a 4 week storage period. The 4 week timeframe was chosen based on the practical length of time during which a mixed drug standard was used after being made in this research. Based on the results, for all but one drug (3-FMC), storage of up to 4 weeks

as a mixed standard in solution at -20 °C is suitable. 3-FMC should preferably be added fresh to the mixed standard on the day of analysis. Based on standard toxicological practice (Cooper, et al., 2010; Saar, et al., 2012), all individual drug stock solutions and mixed drug standards were stored at -20 °C even prior to this investigation. The results therefore confirmed why storage of drugs in solvent at -20 °C is imperative for many drugs. To err on the side of caution, the approach taken in this research was to make new individual drug stock solutions every 6 months from which several vials of mixed standards were immediately made. These 'fresh' working solutions were then evaporated to dryness before storage at -20 °C until further analysis. Drying the mixed standards before storage at -20 °C was thought to be a better way of curbing drug-drug interactions and analyte degradation and save time during future sample preparation. However, the stability of the dried mixed drug standard was not evaluated during the time frame of this research but has been noted for further investigation. All internal standards were stored at -20 °C and added individually to the mixed drug standards on the day of analysis. Mixed drug standards in methanol have reportedly been prepared every 3 months and stored at 4 °C but there is no mention of stability studies conducted to determine if these storage conditions were appropriate (Loganathan, et al., 2009).

No mention of autosampler or storage stability of drug stock standards or mixed standards has been reported for sewage epidemiology. Stability studies are recommended as part of method validation especially for biologically active samples and have been reported in clinical and toxicological studies (Saar, et al., 2012). Although the actual study undertaken needs to be relevant for the method under validation, the stability of any mixed drug standard stored in liquid form should be conducted in order to determine if there is any degradation or interconversion due to drug-drug interactions. Results from stability studies of multianalyte methods depend on the combination of drugs mixed together and hence should form part of the method validation.

For all the stability studies conducted, the author is not aware of similar studies incorporating this combination and number of emerging and classic PFPA-derivatized drugs of abuse being reported.

In addition to derivatization, the extraction method used for isolating the drugs from the matrix played an important role in whether the drugs could be detected. Therefore, the next step in method development was to select and optimise an extraction method.

3.4 ANALYTE EXTRACTION METHODS

Although discussed in separate sections (3.4.1.2 and 3.4.2.1), preliminary LLE and SPE studies were alternately conducted around the same time period in order to compare the results and decide which was the most suitable for the drugs and matrix under analysis. The effectiveness of an extraction method was assessed based on the percentage recovery of a known concentration of a drug spiked into a matrix.

The recovery was calculated according to Equation 3.1 using absolute peak areas (Baker & Kasprzyk-Hordern, 2011a).

$$\% \text{ Recovery} = \left(\frac{\text{Peak area of standard spiked before extraction}}{\text{Peak area of un-extracted standard}} \right) \times 100 \qquad \text{Equation 3.1}$$

The un-extracted mixed drug standard was regarded as representing 100 % recovery.

3.4.1 Liquid-Liquid Extraction (LLE)

Even though the majority of published work in sewage epidemiology had reported SPE, it was worth investigating whether some of the known advantages of LLE would apply to extraction of trace analytes from an aqueous matrix (section 1.5.3.3 and Table 1.7).

For the optimisation of a LLE method, the emphasis was on the selection of an extraction solvent and a suitable sample pH as discussed in the following sections.

3.4.1.1 Selection of Solvent

The initial stage of LLE optimisation investigated the extraction solvent following the procedures as detailed in section 2.4.3.1.1. The results from the comparison of chloroform:isopropyl alcohol ($CHCl_3$:IPA) 3:1 v/v and chloroform:ethyl acetate:ethanol

($CHCl_3$:EtOAC:EtOH) 3:1:1 v/v as extraction solvents are provided in Table 3.7. Highlighted areas indicate the highest recovery obtained for that particular drug.

Table 3.7: Comparison of recovery (%) using $CHCl_3$:IPA 3:1 v/v and $CHCl_3$:EtOAC:EtOH, 3:1:1 v/v.

DRUG	% RECOVERY, pH 10.5, n=2	
	$CHCl_3$:IPA (3:1 v/v)	$CHCl_3$: EtOAC: EtOH (3:1:1 v/v)
PIP	3	1
AMP	16	3
MAMP	14	1
4-FMA	10	1
3-FMC	ND	3
EME	15	11
MEPH	29	30
BZP	19	21
4-FPP	28	34
3-TFMPP	32	38
MDMA-d_5	79	83
MDMA	24	24
MBDB	24	26
4-TFMPP	35	49
4-MPP	20	35
MBZP	35	45
2-MeOPP	35	46
BUTY	43	55
3-CPP	15	19
4-MeOPP	9	23
KET	22	53
AMIT	19	22
COC-d_3	28	25
COC	26	25
MOR-2PFP-d_3	25	10
MOR-2PFP	3	3
MOR-PFP-d_3	42	15
MOR-PFP	463	164
6-MAM-PFP	10	12
DIAZ	45	21
6-MAM	19	36
HER	ND	ND

ND = not detected

A theoretical background to LLE has been described in section 1.5.3.2. There are numerous types and combinations of extraction solvents that can be used for LLE (Table 1.6) but in this research $CHCl_3$:IPA (3:1 v/v) and $CHCl_3$:EtOAC:EtOH (3:1:1 v/v) were used as reported by Tsutsumi (2005) and Raikos (2009), respectively. An initial

low volume of sample matrix (2 mL) and high drug concentration (0.6 to 2.8 µg/mL) was used to improve the chances of recovery and detection.

Recoveries of drugs from both solvent systems were somewhat similar. The majority of drugs had recoveries of between 24 - 55 % with the highest recoveries obtained for MDMA-d_5 with $CHCl_3$:IPA (3:1 v/v) at 79 % and $CHCl_3$:EtOAC:EtOH (3:1:1 v/v) at 83 % . Some drugs such as PIP, AMP, MAMP, 4-FMA, 3-FMC & EME had low recoveries ranging from 0 - 16 % in both solvents. Although it had much lower recoveries for AMP and MAMP, $CHCl_3$:EtOAC:EtOH (3:1:1 v/v) was selected as the extraction solvent as it had higher recoveries for piperazines, cathinones and ketamine, which are included under NPS.

According to the Henderson-Hasselbalch equation (Equations A-1 and A-2, Appendix III), for basic drugs with a pK_a range of 7.5 to 9.9 an optimal extraction pH would range from 9.5 to 12, respectively. At this pH, the drugs would be unionised and hence more amenable to extraction into the organic solvent (Telepchak, et al., 2004; Flanagan, et al., 2007). Therefore, a sample pH of 10.5 was more suitable for some drugs such as BUTY and KET (pK_a 7.5) which had recoveries of 55 and 53 %, respectively. For the first 6 drugs listed in Table 3.7, which had the lowest recoveries, it would appear that some loss of the analytes occurred during sample preparation and/or extraction. Of these 6 drugs, only the pK_a of AMP and MAMP was known i.e. 9.9 for both. In order for drugs with a pK_a of 9.9 to be completely unionised, a more suitable pH would have been pH 12. It would appear that at a pH of 10.5, a portion of the drugs were still present as the protonated conjugate acid (Equation A-2, Appendix III) and hence more soluble in the aqueous phase. Although sample preparation was carefully done, low recoveries could also be attributed to loss during the transfer of samples between vessels, during evaporation or in the case of heroin (which was not detected), hydrolysis to 6-MAM or MOR (Figure 3.17).

As per observations during derivatization studies (section 3.2.3), both MOR-2PFP and MOR-PFP were detected but MOR-PFP had much higher recoveries (> 100 %). This was due to a higher peak area for the extracted standard than for the unextracted standard possibly as a result of loss of analyte during evaporation or contribution

from the deacetylation of MOR-2PFP to MOR-PFP (Figure 3.16). In addition, 6-MAM was also detected at higher recoveries than 6-MAM-PFP possibly due to the hydrolysis of heroin to 6-MAM (Figure 3.17).

As observed from the variable recoveries, with multianalyte methods containing drugs from different classes and with different pK_a values and derivatives formed, compromises will be made and hence some drugs will have better recoveries than others at a particular pH and with a particular solvent (Appendix III).

Therefore the next stage was to optimise the LLE method based on extraction with $CHCl_3$:EtOAC:EtOH (3:1:1 v/v) at different sample pHs to determine if higher recoveries could be attained.

3.4.1.2 Optimisation of pH

Following the procedures as detailed in section 2.4.3.1.2, sample extraction with $CHCl_3$:EtOAC:EtOH (3:1:1 v/v) was conducted at pH 5.0, 7.0 and 10.5. A higher volume of deionised water (150 mL) and low drug concentrations (< 5 µg/mL) were incorporated into the method to correlate with volumes of waste water samples that would be used later. The results are shown in Table 3.8.

Out of the 26 drugs, 22 were detected at pH 10.5 including heroin and 3-FMC which were not detected at the other pHs and in the unextracted standard (hence recovery could not be calculated for them). Only 13 and 15 drugs were detected at pH 5.0 and pH 7.0, respectively. Aside from heroin at pH 10.5, none of the opioids were detected in any of the matrices. Overall, recoveries were less than 20 % for the majority of detected drugs across the 3 pHs (e.g. PIP, 2-FPP, MDMA, and KET) but some drugs had recoveries above 100 % (e.g. MEPH, BUTY and COC). The low recoveries observed could be due to several factors including the use of a 20:1 injection split ratio which meant that only 5 % of the already low concentration of drugs injected (1 µL) was actually available for detection.

Table 3.8: Comparison of extraction pH using $CHCl_3$:EtOAC:EtOH, 3:1:1 v/v.

	% RECOVERY, n=2		
DRUG	pH 5.0	pH 7.0	pH 10.5
PIP	4	< 1	1
AMP	64	1	4
MAMP	222	9	16
4-FMA	162	6	5
3-FMC	NA	ND	ND
EME	16	1	1
MEPH	2304	895	201
2-FPP	< 1	ND	< 1
BZP	2	< 1	ND
4-FPP	ND	ND	ND
3-TFMPP	1	ND	ND
MDMA-d_5	ND	ND	ND
MDMA	2	< 1	< 1
4-TFMPP	< 1	ND	ND
4-MPP	4	ND	ND
MBZP	< 1	ND	ND
2-MeOPP	ND	ND	ND
BUTY	94	198	233
3-CPP	8	5	ND
4-MeOPP	2	2	ND
KET	< 1	17	< 1
AMIT	< 1	< 1	< 1
COC-d_3	612	599	433
COC	402	401	136
MOR-d_3	ND	ND	ND
MOR	ND	ND	ND
6-MAM	ND	ND	ND
DIAZ	2	4	5
HEROIN	NA	ND	ND

ND = not detected; NA = not applicable

Additionally, there could have been analyte loss during the sample preparation and extraction process as stated in section 3.4.1.1. For the deuterated internal standards, only COC-d_3 was detected, most likely due to the higher concentration added (1.7 µg/mL) compared to the lower concentration added for both MDMA-d_5 and MOR-d_3 (0.17 µg/mL). Recoveries > 100 % were due to a higher concentration of the drugs in the extracted standards than the unextracted standards, possibly due to loss during the evaporation steps.

As can be expected based on the pK_a ± 2 rule for selection of extraction pH, the most suitable pH for this combination of drugs with average pK_a ranging from 8-10 was

pH 10-12. Therefore at pH values close to the pK_a of the drugs, such as pH 10.5 for AMP and MAMP which have pK_a of 9.9, the drugs would still mainly be ionized and hence more soluble in the aqueous phase (Appendix III). But for drugs with pK_a less than 9, such as KET, heroin and 3-TFMPP, better recoveries would be expected.

Overall, recoveries for LLE of a 150 mL spiked sample were poor and not reliable. These factors, in addition to the time consuming process, led to the decision to proceed with SPE which had shown much better recoveries under the same experimental conditions (section 3.4.2.1). On the other hand, Mol (2009) had found both SPE and LLE to be suitable for extraction of acidic drugs from 500 mL of waste water. However, they also selected SPE over LLE due to its time efficiency.

3.4.2 Solid Phase Extraction (SPE)

The method optimisation process for SPE focussed on the selection of a suitable sorbent, sample pH and elution solvent following the procedures as detailed in section 2.4.3.2. Preliminary SPE optimisation studies were conducted on various volumes of deionised water, tap water, treated and untreated waste water spiked with the mixed drug standard. Internal standards were spiked together with the drug analytes before extraction to determine their percentage recovery and hence suitability for use for PAR calculations if needed. Method blanks using unspiked samples were also co-currently extracted and analysed together with the spiked analytes. These were checked for analyte peaks or potential co-eluting interferents from the matrix or SPE sorbents.

3.4.2.1 Comparison of the SPE Sorbents, Oasis® MCX and HLB

In order to determine which sorbent type would be most suitable for the types of drugs under investigation, preliminary studies were conducted using two SPE cartridges, MCX and HLB at various pHs, according to the procedures as detailed in section 2.4.3.2.1 and Table 2.9. The same sample volume, concentration, number of drugs and instrumental method as per the LLE method (section 3.4.1.2 above) was used for comparison purposes.

Recovery (%) was calculated using equation 3.1 and the results are listed in Table 3.9. Highlighted areas indicate the highest recovery obtained for that particular drug for each type of cartridge.

Table 3.9: Comparison of Oasis MCX and HLB sorbents at different pH values.

	% RECOVERY, n=2					
DRUG	MCX pH 2.0	MCX pH 5.4	MCX pH 10.0	HLB pH 2.8	HLB pH 7.4	HLB pH 8.5
PIP	93	215	126	1	1	<1
AMP	100	67750	67	1243	56743	47
MAMP	84	261950	100	21850	292350	37
4-FMA	87	19322	22	1691	16373	37
3-FMC	40	ND	ND	ND	ND	20
EME	1	17	17	ND	ND	<1
MEPH	75	152	7	2093	3318	43
2-FPP	97	135	25	1	71	36
BZP	101	61	63	2	12	1
4-FPP	52	31	15	ND	107	6
3-TFMPP	92	56	21	3	59	22
MDMA-d_5	75	48	29	55	127	27
MDMA	95	58	59	24	114	40
4-TFMPP	93	47	44	6	100	27
4-MPP	4	19	54	ND	100	2
MBZP	82	74	93	2	35	10
2-MeOPP	64	37	75	ND	142	11
BUTY	100	32	88	208	165	58
3-CPP	75	19	27	ND	156	15
4-MeOPP	1	ND	100	ND	ND	1
KET	68	15	38	290	170	82
AMIT	101	3	90	2	26	13
COC –d_3	33	ND	ND	ND	ND	43
COC	48	ND	ND	ND	ND	55
MOR-d_3	ND	ND	ND	ND	ND	ND
MOR	ND	ND	ND	ND	ND	ND
6-MAM	ND	ND	ND	ND	ND	ND
DIAZ	100	33	267	593	240	83
HEROIN	ND	ND	ND	ND	ND	ND

ND = detected

Considering that the same experimental conditions were used as per LLE (Table 3.8), the results from SPE show much higher overall recoveries (38 to 152 %), indicating better extraction and concentration of analytes from the sample matrix. In addition, the SPE extraction process was more amenable to conducting multiple extractions at the same time which was seen as an advantage since high sample throughput is part of the process of ensuring a quick turn around with results.

MCX

The MCX (pH 2) sorbent gave higher recovery values for the majority of drugs, ranging from 48 - 101 %. In addition, 3-FMC, COC and COC-d_3 were only detected at pH 2 and

not at pH 5.5 and 10. However, some drugs such as AMP, MAMP, MBZP, 2-MEOP, BUTY and AMIT were recovered at relatively high percentages (> 67 %) at both pH 2 and pH 10. This is because at pH 2, the basic drugs were 100 % ionised but at pH 10, they were still about 50 % ionised as the pH was close to the pK_a value for a number of the drugs (AMP, MAMP). This would still enable some interaction with the negatively charged sulphonic group on the MCX sorbent (Figure 1.2). This indicates that the pK_a ± 2 rule is not as straightforward with SPE or LLE (Hendriks, et al., 2007) and systematic optimisation is needed to ensure the optimal conditions for the drugs under investigation.

The use of two elution solvents in tandem for MCX, i.e. 100 % methanol (pH 5.0) and 2-5 % ammonium hydroxide in methanol (pH 10-11), enables the elution of acidic, basic and neutral drugs. Either solvent could be used alone to improve the specificity of the method depending on the acid-base properties of the compounds under investigation. If interest was only in acidic compounds from the matrix, then only methanol could be used and if basic compounds were the analytes of interest, then basified methanol could be used alone. Both basic and amphoteric drugs were included in this research so both solvents were used.

HLB

For the HLB sorbent, a sample matrix of pH 7.5 gave better recovery values (59 - 165 %) compared with pH 2.8 and 8.5. However, an extra 5 - 9 drugs, including COC and 3-FMC, were detected at pH 8.5 than at pH 2.8 and 7.4. This correlates with published reports where sample pHs of between 7.0 and 8.5 have been used with HLB sorbents (Gonzalez-Marino, et al., 2010; Gracia-Lor, et al., 2010; Tarcomnicu, et al., 2011). Two elution solvents were also used with HLB to target the different types of drugs present in the sample. Ethyl acetate was used to recover phenylethylamines followed by acetone to extract the remaining drugs. The amine group is thought to react with the carbonyl group of acetone leading to interference during derivatization. Therefore, the fractions were eluted separately and the acetone fraction was evaporated to dryness before the ethyl acetate fraction was added to it (Gonzalez-Marino, et al., 2010).

The opioids were not detected for all sorbents and pHs possibly due to their low detector response and degradation reactions mentioned in section 3.2.3.

Oasis MCX and HLB sorbents have been the most widely reported in sewage epidemiology for multianalyte extraction of acidic, basic and neutral pharmaceutical compounds (Boles & Wells, 2010; van Nuijs, et al., 2011a; Wille, et al., 2012). Depending on the extraction protocol and pharmaceuticals under analysis, either the Oasis MCX or HLB sorbent were found to be suitable (Gheorghe, et al., 2008; Gracia-Lor, et al., 2010; Vazquez-Roig, et al., 2013). A background to the chemical structures of these sorbents can be found in section 1.5.3.1.1 (Figure 1.2). MCX has strong cationic exchange properties suitable for bases while HLB is a universal reverse phase sorbent that can retain polar acids and bases and neutral analytes.

In reference to the Henderson-Hasselbalch equation and pK_a ± 2 rule, basic drugs are expected to have better recoveries at acidic pH values at least 2 pH units below the pK_a using the MCX sorbent. This is because basic drugs are protonated under acidic pH thereby forming an ionic bond with the negatively charged sulphonic group on the MCX sorbent during the loading step (Figure 1.2). During elution, the basified organic solvent neutralizes the charge on the basic drugs thereby disrupting the ionic bond and causing the analytes to elute. Acidic and neutral drugs are not ionized at acidic pH but because MCX sorbents also have an HLB backbone, these drugs will be retained by the reverse-phase HLB backbone and be eluted by the methanol which disrupts these non-polar interactions (Table 2.9). As most of the drugs investigated in this research were bases with pK_a between 8 and 10, they were more suited to extraction by the MCX sorbent at a sample pH of 2.

Another observation made from the results in Table 3.9 is that some recoveries for both MCX and HLB extractions were excessively high for drugs such as AMP, MAMP and 4-FMA. This was due to possible loss of analytes during evaporation of unextracted standards. The peak areas of the unextracted standard were sometimes inexplicably lower than those of the extracted standard and this affected recovery calculations by resulting in unrealistically high recoveries (> 1000 %). Interference from co-eluting compounds can also not be ruled out (i.e. from the cartridge or

solvents used). The unextracted standard is supposed to represent 100 % recovery and hence should have higher peak areas than the extracted standard (Richards, 2010). During method validation, use of a post-extracted standard instead of an unextracted standard was expected to reduce some of these anomalies (section 4.5). Overall, MCX (pH 2) gave much better recoveries and number of detected drugs than HLB (pH 7.4 & 8.5). Therefore, the final SPE protocol selected was based on MCX cartridges with a sample pH of 2. This is in line with other published reports in which acidic sample pHs result gave better recoveries on MCX cartridges for basic drugs (Baker & Kasprzyk-Hordern, 2011a). Another advantage of using the MCX cartridge was that pH adjustment of samples was not required before extraction since samples were acidified to pH 2 before storage to prevent the degradation of analytes (section 1.5.2) (Vazquez-Roig, et al., 2013).

It is worth noting that with multianalyte methods, extraction conditions are often a compromise therefore some analytes will have lower recoveries due to a non-ideal pH, sorbent or elution solvents used for their extraction (Couchman & Morgan, 2011). Perhaps some recoveries could have been improved had different elution solvents been used at the various pHs since the acid-base equilibria of the drugs would also be different. In addition, the sample matrix, sample preparation, extraction protocol, instrumental conditions, and analyte type and concentrations used can also affect results and hence intra- and inter-laboratory comparisons can yield different values. Table 3.10 compares the results from the extraction of drugs on MCX (pH 2.0) using different matrices, spiked drug concentrations and instrumental parameters.

The comparison shows varying recoveries of the same drug across different matrices. However, in at least 2 out of the 4 comparisons, recoveries were less than 15 % different for some drugs such as MEPH, BZP, MDMA, 4-TFMPP, AMIT and COC indicating that the method was reproducible for some drugs. The internal standards MDMA-d_5 and COC-d_3 had reproducible recoveries across at least three matrices. Sometimes, drugs were not detected (e.g. MDMA in column D) as a result of loss of sensitivity during analysis but in other cases it was due to degradation or low detector response for the drug (e.g. MOR and heroin). For waste water samples, any peak areas corresponding to the target analytes (from the unspiked sample) were taken

into account during recovery calculations. The manual extraction set-up used during this research also introduced its own set of variabilities such as different average flow-rates used between cartridges and from day to day (5 - 8 mL/min) and different elution solvent compositions which had to be freshly made (average pH of 2.4 - 2.8) This contributed to the variability in reproducibility for intra-day and inter-day analyses.

Table 3.10: Comparison of recoveries of different spiked matrices using Oasis MCX at pH 2.0.

	% Recovery of MCX, pH 2.0, n=2			
DRUG	2 mL DH_20, SCAN, split (0.6 to 2.8 µg/mL) **A**	150 mL DH_20, SIM, split (2.5 to 6.6 µg/mL) **B**	150 mL raw WW, SIM, split (3.8 - 7.0 µg/mL) **C**	150 mL tap water, SIM, splitless (0.8 - 2.0 µg/mL) **D**
PIP	3	93	36	57
AMP-d_6	NA	NA	NA	65
AMP	6	100	229	64
MAMP	6	84	203	63
4-FMA	1	87	176	66
CAT	NA	NA	NA	82
3-FMC	135	40	ND	103
MCAT	NA	NA	NA	88
EME	119	1	1	5
MEPH	89	75	168	91
2-FPP	NA	97	90	2
BZP	48	101	90	86
4-FPP	50	52	110	6
3-TFMPP	48	92	85	1
MDMA-d_5	68	75	72	ND
MDMA	102	95	85	ND
MBDB	66	NA	NA	ND
4-TFMPP	56	92	81	ND
4-MPP	55	4	163	ND
MBZP	56	82	83	70
2-MeOPP	59	64	94	ND
BUTY	71	100	91	16
3-CPP	54	75	63	ND
4-MeOPP	60	1	167	ND
KET	66	68	32	24
AMIT	86	101	89	<1
COC-d_3	70	33	70	71
COC	69	48	54	76
MOR-2PFP-d_3	89	ND	11952	ND
MOR-2PFP	ND	ND	ND	ND
6-MAM	103	ND	ND	ND
DIAZ	78	100	128	70
HEROIN	ND	ND	ND	ND

ND = not detected; NA = not applicable

The low recoveries of some drugs (i.e. < 6 %) could be due to factors related to the SPE protocol such as the sorbent choice, pH of sample in relation to the pK_a of the drug and elution solvent used and/or factors ouside the SPE process such as the

evaporation temperature and time and derivatization process. All these factors have been attributed to the loss of analytes by other authors as well (Bogusz, et al., 1985; Baker & Kasprzyk-Hordern, 2011b; Wille, et al., 2012). During this research, some loss of analytes occurred for the unextracted standards which resulted in much inflated levels of recoveries when using Equation 3.2, as observed in Table 3.10 for AMP, MAMP and 4-FMA. The exact point at which analytes could have been lost is unknown but suggestions by the author include: during the evaporation stages (before and after derivatization), through the use of unsilanised glassware or during analysis.

Aside from matching the sample pH to the drugs under analysis and the SPE sorbent used, the elution solvent also plays a large role in terms of recovery of the drugs (Baker & Kasprzyk-Hordern, 2011b). Therefore, the next stage in SPE optimisation was to evaluate two elution solvents used for the MCX cartridge which was selected as the most suitable sorbent for the drugs under analysis.

3.4.2.2 Comparison of Elution Solvents for MCX at pH 2.0

The most reported basified organic elution solvent in literature for the MCX sorbent is 2-5 % (v/v) ammonium hydroxide in methanol (Waters, 2006b; Lai, et al., 2011; Tarcomnicu, et al., 2011). Very few alternatives, such as 5 % (v/v) ammonium hydroxide in acetone:ethyl acetate (1:1 v/v) have been used (Bones, et al., 2007). As mentioned in section 3.4.2.1, a basified organic solvent is essential for the elution of basic drugs. Although 5 % (v/v) ammonium hydroxide in methanol was used in earlier SPE optimisation studies, it was decided to evaluate whether there would be a difference in recovery if 5 % (v/v) ammonium hydroxide in acetone/ethyl acetate (1:1 v/v). Following the procedure as detailed in section 2.4.3.2.2, the results are presented in Figure 3.25.

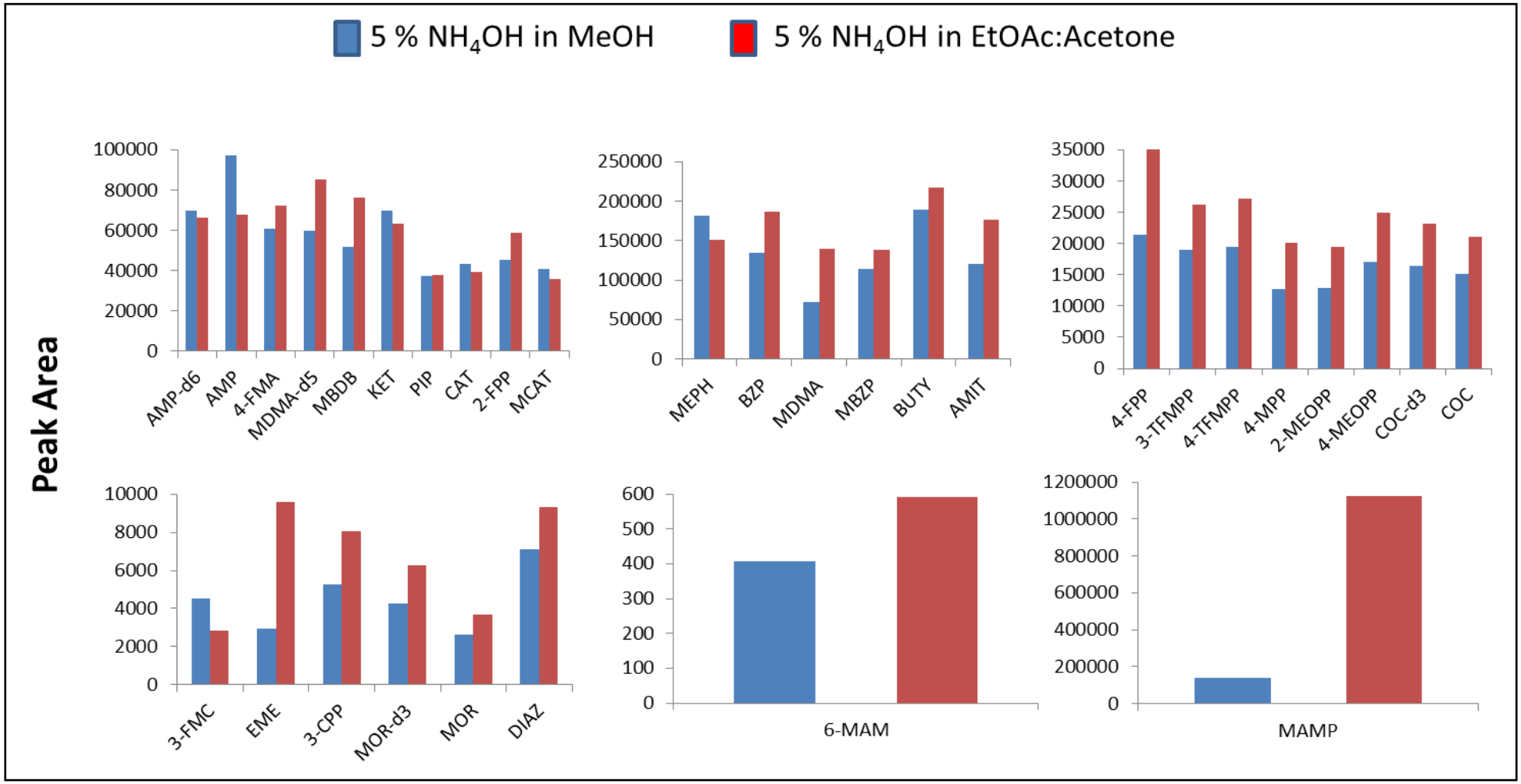


Figure 3.25: Comparison of peak area using 5 % (v/v) ammonium hydroxide in methanol versus 5 % (v/v) ammonium hydroxide in acetone:ethyl acetate (1:1 v/v) as elution solvents, n=2.

Results show that elution with 5 % (v/v) ammonium hydroxide in acetone:ethyl acetate (1:1 v/v) resulted in higher peak areas than elution with 5 % (v/v) ammonium hydroxide in methanol for 24 out of the 32 drugs and internal standards. In particular MAMP and EME showed much larger increases in peak areas. A few drugs such as AMP, MEPH and 3-FMC had higher peak areas in 5 % (v/v) ammonium hydroxide in methanol but these were in the minority. Therefore, 5 % (v/v) ammonium hydroxide in acetone:ethyl acetate (1:1 v/v) was selected as the elution solvent for further studies due to the stronger elution properties of a combined acetone and ethyl acetate solvent mixture as well as the quicker evaporation of the two solvents as compared to methanol alone (Table 1.6) (Bones, et al., 2007; Richards, 2010).

The findings from this research correlate with results as reported by Bones (2007) with different drugs having higher recoveries in the different solvents. However, MDMA, COC, DIAZ and MOR, which had higher recoveries in this research with 5 % (v/v) ammonium hydroxide in acetone:ethyl acetate (1:1 v/v), had lower recoveries in Bones's (2007) study. However, a different SPE cartridge from a different manufacturer, but with similar chemical characteristics (mixed-mode strong cation exchange), was used and this could account for some differences.

Optimisation of elution solvents for MCX cartridges has only been reported in a few published manuscripts as the premise has been to just use what has been reported before or manufacturers' recommendations (Bones, et al., 2007). However, as this study has shown, it is worth comparing different solvents for drugs under investigation because inter-laboratory differences will always exist which can have a profound influence on the results obtained.

Therefore, the final SPE method used in this research for method validation was as presented in Table 2.10

3.4.3 Selectivity through Extraction and Recovery

Although the extraction and recovery studies served primarily to determine the efficiency of the SPE process, they also verified whether the optimised derivatization protocol (section 3.2.4) and selected diagnostic ions (Table 3.3), especially the quantifier ion, were suitable and selective enough for application in a complex matrix.

Figure 3.26 shows the TICs of unspiked and spiked (5 µg/mL mixed drug standard) untreated waste water samples. Both chromatograms show the complexity of the waste water samples with regard to the presence of high concentrations of non-targeted matrix components. However, in the TIC for the spiked sample, the internal standards MDMA-d_5 and COC-d_3 can be detected among other matrix components. The remaining drugs were detected through SIM. Although other drug standards were spiked into the waste water sample, MDMA-d_5 and COC-d_3 are used here for illustration purposes since their peaks could clearly be seen above the baseline. Both internal standards were spiked at 5 µg/mL. A further illustration of the complexity of untreated waste water samples can be found in Appendix VIII where a comparison with the chromatogram of a mixed drug standard is shown.

In spite of the complexity of the waste water matrix, some of the target drugs could still be detected in unspiked waste water samples during preliminary studies and method optimisation. Figure 3.27 shows the SIM spectra of MOR (*m/z* 414) and KET (*m/z* 320) detected in the unspiked, untreated waste water sample shown in Figure 3.26. The RT and selected diagnostic ion was used for confirmation of identity when compared to a spiked waste water sample. An ethyl acetate blank analysed directly before the unspiked waste water sample was used to check for any carryover. In the unspiked waste water sample (Figure 3.27), the MOR peak at a RT of 20.41 and the KET peak at RT of 16.45 have ion counts of 293 and 130, respectively. Corresponding MOR and KET peaks in the ethyl acetate blank have ion counts of 3 and 6, respectively, indicating negligible carryover at less than 5 % (van Nuijs, et al., 2012). In addition, Figure 3.27 further highlights the selectivity of the method as there was minimal interference from other matrix components for the selected diagnostic ions.

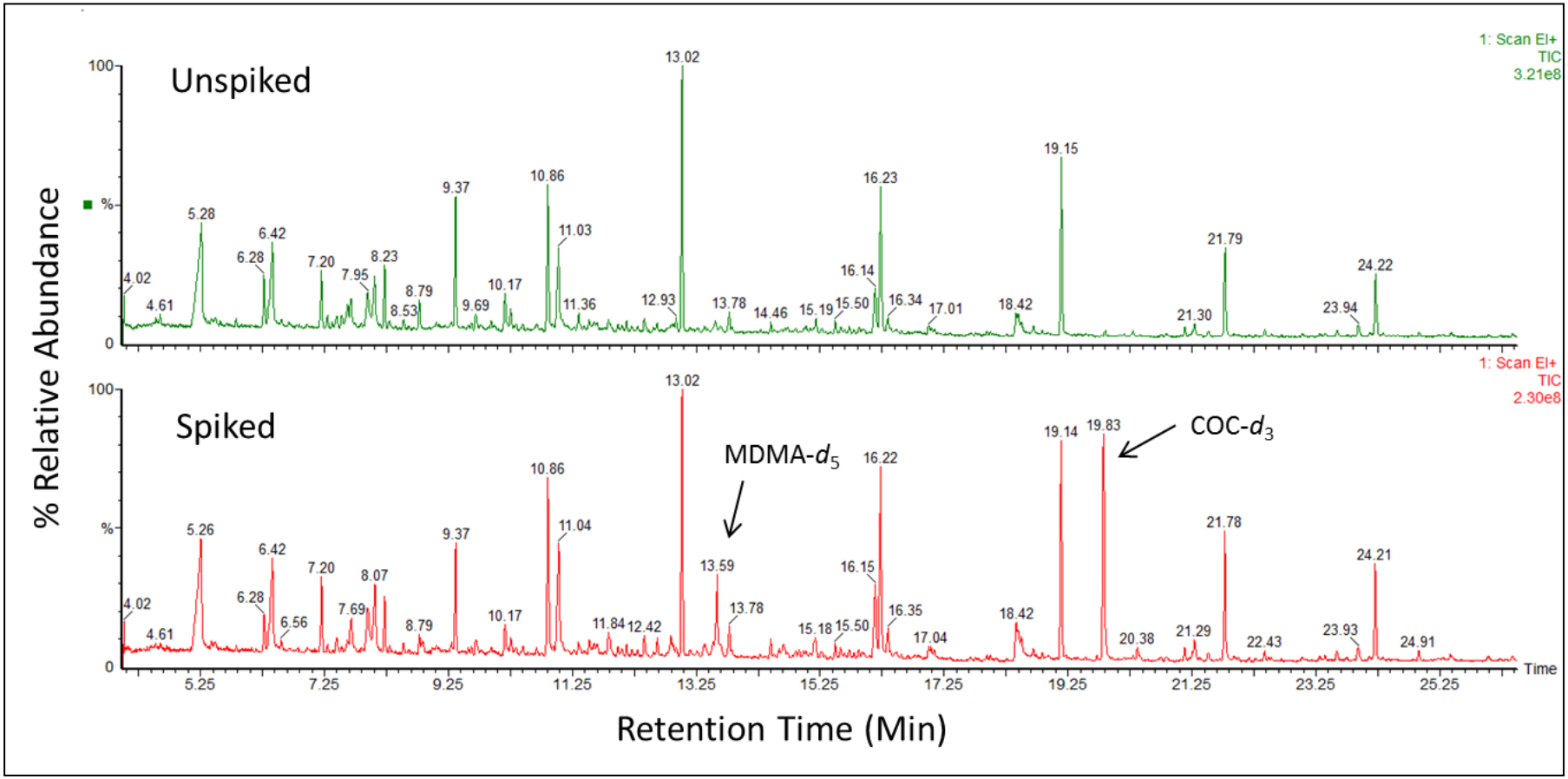


Figure 3.26: Total ion chromatograms of spiked and unspiked untreated waste water samples.

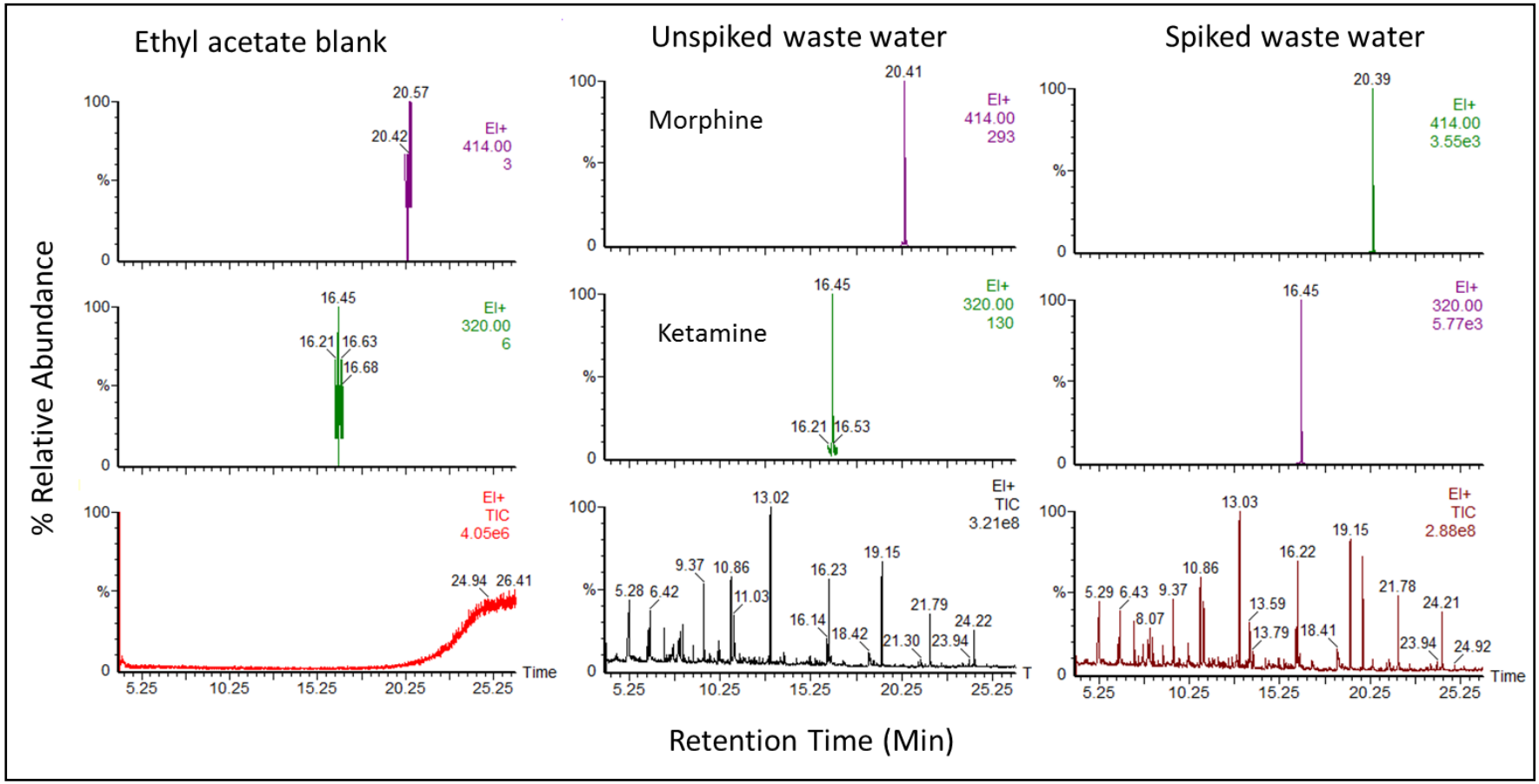


Figure 3.27: Detection of KET (RT 16.45) and MOR (RT 20.41) in an unspiked waste water sample.

As the method development progressed, 3 diagnostic ions and at least 1 ion ratio (EC, 2002; Cooper, et al., 2010; Migowska, et al., 2012; Kumirska, et al., 2013) were used in confirming the detection of the target analytes in spiked and unspiked waste water samples (Table 3.3, page 102). Appendix IX a&b shows, as an example, SIM spectra and ion ratios used in detecting and confirming the presence of MOR in waste water samples collected in different months and years using different sampling methods i.e. grab and 48 h composite. This indicates that perhaps MOR is consistently present in waste water collected from a WWTP serving the Cambridge, UK, area.

Figure 3.28 shows the quantifier and confirmation ions for AMP and 4-TFMPP recovered from an untreated waste water sample spiked with 1 µg/mL mixed drug standard. The SIM spectra of the quantifier ions for the rest of the target drugs can be found in Appendix X a-d.

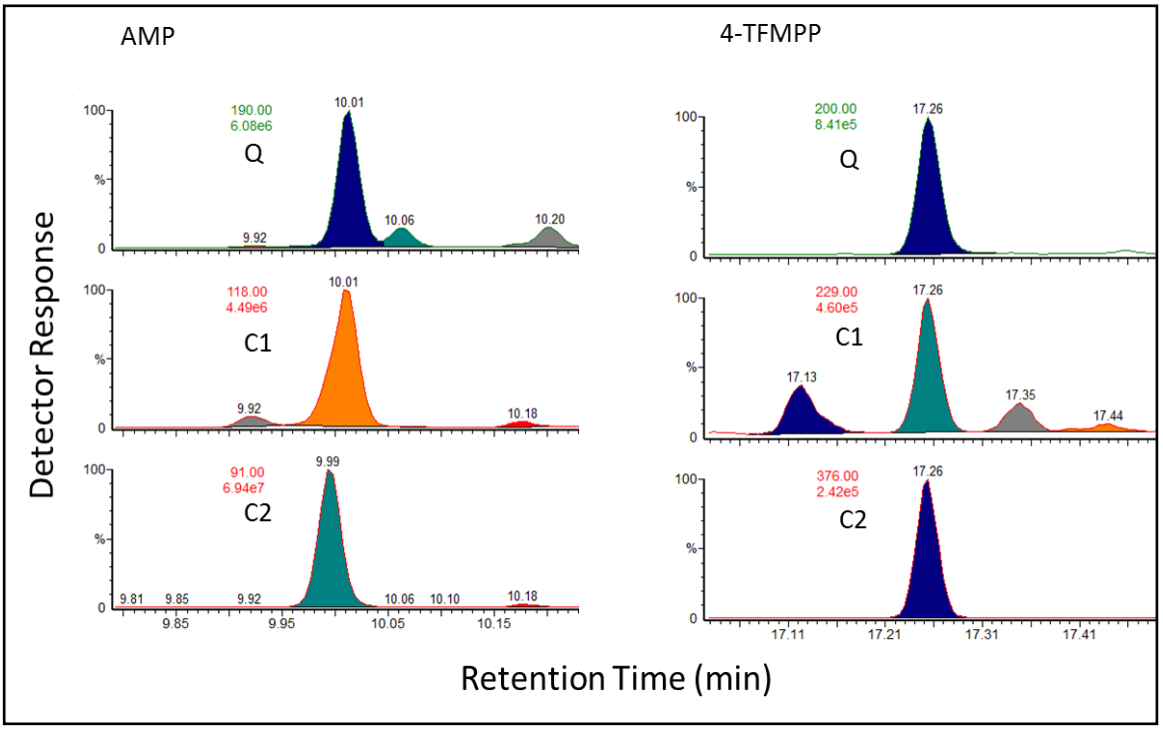


Figure 3.28: Quantifier (Q) and confirmation ions (C1 and C2) for AMP and 4-TFMPP.

Based on the detection of all diagnostic ions during various preliminary investigations as detailed in sections 3.4.1 and 3.4.2, the derivatization protocol optimised for mixed drug standards also resulted in the complete derivatization of all drugs within a waste water sample. All relevant m/z values for derivatized drugs were detected and no underivatized m/z values were detected (aside from MOR and 6-MAM as explained in

section 3.2.3). Therefore, the chosen derivatization reaction conditions were also suitable for the analysis of spiked waste water samples.

The challenge with derivatizing waste water samples is the unknown amount and variety of compounds within the sample matrix, both desired and undesired, polar and non-polar. As mentioned in section 1.5.3.1, page 32, other components in the matrix can have a negative influence on the detection of target drugs. For derivatized drugs, the effect is two-fold: signal suppression or enhancement and incomplete derivatization process (Mol, et al., 2000). The presence of other components within the matrix can affect the completion of the derivatization reaction by competing for the reagent and also influence which derivative is formed, especially for analytes with multiple derivatization sites such as PIP and MOR (Jimenez, et al., 2002; Racamonde, et al., 2013). Incomplete derivatization would result in lower amounts of the derivatized drug since some of it would still be in underivatized form and lead to inaccurate quantification.

However, in the examples shown in Figures 3.27 & 3.28 there is a lack of noticeable interference from other matrix components on quantifier ions. Although the presence of other matrix components varied from sample to sample, and their detector response varied depending on the concentration of the target drugs, the quantifier ions were always distinguishable from other peaks and confirmation was made by using 2 to 3 diagnostic ions (quantifier and confirmation) and respective ion ratios.

This adds credence to the importance of carrying out derivatization optimisation studies on mixed drug standards to determine the chromatographic and mass spectral properties of the completely derivatized drug (i.e. mass spectrum, ion ratios, RI). The results are then used to compare with chromatographic and mass spectral parameters from analysis of field samples which should be similar.

During this research, the optimised derivatization and recovery protocols ensured that identification of the target drugs in waste water samples could be made with

reliability and confidence during method validation (Chapter 4) and application of the method (Chapter 5).

Whilst conducting the extraction optimisation studies, various aspects of the protocol were fine-tuned in order to maximise the recovery of the drugs. These are discussed in the following sections.

3.4.4 Maximising Analyte Recovery

A series of measures were put in place during the SPE process in order to maximise the recovery of the target drugs. These included assessing the optimal sample volume to use depending on the matrix and cartridge as well as ensuring minimal loss of analytes during the rinsing and elution stages.

3.4.4.1 Sample Volume

For the cartridge and sorbent size used in this research (Oasis MCX 60 mg, 3 mL), it was found that 200 mL of filtered untreated waste water could be analysed before clogging started occurring. Therefore sample sizes were confined to 50 to 150 mL per cartridge during extraction studies. This was also in line with sample volumes used in published literature (Hernandez, et al., 2011; Bijlsma, et al., 2013a&b) although volumes up to 1500 mL have reportedly been used without sample breakthrough (Rodriguez, et al., 2003). Breakthrough occurs when analytes pass through the SPE cartridge unretained due to overloading of the sorbent and can result in an underestimation of the percentage recovery. Composite and passive sampling offer an advantage over large volume grab sampling in that less sample volume can be used (50 mL to 100 mL) which can also be regarded as a compromise between achieving sensitivity and minimizing matrix effects (Postigo, et al., 2008b).

3.4.4.2 Rinsing Stage

Another stage in the SPE process where loss of analytes can potentially occur is during the rinse stage (Table 2.10). A careful balance needs to be made between using solvents that are strong enough to remove interferences without causing premature elution of target analytes (Telepchak, et al., 2004). Results from the evaluation of the rinse solvent during extraction of a 150 mL deionised water (DH_2O) sample spiked

with 5 µg/mL of a mixed drug standard are presented in Table 3.11. The rinse solutions (Tables 2.9 and 2.10) were collected and analysed separately.

Table 3.11: Analyte (%) in rinse solvent from extraction of 150 mL spiked DH_2O.

DRUG	PERCENTAGE IN RINSE SOLVENT (5µg/ml, 150ml DH_2O, SIM/SCAN; 20:1) n=2
PIP	< 1
AMP-d_6	NA
AMP	< 1
MAMP	< 1
4-FMA	< 1
CAT	NA
3-FMC	< 1
MCAT	NA
EME	22
MEPH	< 1
2-FPP	< 1
BZP	< 1
4-FPP	< 1
3-TFMPP	< 1
MDMA-d_5	< 1
MDMA	< 1
MBDB	NA
4-TFMPP	< 1
4-MPP	< 1
MBZP	< 1
2-MEOPP	< 1
BUTY	1
3-CPP	< 1
4-MEOPP	< 1
KET	< 1
AMIT	< 1
COC-d_3	< 1
COC	< 1
MOR-d_3	ND
MOR	ND
6-MAM	ND
DIAZ	< 1
HEROIN	ND

NA = not applicable; DH_2O = deionised water

The % loss was calculated by dividing the absolute peak area of the analyte in the rinse solvent by the absolute peak area of the analyte in the elution solvent and multiplying by 100. The % loss of the majority of analytes was less than 5 % and this was considered to be a negligible loss (van Nuijs, et al., 2012). Even though EME showed a loss of 22 %, the rinse step was included in this research since having cleaner extracts was worth the loss in one analyte. These are some of the compromises that need to be made with multianalyte methods. However, this didn't seem to affect recovery of EME which was 109 % during validation studies (Table 6.7).

In addition, other authors also report no negative effect of the rinse step on the recovery or sensitivity of analytes (Bijlsma, et al., 2009; Baker & Kasprzyk-Hordern, 2011b).

3.4.4.3 Elution Solvent Volume

To ensure the volume of elution solvent used was sufficient to elute all target analytes and hence increase recovery, two lots of eluants were collected from each cartridge consecutively. These were referred to as eluant A and eluant B. Eluant A was collected immediately after the rinse and drying stage as per the SPE protocol. The same cartridge was then eluted a second time with the same solvents and volume as in the first elution. This was eluant B. Both eluants A and B were analysed separately with the premise that elution B should contain none or a negligible percentage of analytes as found in eluant A. The percentage of analytes in eluant B, relative to those in eluent A, was calculated by dividing the absolute area of the analytes in eluant B by the absolute area of the analytes in eluant A and multiplying by 100.

Results from the evaluation of the elution solvent during extraction of various water samples are presented in Table 3.12.

The percentage of analytes present in eluant B was found to be less than 5 % of that found in eluant A, for the majority of drugs, indicating that the volume of solvent used was sufficient in eluting the majority of analytes. However, to err on the side of caution and further optimise the elution, the solvent was allowed to stand in the cartridge for a few minutes prior to elution to maximise the disruption of bonds between the analyte and the sorbent (Metcalfe, et al., 2010). In addition, for a 4 mL volume of elution solvent, five fractions of 0.8 mL were used with the first one being allowed to stand in the cartridge or 5 minutes before elution while the others were left for about 2 minutes. AMP, EME and 4-FPP showed percentage losses ranging from 26 to 41 % in a few of the samples but since the losses were not consistent across the various matrices, this was regarded as possible random error introduced during the extraction process.

During the preliminary studies as described above (i.e. derivatization, stability and extraction), various instrumental parameters were simultaneously being optimised to ensure the reliability of the results obtained. These are discussed in the next section.

Table 3.12: Analyte (%) in elution solvent B from extraction of various water samples.

PERCENTAGE LOSS DURING ELUTION, n=2				
DRUG	5µg/mL 150ml Raw WW SIM/SCAN; 20:1	5µg/mL 150ml DH_2O SIM/SCAN; 20:1	1µg/mL 150ml Tap Water SIM; splitless	1µg/mL 50ml Raw WW SIM; splitless
PIP	< 1	< 1	2	2
AMP-d_6	NA	NA	2	1
AMP	1	< 1	2	31
MAMP	3	1	4	1
4-FMA	1	< 1	3	1
CAT	NA	NA	< 1	1
3-FMC	NA	< 1	< 1	ND
MCAT	NA	NA	1	1
EME	< 1	< 1	26	ND
MEPH	< 1	< 1	1	< 1
2-FPP	< 1	< 1	ND	1
BZP	< 1	< 1	2	1
4-FPP	< 1	< 1	41	< 1
3-TFMPP	< 1	< 1	ND	1
MDMA-d_5	6	< 1	ND	3
MDMA	1	< 1	ND	2
MBDB	NA	NA	ND	1
4-TFMPP	< 1	< 1	ND	< 1
4-MPP	< 1	< 1	ND	< 1
MBZP	< 1	< 1	2	1
2-MEOPP	< 1	< 1	ND	< 1
BUTY	< 1	2	2	< 1
3-CPP	< 1	< 1	ND	1
4-MEOPP	< 1	< 1	ND	< 1
KET	< 1	< 1	5	< 1
AMIT	< 1	< 1	ND	ND
COC-d_3	< 1	< 1	1	1
COC	< 1	< 1	4	7
MOR-d_3	< 1	ND	ND	ND
MOR	< 1	ND	ND	ND
6-MAM	< 1	ND	ND	ND
DIAZ	ND	< 1	5	1
HEROIN	ND	ND	ND	ND

NA = not applicable; DH_2O = deionised water; WW = waste water

3.5 OPTIMISATION OF INSTRUMENTAL PARAMETERS

The instrumental parameters that were optimised for method validation were SIM analysis with splitless injection (which followed on from derivatization studies and the

selection of suitable diagnostic ions, section 3.2), the GC oven temperature program (Table 2.4), and the photomultiplier tube (PMT) (section 3.5.3).

3.5.1 SIM Analysis

For method validation studies, SIM was used in conjunction with splitless injection. Various combinations of dwell time and number of ions per window were investigated resulting in the final parameters that were used in this research (Table 2.4). For the 29 drugs and 4 internal standards under investigation 14 retention time windows containing 3 to 12 ions were used. Windows with more ions were as a result of the proximity of the RTs of drugs to each other within a particular section of the TIC. Dwell times for each ion within a RT window ranged from 20 to 60 ms depending on the number of other ions present. Setting up the retention time windows and dwell times was time-consuming but it contributed to increased sensitivity (González-Mariño, et al., 2010; Migowska, et al., 2012). In Migowska (2012) study, the sensitivity increased at least two-fold through the use of SIM and retention time windows. Since at least 3 diagnostic ions were monitored (Table 3.3), this resulted in at least 87 ions being monitored. While this appears to be a large number, it shows the capability of modern instruments, especially those with mass analysers, to facilitate multianalyte method development while maintaining sensitivity and producing reliable, quantifiable results. This has led to regions of 40 to > 100 pharmaceuticals being monitored in multianalyte methods using mass analysers (Kolpin, et al., 2004; Nodler, et al., 2010; Baker & Kasprzyk-Hordern, 2011a&b; Hernandez, et al., 2011).

3.5.2 GC Oven Temperature Program

Various GC oven programs were trialled during the course of the method development resulting in the final method with a run-time of 31.17 min (Table 2.4). Published GC-MS methods for waste water analysis range from 28 - 45 min (Gonzalez-Marino, et al., 2010, Racamonde, et al., 2012) which seem long compared to average run times of 6-13 min on LC-MS systems (Gracia-Lor, et al., 2010). However this depends on the combination of drugs being analysed and the column used. When a wider selection of drugs were analysed, run times of 34 min were also reported for LC-MS (Baker & Kasprzyk-Hordern, 2011a&b). Although shorter run times are desired

for increased sample throughput, for multianalyte methods a compromise has to be made with regards to analysis time versus peak separation and increased sensitivity.

3.5.3 Photomultiplier Tube (PMT) Voltage

The voltage at which the PMT on the mass spectrometer was set also had a significant impact on the detector response. During preliminary studies the PMT voltage was automatically set at a range of 350 - 450 V (dictated by the results of tuning). This was then manually adjusted to 500 V and ultimately 600 V when lower drug concentrations were used during SIM analysis. The results from a comparison of peak areas for selected drugs using PMT settings of 500 and 600 V are shown in Table 3.13.

Table 3.13: Comparison of peak area at PMT settings of 500 and 600 V.

Drug & Quantifier Ion (m/z)	RT (min)	Peak Area		% Peak Area Increase
		PMT 500 V	PMT 600 V	
PIP, 259	9.54	209	1404	572
AMP, 190	9.92	263	1872	612
MAMP, 204	11.52	366	2238	511
4-FMA, 204	11.67	115	960	735
MDMA, 204	16.32	40	414	935

A PMT setting of 600 V led to over a 500 % increase in the peak area compared with a setting at 500 V as depicted for selected drugs. In this regard, a PMT voltage of 600 V was the final setting used for validation studies as it resulted in a significant increase in detector response to the target analytes. This was a key advantage in being able to detect trace amounts of these drugs in a complex waste water sample.

Although the number of drugs analysed during preliminary investigations varied in a few studies, for method validation studies, all optimised methods and instrumental parameters were applied to 29 drugs and 4 internal standards as discussed in Chapter 4. As mentioned earlier, although MOR-d_3 was included in the mixed drug standards used, it was not used in the calculation of PAR (section 3.3.1, pages 108-109).

CHAPTER 4

RESULTS & DISCUSSION – METHOD VALIDATION

This chapter discusses the results from the key performance tests undertaken in validating the method. The selectivity of the method, extraction recovery and stability of the drugs have been covered to a large extent in chapter three but the results discussed in this chapter are based on the final optimised derivatization, extraction and recovery and instrumental methods.

4.1 INSTRUMENTAL LINEAR RANGE

The linear range was assessed between 2.0 x 10^{-4} and 1.4 µg/mL as per the procedure in section 2.5.1. Figure 4.1 shows an example of the linear regression plot of PAR versus concentration for AMP. As already established in section 3.22 (page 91) and Appendix IV the PAR was used in the establishment of linear range and other validation parameters. This was in order to compensate for fluctuations during sample preparation and instrumental response.

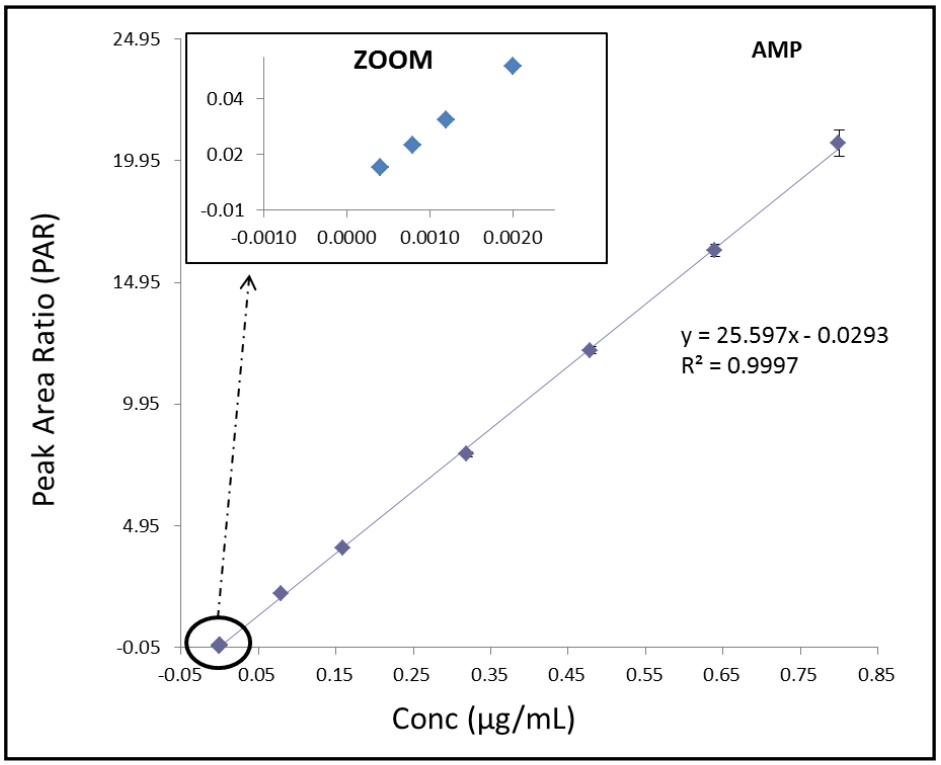


Figure 4.1: Linear regression plot of PAR versus concentration for AMP, n = 3.

The linear regression plots for each drug investigated in this research can be found in Appendix XI a-e while the correlation coefficients and linear range are listed in Table 4.1.

Table 4.1: Linear range and correlation coefficients of target drugs.

DRUG STANDARD	Linear Range, µg/mL	R^2
PIP	1.1×10^{-3} to 1.1	0.9983
AMP	4.0×10^{-4} to 0.8	0.9997
MAMP	1.2×10^{-3} to 1.2	0.9995
4-FMA	8.0×10^{-4} to 0.8	0.9998
CAT	1.2×10^{-3} to 0.8	0.9980
3-FMC	1.1×10^{-1} to 1.1	0.9959
MCAT	2.2×10^{-3} to 1.1	0.9965
EME	1.1×10^{-2} to 1.1	0.9900
MEPH	1.8×10^{-3} to 1.2	0.9967
2-FPP	2.5×10^{-3} to 1.0	0.9963
BZP	2.0×10^{-3} to 1.0	0.9958
4-FPP	1.4×10^{-3} to 1.4	0.9988
3-TFMPP	1.5×10^{-3} to 1.0	0.9977
MDMA	1.0×10^{-1} to 1.0	0.9985
MBDB	2.0×10^{-3} to 1.0	0.9983
4-TFMPP	2.4×10^{-3} to 1.2	0.9990
4-MPP	1.7×10^{-3} to 1.1	0.9999
MBZP	1.4×10^{-1} to 1.4	0.9931
2-MeOPP	1.7×10^{-3} to 1.1	0.9995
BUTY	1.3×10^{-1} to 1.3	0.9991
3-CPP	1.1×10^{-1} to 1.1	0.9993
4-MeOPP	2.0×10^{-3} to 1.3	0.9996
KET	2.2×10^{-1} to 1.1	0.9933
AMIT	1.2×10^{-1} to 1.2	0.9990
COC	6.5×10^{-3} to 1.3	0.9981
MOR	6.7×10^{-2} to 1.1	0.9912
6-MAM	Not determined	Not determined
DIAZ	1.1×10^{-1} to 1.1	0.9985
HEROIN	Not determined	Not determined

A correlation coefficient of ≥ 0.9900 is considered an acceptable measure of linearity (UNODC, 2009; Tarcomnicu, et al., 2011; Ammann, et al., 2012). Therefore these results show good linearity for all drugs assessed. The linearity was further evaluated by plots of the relative response/concentration against the log of concentration as

recommended by ISO 17025, QA/QC (Huber, 2010). This has been depicted in Appendix XII using AMP as an example.

Since the multianalyte method under development encompassed 29 drugs and metabolites from different classes the linear range was not surprisingly different for various drugs. Some drugs, including phenylethylamines, cathinones and piperazines had wide linear ranges from 4.0 x 10^{-4} to 0.8 µg/mL (AMP) to 1.8 x 10^{-3} to 1.2 µg/mL (MEPH). Other drugs, such as 3-FMC, MDMA, 3-CPP and AMIT, had a narrower linear range at 1.1 x 10^{-1} to 1.2 µg/mL. The linear ranges for 6-MAM and heroin could not be established within the chosen concentration range. A much wider range encompassing even more orders of magnitude towards the higher concentration end (1.0 – 5.0 µg /ml) was needed but this would have resulted in prolonged analysis time which could compromise on the stability of the samples. In addition, based on published studies (Table 1.2, page 9) the expected concentration of the target analytes in waste water samples would be towards the lower concentration end which was the focus of the linearity study. A separate linear test for opioids should have been assessed but this was seen as defeating the purpose of a multianalyte method. However, other researchers also reported different linear ranges for different classes of drugs (Gracia-Lor, et al., 2010; Baker & Kasprzyk-Hordern, 2013). As discussed during preliminary investigations, challenges were experienced during stability and recovery studies for 6-MAM and heroin where these two drugs could not be detected, especially below 1 µg/ml (Table 3.4 and 3.9, sections 3.3.2 & 3.3.3). This was also observed during precision studies (section 4.2) and LOD/LOQ (section 4.3). In addition, challenges were also experienced with these two analytes during derivatization studies (section 3.2.3). In sections 4.5 and 5.4.1.5, further challenges experienced by other researchers and in this thesis with regard to stability and detection of opioids are discussed. Therefore, since the linear range was established for the majority of target analytes below 1 µg/ml, especially NPS, a decision was made to proceed with the established linear ranges with a compromise on 6-MAM and heroin. Once the linear range was established the precision of the instrument could then be assessed.

4.2 PRECISION

Both intra-assay and intermediate precision were assessed according to the procedure as detailed in section 2.5.2. The results are discussed below.

4.2.1 Intra-assay Precision (Instrument)

The intra-assay precision of the instrument was evaluated at low (0.005 µg/mL), medium (0.1 µg/mL) and high (1.0 µg/mL) drug concentrations due to the wide linear range encompassing several orders of magnitude (Hartmann, et al., 1998). The results are listed in Table 4.2.

RSDs across the 3 concentrations were well below the ≤ 15 % criteria normally set for higher concentrations and the ≤ 20 % criteria normally set for lower concentration ranges, indicating good repeatability of the method (FDA, 2001; Tarcomnicu, et al., 2011). The RSD for 0.005 µg/mL ranged from 5.4 % (AMP) to 18.7 % (MCAT); for 0.1 µg/mL, 1.8 % (4-FPP) to 10.9 % (6-MAM); for 1.0 µg/mL, 0.7 % (CAT) to 11.2 % (MOR). The exception was 2-FPP with an RSD of 24.2 % at 0.005 µg/mL, most likely due to higher fluctuations in detector response normally experienced at low concentrations since respective RSDs at 0.1 and 1.0 µg/mL were less than 3.00 %.

Table 4.2: PAR and RSD from intra-assay precision (instrument).

DRUG	INTRA-ASSAY PRECISION (n = 7, 18 h analysis time)					
	0.005 µg/mL		0.1 µg/mL		1.0 µg/mL	
	Av. PAR	RSD (%)	Av. PAR	RSD (%)	Av. PAR	RSD (%)
PIP	0.05	5.9	2.56	2.7	21.20	1.0
AMP	0.15	5.4	3.37	2.7	36.79	1.3
MAMP	0.18	6.4	4.47	3.1	49.93	0.9
4-FMA	0.16	5.8	4.31	3.1	48.62	0.9
CAT	0.07	14.3	1.20	2.7	6.97	0.7
3-FMC	0.01	25.3	0.18	2.7	1.24	0.8
MCAT	0.12	18.7	2.01	2.7	14.32	0.7
EME	0.13	21.3	2.05	6.8	11.33	8.9
MEPH	0.19	7.8	4.31	2.9	34.20	1.1
2-FPP	0.03	24.2	1.10	2.5	9.29	2.7
BZP	0.60	8.8	4.25	6.1	34.66	2.0
4-FPP	ND	ND	0.50	1.8	9.38	2.0
3-TFMPP	ND	ND	1.01	3.3	10.53	2.6
MDMA	0.09	11.7	2.64	2.7	22.97	2.0
MBDB	0.09	10.0	2.23	2.6	21.01	2.8
4-TFMPP	ND	ND	0.87	3.8	8.94	2.2
4-MPP	ND	ND	0.11	7.2	7.19	3.7
MBZP	0.32	6.3	3.26	6.4	33.18	2.7
2-MEOPP	ND	ND	0.28	6.7	5.68	3.7
BUTY	0.39	8.0	6.18	1.8	46.69	2.9
3-CPP	ND	ND	0.13	6.5	2.18	3.2
4-MEOPP	ND	ND	0.02	10.0	4.30	3.7
KET	0.11	8.4	1.66	2.6	12.24	1.8
AMIT	0.45	7.6	7.15	6.5	53.92	3.2
COC	0.31	13.4	2.66	5.7	17.25	7.6
MOR	ND	ND	0.07	8.5	1.70	11.2
6-MAM	ND	ND	0.05	10.9	0.71	9.1
DIAZ	ND	ND	0.37	7.2	3.61	5.2
HEROIN	ND	ND	ND	ND	ND	ND

ND = not determined

4.2.2 Intra-assay Precision (Analytical Method)

The intra-assay precision of the analytical method was assessed on spiked extracted waste water samples. Only the highest concentration (1 µg/mL) was assessed to maximise detection of the drugs. The results are listed in Table 4.3.

RSDs for the majority of the drugs (20 out of 27 detected) ranged from 4.9 % (MAMP) to 18.6 % (3-TFMPP), indicating good repeatability especially in a complex sample (Karolak, et al., 2010; Tarcomnicu, et al., 2011). The rest of the drugs had RSDs ranging from 27.8 % (3-FMC) to 51.1 % (2-MEOPP). The high RSD for MOR (38.1 %) is

not surprising considering that it undergoes degradation reactions (sections 3.2.3.1). For the other drugs high RSDs could be attributed to errors introduced during the sample preparation and manual extraction process such as interfering co-eluants and possible variabilities with the concentration of spiked (and ultimately extracted) drugs and SPE sorbent material. Heroin and 6-MAM were not detected as per preliminary extraction experiments.

Table 4.3: PAR and RSD from intra-assay precision (analytical method).

DRUG, 1 µg/mL	INTRA-ASSAY PRECISON TREATED WASTEWATER, (n = 5, 6 h analysis time)	
	Av. PAR	RSD (%)
PIP	0.03	11.6
AMP	1.18	5.1
MAMP	1.56	4.9
4-FMA	1.54	3.9
CAT	0.18	33.3
3-FMC	0.07	27.8
MCAT	0.51	14.0
EME	0.06	15.8
MEPH	1.90	11.4
2-FPP	0.13	16.5
BZP	2.39	8.4
4-FPP	0.02	39.7
3-TFMPP	0.13	18.6
MDMA	1.04	3.0
MBDB	1.20	4.3
4-TFMPP	0.18	12.8
4-MPP	0.00	32.0
MBZP	2.46	7.8
2-MEOPP	0.03	51.1
BUTY	3.03	6.5
3-CPP	0.00	28.8
4-MEOPP	0.00	15.6
KET	0.14	12.8
AMIT	2.64	8.6
COC	0.74	3.8
MOR	0.00	38.1
6-MAM	ND	ND
DIAZ	0.18	6.9
HEROIN	ND	ND

ND = not determined

4.2.3 Intermediate Precision (Instrument)

The intermediate precision of the instrument was also conducted at low (0.005 µg/mL), medium (0.1 µg/mL) and high (1.0 µg/mL) drug concentrations and the results are in Table 4.4.

Table 4.4: PAR and RSD from intermediate precision (instrument).

DRUG	INTERMEDIATE PRECISION (n = 3, over 3 days)					
	0.005 µg/mL		0.1 µg/mL		1.0 µg/mL	
	Av. PAR	RSD (%)	Av. PAR	RSD (%)	Av. PAR	RSD (%)
PIP	0.05	3.7	2.48	3.8	20.27	5.4
AMP	0.17	11.5	3.55	3.7	37.59	1.5
MAMP	0.22	15.7	4.71	4.2	49.61	1.3
4-FMA	0.20	16.3	4.46	2.5	48.11	1.4
CAT	0.07	0.5	1.26	4.6	7.14	3.1
3-FMC	0.02	7.4	0.19	5.8	1.22	1.8
MCAT	0.15	13.0	2.17	6.4	14.58	3.2
EME	0.15	13.5	2.04	5.3	11.27	1.6
MEPH	0.23	13.5	4.60	6.2	35.02	5.0
2-FPP	0.04	23.5	1.07	2.1	9.22	3.9
BZP	0.76	15.3	4.73	6.1	37.78	10.0
4-FPP	ND	ND	0.48	3.0	9.43	3.8
3-TFMPP	ND	ND	0.97	4.3	10.41	2.7
MDMA	0.11	5.0	2.58	1.9	22.92	3.2
MBDB	0.11	9.3	2.19	2.5	20.59	2.8
4-TFMPP	ND	ND	0.84	3.4	8.90	3.7
4-MPP	ND	ND	0.10	8.6	6.39	10.4
MBZP	0.36	3.6	3.53	3.0	35.60	8.1
2-MEOPP	ND	ND	0.24	13.2	4.96	11.9
BUTY	0.43	4.1	6.23	0.7	47.50	6.3
3-CPP	ND	ND	0.12	7.4	1.97	8.5
4-MEOPP	ND	ND	ND	ND	3.78	11.1
KET	0.12	5.0	1.73	3.7	12.76	7.4
AMIT	0.55	11.1	7.39	0.9	55.32	6.2
COC	0.37	7.6	2.63	4.3	17.58	2.5
MOR	ND	ND	0.08	12.3	1.43	16.5
6-MAM	ND	ND	0.04	8.3	0.58	20.3
DIAZ	ND	ND	0.37	2.7	3.46	2.8
HEROIN	ND	ND	ND	ND	ND	ND

ND = not detected

The majority of RSDs across the 3 concentrations were well below the ≤ 15 % criteria normally set for higher concentrations and the ≤ 20 % criteria normally set for lower concentration ranges, indicating good repeatability of the method over different days of analysis (FDA, 2001; Tarcomnicu, et al., 2011). The RSD for 0.005 µg/mL ranged from 0.5 % (CAT) to 16.3 % (4-FMA); for 0.1 µg/mL, 0.7 % (BUTY) to 13.2 % (2-MEOPP); for 1.0 µg/mL, 1.3 % (MAMP) to 20.3 % (6-MAM). As observed with intra-assay precision, 2-FPP was the exception with an RSD of 23.5 % at 0.005 µg/mL indicating a greater variability with the detector response to 2-FPP at low concentrations. RSDs at 0.1 and 1.0 µg/mL, for 2-FPP, were less than 4 %.

The next stage with method validation was the determination of instrumental LOD and LOQ in order to determine whether the analytical method could detect trace amounts of target analytes, as normally found in waste water samples, and reliably quantify them.

4.3 INSTRUMENTAL DETECTION AND QUANTIFICATION LIMITS

Following the procedure as described in section 2.5.3, the instrumental LOD and LOQ for the drugs under investigation are presented in Table 4.5. The LOD and LOQ were calculated according to empirical methods i.e. analysing decreasing concentrations of a mixed standard and measuring the detector response (Baker & Kasprzyk-Hordern, 2011a). The LOD was taken as the concentration that resulted in a peak (measured from its maximum height) with a signal-to-noise (S/N) ratio of 3:1 while the LOQ was based on a peak with a S/N ratio of 10:1. Root mean square (RMS) integration, rather than peak-to-peak, was used to calculate the S/N ratio. RMS is based on the standard deviation of the fluctuation in the baseline rather than just on a maxima and minima as with peak-to-peak (Shimadzu, 2013). The results are presented in Table 4.5.

The LOD of the majority of drugs ranged from 1.36 x 10^{-4} µg/mL (e.g. AMP, BZP) to 5.33 x 10^{-3} µg/mL (e.g. 4-MEOPP) and LOQs ranged from 3.33 x 10^{-4} (e.g. AMP) to 5.33 x 10^{-3} µg/mL (e.g. KET). Some drugs such as 6-MAM and heroin had relatively high LODs of 1.33 x 10^{-1} and 5.62 x 10^{-1} µg/mL, respectively which also meant that their LOQs were also relatively high. In terms of drug groups, phenylethylamines and cathinones had lower LODs/LOQs than piperazines and opioids. Opioids are thought to undergo degradation during GC-MS analysis so their high LOQs for the developed method are not surprising (section 5.2.3.1). Although the LOD for heroin was observed to be 5.62 x 10^{-1} µg/mL, at times it was not detected at 1.0 µg/mL (Tables 4.2 & 4.4) further indicating the unpredictable behaviour of this drug (and other opioids) during this research.

Table 4.5: Instrumental LOD and LOQ

DRUG	LOD, S/N = 3, RMS		LOQ, S/N = 10, RMS	
	µg/ mL	pg on column	µg/ mL	pg on column
PIP	1.36×10^{-4}	0.14	6.67×10^{-4}	0.67
AMP	1.36×10^{-4}	0.14	3.33×10^{-4}	0.33
MAMP	1.36×10^{-4}	0.14	3.33×10^{-4}	0.33
4-FMA	1.36×10^{-4}	0.14	3.33×10^{-4}	0.33
CAT	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
3-FMC	1.36×10^{-4}	0.14	2.67×10^{-3}	2.67
MCAT	1.36×10^{-4}	0.14	6.67×10^{-4}	0.67
EME	6.67×10^{-4}	0.67	1.33×10^{-3}	1.33
MEPH	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
2-FPP	6.67×10^{-4}	0.67	2.67×10^{-3}	2.67
BZP	1.36×10^{-4}	0.14	3.33×10^{-4}	0.33
4-FPP	6.67×10^{-4}	0.67	2.67×10^{-3}	2.67
3-TFMPP	6.67×10^{-4}	0.67	2.67×10^{-3}	2.67
MDMA	3.33×10^{-4}	0.33	1.33×10^{-3}	1.33
MBDB	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
4-TFMPP	1.33×10^{-3}	1.33	5.33×10^{-3}	5.33
4-MPP	2.67×10^{-3}	2.67	5.33×10^{-3}	5.33
MBZP	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
2-MEOPP	2.67×10^{-3}	2.67	1.07×10^{-2}	10.67
BUTY	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
3-CPP	1.07×10^{-2}	10.67	2.13×10^{-2}	21.33
4-MEOPP	5.33×10^{-3}	5.33	1.07×10^{-2}	10.67
KET	1.33×10^{-3}	1.33	5.33×10^{-3}	5.33
AMIT	1.07×10^{-2}	10.67	2.13×10^{-2}	21.33
COC	3.33×10^{-4}	0.33	6.67×10^{-4}	0.67
MOR	6.67×10^{-2}	66.67	1.41×10^{-1}	141.39
6-MAM	1.33×10^{-1}	133.33	5.33×10^{-1}	533.33
DIAZ	1.07×10^{-2}	10.67	2.13×10^{-2}	21.33
HEROIN	5.62×10^{-1}	562.40	1.12	1124.80

RMS = root mean square; 'µg/ mL to pg on column' conversion takes into account a 1µl injection volume and splitless injection mode.

Due to different injection volumes used on different instruments i.e. 1 - 2 µl in this research (Table 2.4) and 20 µl for LC-MS (Bagnall, et al., 2012), pg on column was used to compare LOD and LOQ values obtained in this research with those from published literature (normalized to a 1 µl injection). The comparisons are shown in Table 4.6.

Table 4.6: Comparison of LOD and LOQ with literature (pg on column).

DRUG	GC-MS[1]		GC-MS/MS[2]		LC-MS/MS[3]		LC-MS/MS[4]		LC-QTOF-MS[5]	
	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]	LOD[a]	LOQ[b]
AMP	0.14	0.33	0.8	-	2	10	-	380	20	52
MAMP	0.14	0.33	1.45	-	0.5	2	-	208	50	200
MDMA	0.33	1.33	2.95	-	0.5	2	-	278	80	300
MCAT	0.14	0.67	-	-	1.5	10	-	-	-	-
BZP	0.14	0.33	-	-	10	2	-	-	-	-
3-TFMPP	0.67	2.67	-	-	0.5	2	-	-	-	-
KET	1.33	5.33	-	-	0.5	2	-	-	-	-
COC	0.33	0.67	1.4	-	-	-	-	18	-	-
MOR	66.67	141.39	2.05	-	-	-	-	250	-	-
Heroin	562.40	-	17.25	-	-	-	-	-	-	-

a = 3 x (S:N); b = 10 x (S:N); [1]Mwenesongole, et al., 2013; [2]Gonzalez-Marino, et al., 2010; [3]Baker & Kasprzyk-Hordern (2011a); [4] Castiglioni, et al., 2006; [5]Bagnall, et al., 2012.

For AMP and MAMP, the LOD values (0.14 and 0.33 pg, respectively) reported in this thesis are lower than those for GC-MS/MS i.e. 1.6 pg and 2.9 pg, respectively, from published literature (Gonzalez-Marino, 2010). However, for MOR and heroin, the LOD values obtained in this research (66.67 and 562.40, respectively) were much higher than those reported by Gonzalez-Marino (2010) at 2.05 pg and 17.25 pg, respectively. However, silylation was used and this could have been a better derivatization method for the opioids especially with regards to degradation during injection or in solvents which was experienced with the PFPA derivatives (section 5.2.3.1).

In a separate comparison, LOQs from this research were much lower than those reported by Castiglioni (2006) on an LC-MS/MS instrument for MOR (250 pg), AMP (380 pg), MAMP (208 pg), and MDMA (278 pg) but was higher than that reported for COC (18 pg). This shows that the analytical method used in this research (based on derivatization with PFPA and GC-MS) is more sensitive than GC-MS/MS or LC-MS/MS for some drugs. Therefore, GC-MS is just as sensitive for trace analysis in complex

matrices as triple quadrupole instruments and hence can be used routinely for sewage epidemiological studies (Vazquez-Roig, et al., 2013).

4.4 SPE EXTRACTION AND RECOVERY USING OPTIMISED INSTRUMENTAL METHOD

Following the procedures as detailed in section 2.5.4, the recoveries of drugs in spiked treated waste water are listed in Table 4.7. For validation purposes, Equation 4.1 (Chambers, et al., 2007; Karolak, et al., 2010) was used to calculate the recovery as it was expected to give more representative results than Equation 3.1 that was used during preliminary investigations (section 3.4, page 116).

$$\% \text{ Recovery} = \left(\frac{\text{Peak area of drug standard spiked before extraction}}{\text{Peak area of drug standard spiked after extraction}} \right) \times 100$$

Equation 4.1

The treated waste water samples were evaluated for the presence of target analytes and any found to be present were accounted for during calculations.

The RSDs for over half of the extracted analytes ranged from 4.4 % (AMP-d_6) to 19.3 % (MEPH), which are within the ≤ 20 % criteria (FDA, 2001; Tarcomnicu, et al., 2011). The exceptions with RSDs ranging from 21.7 to 60.6 % included PIP, 3-FMC, MOR and 2-MEOPP. This indicates that there was a combination of low and high extraction precision for different drugs as observed in Table 4.3 (the same matrix and experimental conditions were applied). RSD values for the post-extracted spiked sample were much better, with the majority ranging from 1.7 % (AMP-d_6) to 18.2 % (MOR-d_3). The exceptions, with RSDs ranging from 21.4 to 52.0 %, were 2-FPP, 3 and 4-TFMPP, 4-MPP and 2-MEOPP. High RSDs can be attributed to errors during the sample preparation and manual extraction process as discussed in section 3.4.2.1. This suggestion is further confirmed by the much lower overall RSDs for the drugs spiked into the post-extracted sample. For instance the RSD for CAT was 46.1 % in the extracted spiked sample but reduced to 3.5 % in the post extracted spiked sample.

Table 4.7: Recovery (%) of a treated waste water sample.

DRUG	EXTRACTED SPIKED SAMPLE (100 mL, n = 5)		POST-EXTRACTED SPIKED SAMPLE (100 mL, n = 3)		Recovery (%)
	Av. Peak Area	RSD (%)	Av. Peak Area	RSD (%)	
PIP	6942	21.7	89890	3.7	8
AMP-d6	195347	4.4	245431	1.7	79
AMP	241577	14.8	282469	4.3	84
MAMP	354956	26.9	439367	3.4	81
4-FMA	301670	7.3	378332	3.5	80
CAT	33246	46.1	22453	3.5	148
3-FMC	14823	44.7	3400	13.0	436
MCAT	97149	24.5	45060	7.7	216
EME	3124	9.2	2861	11.1	109
MEPH	216331	19.3	133429	2.4	162
2-FPP	14626	23.4	11276	31.9	92
BZP	269576	6.4	326295	4.2	78
4-FPP	1922	48.6	1198	17.0	160
3-TFMPP	15349	24.6	8717	36.4	161
MDMA-d5	113540	10.8	177209	7.3	64
MDMA	120313	9.1	164428	6.1	70
MBDB	137076	9.0	160676	6.6	85
4-TFMPP	19977	17.9	16005	25.3	100
4-MPP	114	39.3	65	21.4	176
MBZP	277390	7.3	348380	4.0	74
2-MEOPP	3826	60.6	3290	52.0	115
BUTY	343907	10.4	336096	1.7	100
3-CPP	229	33.9	203	7.8	113
4-MEOPP	297	30.7	286	8.1	104
KET	15439	13.3	25278	9.6	44
AMIT	298616	6.9	272235	10.3	107
COC-d3	53931	11.2	33954	13.6	156
COC	39339	7.7	24152	15.0	162
MOR-d3	79	33.3	179	18.2	44
MOR	90	46.2	119	13.9	76
6-MAM	ND	ND	ND	ND	ND
DIAZ	20664	7.6	22684	3.7	90
HEROIN	ND	ND	ND	ND	ND

ND = not detected

Since the drugs spiked into the post-extracted sample have not been through the extraction process, RSDs are expected to be less than for the extracted standards as the drugs are exposed to less intrinsic variables associated with the extraction process. The exceptions were for 2-FPP, 3-TFMPP and 4-TFMPP which saw an increase in RSDs in the post extracted spiked sample possibly due to errors during drying, derivatization and analysis.

Most of the drugs had recoveries falling between 64 and 162 %. PIP exhibited a very low recovery (8 %) possibly due to signal suppression by co-eluting interferents as higher recoveries (36 to 93 %) had previously been obtained (Table 3.10). The recoveries greater than 100 % are not unusual in sewage epidemiology (Vazquez-Roig, et al., 2013) and can be due to many factors such as the higher concentration of extracted standard compared with the post-extracted standard (section 3.4.2.1), loss of sensitivity during analysis (Bogusz, et al., 1985) or interfering co-eluants from the matrix or SPE cartridges (Gonzalez-Marino, et al., 2010; Gracia-Lor, et al., 2010; Baker & Kasprzyk-Hordern, 2011b). Low recoveries can be due to loss during extraction or evaporation (Mol, et al., 2000; Burgard, et al., 2013), unsilanised glassware (Baker & Kasprzyk-Hordern, 2011b) or signal suppression due to matrix effects (Kasprzyk-Hordern, et al., 2007).

However, use of Equation 4.1 to calculate recovery gave more realistic results than Equation 3.1 used during preliminary investigations (section 3.4, page 116). Using the peak area of the sample spiked after the extraction process (added to the eluant) as the denominator ensured that the drugs were exposed to relatively similar solvents and sample preparation post extraction to those spiked before extraction (i.e. longer evaporation, matrix components and derivatization). The neat unextracted standard, as used in Equation 3.1 during preliminary investigations (section 3.4, page 116), was not exposed to the post elution process (i.e. longer evaporation and matrix components) and hence greater errors could be introduced in recovery calculations resulting in unusually high recoveries [Table 3.8 (LLE) & Table 3.9 (SPE)]. In addition, for raw waste water samples, any drugs already present in the sample would be accounted for with the post-extraction standard leading to more accurate recovery calculations. Therefore, only Equation 4.1 was used in extraction and recovery studies for method validation.

In this study an EME recovery of 109 % was obtained which is an improvement on the recovery of 35 % reported by van Nuijs (2009b). Baker & Kasprzyk-Hordern (2011a) also report recoveries of drugs greater than 60 % in raw waste water but acknowledge that some drugs such as EDDP and EMDP showed highly variable recoveries across the different matrices studied, as was also observed for some drugs in preliminary

investigations during this research (Table 3.10). 6-MAM and heroin were not detected in this study and during preliminary investigations which correlates with findings from published reports where these drugs were below the LOQ or were not detected (Boleda, et al., 2009; Baker & Kasprzyk-Hordern, 2011a). This is attributed to possible deacetylation of heroin to 6-MAM which then hydrolyses to morphine (Boleda, et al., 2009).

Internal standards were included in the study to evaluate their recovery especially when used for calculation of PAR where 100 % recovery is assumed. From Table 4.7, one can see that the recoveries for AMP-d_6, MDMA-d_5 and COC-d_3 were 79, 64 and 156 %, respectively. The recovery for COC-d_3 is due to a higher concentration of extracted standard compared with the post-extracted standard due to a number of factors as described above.

A comparison of the recoveries with column C from Table 3.10 (similar type matrix) shows a much lower recovery for PIP (8 versus 36 %) but recoveries > 70 % for some drugs such as MBZP, MDMA , AMP and 2-FPP. This indicates the potential of the method to give reproducible recoveries for some drugs even though the sample matrices were different (untreated versus treated waste water) collected on different days and using a different GC oven program. No two waste water samples have the same composition and hence the matrix components present will also differ and give rise to the different recoveries obtained. As a result of this, with multianalyte methods, the analytical process used is a compromise for the different analytes and therefore it would be unrealistic to expect high recovery and low RSDs for all compounds in the mixture (Jimenez, et al., 2002). In this regard, the acceptance criteria applied to recovery is that the values should be reproducible and precise regardless of the percentage value obtained (FDA, 2001; UNODC, 2009; Tarcomnicu, et al., 2011).

Considering the different variables that are introduced with manual extraction and different sample matrices, the recovery values and RSDs achieved in this research showed that the method was suitable and reproducible for extracting the majority of targeted drugs.

4.5 MATRIX-BASED STABILITY

Following the procedures as detailed in section 2.5.5, Table 4.8 lists the data from the stability study of drugs in untreated waste water stored at 5 °C over 7 days . Stability was assessed as stated in section 3.3.2, page 110. This stability study incorporated 29 emerging and classic drugs of abuse and metabolites. As far as the author is aware, no other waste water stability study has incorporated this combination and number of illicit drugs to date, especially for cathinones and piperazines.

The majority of the drugs were stable to moderately stable after 3 days of storage (see Table 4.8 footnote). EME, MOR and 6-MAM showed instability. After 7 days of storage, the majority of drugs were still stable to moderately stable with only 3-FMC showing instability in addition to the drugs already mentioned. However, overall percentage change in PAR over the 7 day study period was less than the 30 % criteria for stability with the majority of drugs experiencing a change in PAR of < 18 %.

MOR and 6-MAM had percentage increases > 100 % over the 7 day period and heroin was not detected. Based on the proposed hydrolysis and deacetylation reactions as depicted in Figures 3.12 to 3.15, it can be postulated that perhaps the hydrolysis of heroin led to the increase in the concentrations of 6-MAM and MOR. There is also a possibility that the increase in MOR concentration was due to the deconjugation of morphine-3-glucuronide and morphine-6-glucuronide back to MOR, which naturally occurs in waste water (Melis, et al., 2011; van Nuijs, et al., 2011a; Castiglioni, et al, 2013b; Senta, et al., 2014) as mentioned in section 1.4.3, page 26.

Table 4.8: Matrix-based stability of drugs stored at 5 °C.

DRUG	Day 3		Day 7	
	% PAR[a]	Stability	% PAR[a]	Stability
PIP	1	1	-6	1
AMP	-25	2	-27	2
MAMP	-5	1	-6	1
4-FMA	-3	1	-5	1
CAT	-5	1	-3	1
3-FMC	-7	1	-50	3
MCAT	4	1	3	1
EME	-64	3	-130	3
MEPH	-14	1	-5	1
2-FPP	17	2	16	2
BZP	11	1	8	1
4-FPP	18	2	9	1
3-TFMPP	14	1	16	2
MDMA	-14	1	7	1
MBDB	13	1	8	1
4-TFMPP	16	2	13	1
4-MPP	16	2	11	1
MBZP	8	1	6	1
2-MeOPP	18	2	11	1
BUTY	14	1	14	1
3-CPP	25	2	25	2
4-MeOPP	16	2	11	1
KET	-28	2	-29	2
AMIT	11	1	11	1
COC	9	1	6	1
MOR-d3	-109	3	-103	3
MOR	-113	3	-103	3
6-MAM	-141	3	-153	3
DIAZ	24	2	28	2
HEROIN	ND	ND	ND	ND

1 = stable; 2 = moderately stable; 3 = unstable (Saar, et al., 2012); ND = not detected;
a = 100 – ((PAR day n/PAR day 0)x100)

In contrast to the stability studies in sections 3.3.1, 3.3.2 and 3.3.3 which were conducted on mixed drug standards in an organic solvent, this study investigated stability in a real waste water sample in order to determine the extent of the degradation of drugs after sample collection and establish how long they can be stored for before extraction.

Marix based stability results for EME, 6-MAM and heroin correlate with earlier findings in sections 3.3.1, 3.3.2 and 3.3.3 where these drugs were the most unstable in relation to the other drugs. Another observation made was that 3-FMC was highly unstable when stored as the underivatized drug (section 3.3.3) but quite stable when stored as the derivatized drug (sections 3.3.1. and 3.3.2). In this study, 3-FMC was spiked into the matrix as the underivatized drug and than only derivatized on the day of analysis and the results correlate with those in section 3.3.3, i.e. unstable when underivatized.

The results from this study correlate with findings from Baker & Kasprzyk-Hordern (2011b) for similar drugs in acidified waste water stored for over 72 h at 2 °C and with Chen (2013) for BZP, 3-TFMPP and MEPH stored at 4 °C, which were also stable. Although in Baker & Kasprzyk-Hordern‘s (2011b) study, AMP, KET, MOR, 6-MAM and heroin were all stable under the conditions of testing (with no derivatization).

There is little in-sewer stability data on NPS but some biodegradation of KET, MEPH, and MDMA has been reported from stability studies conducted at pH 7.2 (Reid, et al., 2014 a&b). However, as presented in Table 4.8, the majority of drugs analysed in this thesis, in particuar the ATS and NPS (cathinones and piperazines) were stable to moderately stable at pH 2.5, with the exception of 3-FMC. The stability of emerging drugs such as PIP, 4-FMA, CAT, 3-FMC, 2-FPP, 4-FPP, 4-MPP, MBZP, 2-MEOPP, and 4-MEOPP in waste water has not been reported before.

Although a number of matrix-based stability studies from published literature were conducted after sample collection (as in this research), a handful were also conducted in simulated sewage conditions to assist with a better understanding of the degradation process from their points of excretion to the sampling point (van Nuijs, et al., 2012; Senta, et al., 2014; Thai, et al., 2014). Results between the two types of stability studies correlate to a large extent especially with regards to the stability of ATS and BZE but differ on the stability of COC, MOR, EME and 6-MAM (Castiglioni, et al., 2013; Vazquez-Roig, et al., 2013). In Thai’s (2014) stability study conducted in simulated sewerage conditions (pH 7.5), a significant degradation of COC and 6-MAM was observed, while MAMP, MDMA and BZE did not undergo any significant

degradation. Significant changes were also observed for 6-MAM, COC, and MOR in Senta's (2014) study while ATS were stable. van Nuijs (2012) also reports the stability of ATS and BZE under in-sewer conditions but the degradation of COC, EME and 6-MAM. However, in results from this thesis, COC did not undergo significant degradation but 3-FMC, EME, MOR and 6-MAM did (Table 4.8). This indicates that differences in results from stability studies between research groups is expected due to the varied composition and pH of the waste water samples, the drug mix combination and spiking concentration, sample extraction and preparation, stability study conditions and instrumental methods used (Baker, et al., 2012; Castiglioni, et al., 2013; Senta, et al., 2014). However, few studies have been able to detect heroin and 6-MAM during sewage epidemiological studies owing to their significant degradation, which results in concentration levels below the LOD of most methods including LC-MS which do not involve a derivatization step (Baker, et al., 2014; Thai, et al., 2014; Vuori, et al., 2014; Yargeau, et al., 2014). Therefore, the challenges experienced in this thesis with regards to the analysis of opioids are not unique and occur whether derivatization is included in the method (GC-MS) or not (LC-MS).

Simulated in-sewer stability tests help with understanding the degradation process of drugs between their points of excretion and sampling. For example, in urban sewerage networks, it can take anything from 0.5 to 15 h for waste water to reach the sampling point, depending on the size of the WWTP (Castiglioni, et al., 2013; Chen, et al., 2013). Drugs that show significant degradation within hours, therefore, can be detected at levels below LOD or not at all. In addition to the stability in the sewer system, the length of sampling is also a potential source of error, especially when it is extended. Since samples collected in composite autosamplers (as in this thesis) can stay at the sampling point from 12 to 72 h, the stability of drugs during sampling needs to also be considered. However, while 24 h composite sampling has been regarded as a potential source of error in one study (Baker, & Kasprzyk-Hordern, 2011b), in a separate study most of the target analytes were highly stable when stored for 72 h at 4 °C, except 6-MAM and COC which exhibited significant degradation (Senta, et al., 2014). Therefore, even if the target analytes can be stabilised after collection, analyte loss throughout the sewage network and in the

autosamplers at WWTPs can be difficult to estimate especially for target analytes which experience significant degradation within hours (Chen, et al., 2013).

Reported stability studies in waste water have been conducted over 12 h (Thai, et al., 2014), 3 days (Castiglioni, et al., 2006; Baker & Kasprzyk-Hordern, 2011b; Senta, et al., 2014), 5 days (Gheorge et al., 2008) and 7 days (Castiglioni, et al., 2006). Longer term stability studies on MCX cartridges loaded with spiked acidified sample matrix, wrapped in aluminium foil and stored at -20 °C for 6 weeks before elution found all analytes to be stable (Baker & Kasprzyk-Hordern, 2011b). A similar study on HLB cartridges loaded with non-acidified spiked waste water samples and stored at -20 °C for up to 3 months prior to elution also found all analytes to be reasonably stable (Gonzalez-Marino, et al., 2010). Therefore, for storage space considerations, cartridges loaded with samples are a worthy alternative to storage of bottles containing waste water samples (Baker & Kasprzyk-Hordern, 2011b).

It has already been established that acidification and storage at -20 °C is thought to curb any biological activity, preserve the analytes and allow for longer time periods between sample collection and extraction (months versus days or weeks) (section 1.4.4). Although the majority of drugs in this study were stable in acidified waste water for up to 7 days at 5 °C, precautions were taken to curb the observed degradation of some of the drugs. This involved filtration of the sample, then acidification to pH 2.5 followed by storage at 5 °C for extraction within 72 h or at -20 °C for longer storage before extraction.

Overall the majority of target drugs studied were stable in acidified untreated waste water (pH 2.5) for up to 7 days when stored at 5 °C. However, the sampling method, the type of sample preservation technique, e.g. acidification, all play a crucial role in the levels of drugs detected in waste water and this needs to be taken into account during method development, especially with multianalyte methods containing drugs with different physico-chemical properties (Senta, et al., 2014). For drugs that show significant degradation during stability studies, i.e. 3-FMC, 6-MAM, this would need to be taken into account when estimating the relevant drug consumption. In addition, knowledge about the HRT is required for each target analyte in order to more

accurately estimate the level of degradation (Thai, et al., 2014). It could be that any results obtained (quantification or linking to community consumption) could be a gross under- or over- estimation of the actual levels of the drugs in waste water or their consumption (van Nuijs, et al., 2012). A full understanding of the different chemical interactions that drugs are subject to whilst in waste water was beyond the realm of this research. However, the matrix-based stability study undertaken during this research, and in published studies, provides a general approach for determining the stability of drugs of similar classes to those used here. Further studies on the effect of the attenuation processes (section 1.3.4.2) on the target analytes, in conjunction with the type of sewer system and treatment processes applied, is required in order to provide a more accurate estimation of drug consumption using the sewage epidemiological approach (Thai, et al., 2014).

Stability is, therefore, an important criterion when selecting a target analyte to be used in estimating illicit drug consumption. As a result, stability estimations have recently started being incorporated into back-calculations for drug consumption (Baker, et al., 2012 & 2014).

The various tests discussed in this chapter showed that the developed method was suitable and ready for application on untreated waste water samples.

In Chapter 5, the results from the application of the validated method to untreated waste water samples collected from a WWTP serving the Cambridge, UK, area are discussed in more detail with respect to detection and linking to community consumption.

CHAPTER 5

RESULTS AND DISCUSSION - APPLICATION OF THE VALIDATED METHOD

This chapter presents results from the application of the validated method to a 72 hour composite sample collected from Anglian Water in Cambridge, UK, using standard addition for quantification.

5.1 STANDARD ADDITION PLOTS

Standard addition was carried out following the procedures as detailed in section 2.5.6. Figure 5.1 depicts an example of the standard addition plot of the PAR against the concentration for COC.

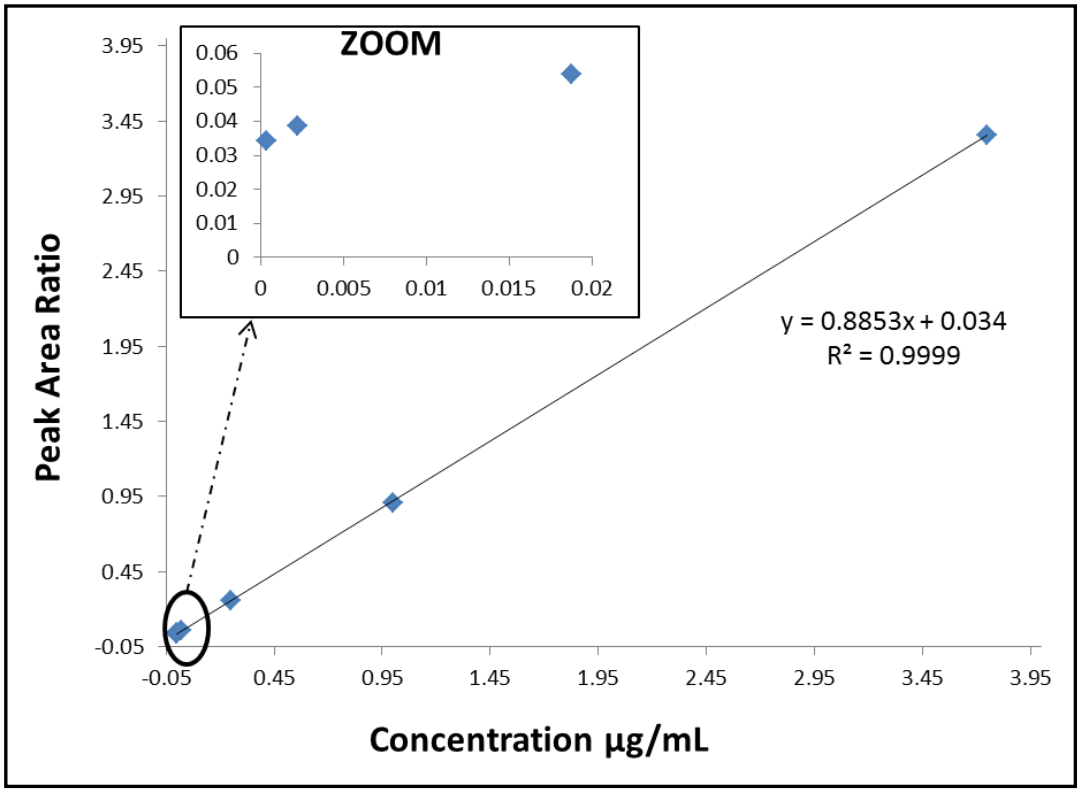


Figure 5.1: Standard addition plot of PAR versus concentration for COC, n = 3.

With standard addition, the y intercept is 0 when no drug standard has been spiked into the sample i.e. 0 = mx + c. When the sample matrix is spiked with a mixed drug standard, the intercept becomes positive from the response of the analyte already present in the sample. Standard addition plots for the rest of the detected drugs are in Appendix XIII a&b. All plots were linear with $R^2 \geq 0.9900$ (UNODC, 2009; Cooper, et al., 2010; Ammann, et al., 2012).

5.2 DETECTED DRUGS

Table 5.1 lists the correlation coefficients and concentrations of the drugs detected in untreated waste water.

Table 5.1: Correlation coefficients (R^2) and concentration (µg/mL) of drugs detected in a 72 h composite waste water sample from Cambridge, UK, n=3.

DRUG	R^2	Conc. (µg/mL) ± StdDev
AMP	0.9998	0.023 ± 0.015
MAMP	0.9954	0.072 ± 0.050
MCAT	0.9999	0.253 ± 0.083
EME	0.9904	0.114 ± 0.056
MEPH	0.9993	0.549 ± 0.046
3-TFMPP	0.9966	0.098 ± 0.004
BUTY	0.9999	0.004 ± 0.002
4-MeOPP	0.9997	0.008 ± 0.006
KET	0.9999	0.097 ± 0.007
COC	0.9999	0.038 ± 0.005
MOR	0.9982	0.256 ± 0.058

Out of the 29 target analytes, 11 drugs and metabolites were detected in waste water within their established linear range and LOQ (Tables 4.1 and 4.5, respectively). These include the emerging drugs of abuse belonging to the cathinone group, MCAT, MEPH and BUTY; the piperazine group, 3-TFMPP and 4-MEOPP as well as classic drugs of abuse such as AMP, MAMP and COC. Also detected were EME (COC metabolite) and the therapeutic drugs KET and MOR. Appendix XIV a&b gives an example of the calculation for the concentration of COC in waste water using standard addition.

The highest concentration levels detected were for MCAT (0.253 ± 0.083), MEPH (0.549 ± 0.046) and MOR (0.256 ± 0.058), all in µg/mL as expounded upon in sections 5.4.1.5, 5.4.2 and 5.4.2.1.

5.2.1 Confirmation of Detected Drugs

With complex matrices such as waste water, analytes are present at trace levels and therefore the chances of observing co-eluting interferents of similar or higher concentrations are greatly increased. In addition waste water is hugely variable in composition and hence no one blank sample exists from which the background can be subtracted for all future analyses (Bijlsma, et al., 2009). It is therefore realistic to expect that with complex and unpredictable matrices, matrix components that share a particular *m/z* value with an analyte may be present and could offset the ion ratios (Gracia-Lor, et al., 2010; Portoles, et al., 2011). This problem is even more pronounced when only one ion is monitored since any matrix component sharing that *m/z* value can lead to a false positive result (Pozo, et al., 2006). In this research, throughout the method development, confirmation using 3 or more diagnostic ions (for the majority of analytes) could be reliably made. The exception was AMIT which only gave one diagnostic ion (*m/z* 58) of relative abundance greater than 10% (Appendix VI). When at least three diagnostic ions (and hence at least one ion ratio) are monitored in a sample matrix with respect to the analytical standard, it is highly unlikely that another matrix component will share the same three ions at a particular RT (so-called isobaric effects) (Rivier, 2003). However, its presence in even one of the monitored ions will offset the ion ratios when compared to the unextracted standard (Gracia-Lor, et al., 2010). These so-called isobaric effects are more likely to be a factor in single quadrupole GC-MS or LC-MS and hence the more diagnostic ions that are chosen for SIM analysis the less likely the errors when reporting findings (Couchman & Morgan, 2010; Vogeser & Seger, 2010). This also gives the opportunity of maintaining at least one ion ratio if one is out of acceptable limits due to matrix interferents (Pozo, et al., 2006).

In this research, at least one ion ratio was within acceptable limits for all detected compounds i.e. ± 20 % (EC, 2002; Cooper, et al., 2010; van de Steene, et al., 2010; Baker & Kasprzyk-Hordern, 2011a; Trinh, et al., 2011; Migowska, et al., 2012). In addition to diagnostic ions and ion ratios, other parameters used to confirm the identity of target analytes were the RT and RI, thereby exceeding the criteria of three identification points (IPs) as recommended by the applicable guideline adopted for this research i.e. EC 2002. [IPs used in this research include at least 3 diagnostic ions

(equivalent to 1 IP per ion), at least 1 ion ratio (equivalent to 1 IP per ion ratio falling within specifications), the RT (equivalent to 1 IP), the RI (equivalent to 1 IP)]. It is worth mentioning that there are no specific guidelines for criteria used in the identification and confirmation of analytes in variable and complex waste water samples. The EC, 2002 guidelines have been 'adopted' by some researchers within sewage epidemiology, including this author, but they are in no way prescriptive or regulatory and aim only to guide individual laboratories in achieving future goals (Cooper, et al., 2010).

While some researchers commented on the lack of specificity for some of the diagnostic ions produced by their method (Gonzalez-Marino, 2010), derivatization with PFPA ensured specificity with regards to diagnostic ions. In addition, GC-MS has been widely applied for confirmation of analytes in complex matrices due to the wealth of valuable information obtainable by using EI ionization and comparison of spectra with databases (Pozo, et al., 2006; Vazquez-Roig, et al., 2013). When using SIM mode, the monitoring of 3 or 4 ions is normally considered sufficient for the confirmation of trace analytes (Pozo, et al., 2006). However, to further aid in confirming the identity of detected drugs as well as to increase the sensitivity of the method, simultaneous SIM and SCAN mode was used for this thesis (Migowska, et al., 2012). The ions monitored for this research have already been mentioned in an earlier chapter (Table 3.3, page 102).

As an illustration, Figures 5.2 and 5.3 depict the SIM and SCAN spectra used to confirm the identity of KET, in the 72 h composite waste water sample. In Figure 5.2, the RTs of the diagnostic ions of the KET peak in the untreated waste water sample, column B (19.36 min), corresponds with the RTs of the diagnostic ions of the mixed drug standard, column A (19.32 min). An ethyl acetate blank, run directly before the waste water sample, acted as quality control to check for any carryover from the drug standards or other contamination. No peaks were present at the corresponding RT indicating that the presence of the KET peak in the unspiked waste water sample was not as a result of contamination.

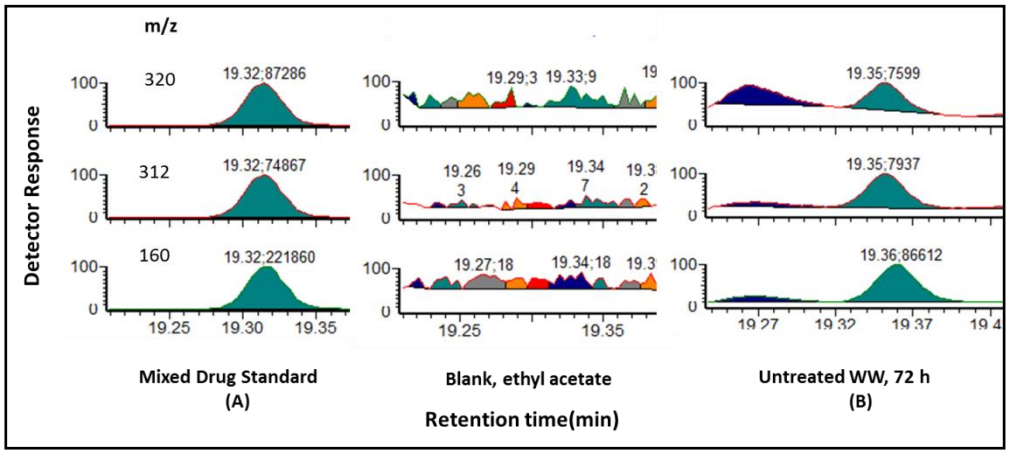


Figure 5.2: Identification and confirmation of ketamine in a 72 h composite waste water sample (SIM).

Table 5.2 lists the corresponding diagnostic ions, peak areas and ion ratio of the mixed drug standard (column A) in reference to that of the unspiked and untreated waste water sample (column B).

Table 5.2: Diagnostic ions, peak areas and ion ratio for ketamine in an untreated waste water sample and mixed drug standard.

	Peak Area			Ion Ratios		
m/z	Drug Std (A)	WW (B)		Drug Std (A)	72 h WW (B)	% Difference 72 h WW/Drug Std
320, C1	87286	7599	C1/C2	1.17	0.96	18
312, C2	74867	7937				

Q = quantifier ion; C = confirmation ion; Std. = standard; WW = waste water

The percentage difference between the ion ratios was 18 %, thereby meeting the criteria for at least one ion ratio being within ± 20 % of the respective standard (EC, 2002; Cooper, et al., 2010). Therefore, the presence of KET could be reliably confirmed in the waste water sample.

To further confirm the presence of KET in the unspiked untreated waste water sample, an extracted ion spectrum of the KET diagnostic ions was conducted (Figure 5.3). The RT (19.32 min) and ion ratios between the unspiked waste water sample and mixed standard corresponded indicating the presence of the same compound.

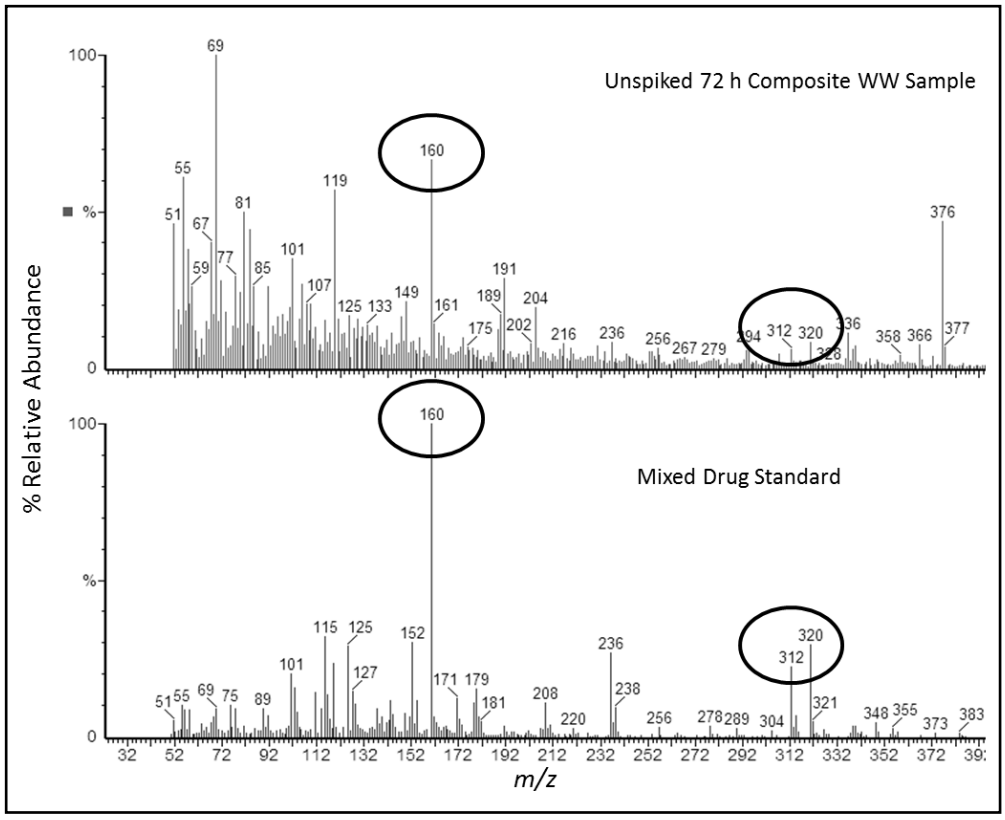


Figure 5.3: Identification and confirmation of ketamine in a 72 h composite waste water sample (SCAN) in relation to a mixed drug standard, showing corresponding diagnostic ions.

Figure 5.4 shows the corresponding TIC spectra of the waste water sample depicting the position of KET in relation to other matrix components. The selectivity of the GC-MS method with PFPA derivatization developed in this research is demonstrated through the positive identification of KET in the presence of much higher concentration levels of numerous unwanted co-extracted matrix components. This was also discussed earlier in section 3.4.3 where KET and MOR were detected in independent untreated waste water samples during preliminary investigations (Figure 3.27 & Appendix IX). Appendix IX is mentioned here again as it further reiterates the detection of MOR in various untreated waste water samples, with confirmation through corresponding RTs and ion ratios in comparison to a drug standard.

Not all quantifiable drugs could be detected in SCAN mode due to much lower concentration levels but they were identified through their SIM spectra and ion ratios (Section 3.4.3, page 130-134; Appendix X).

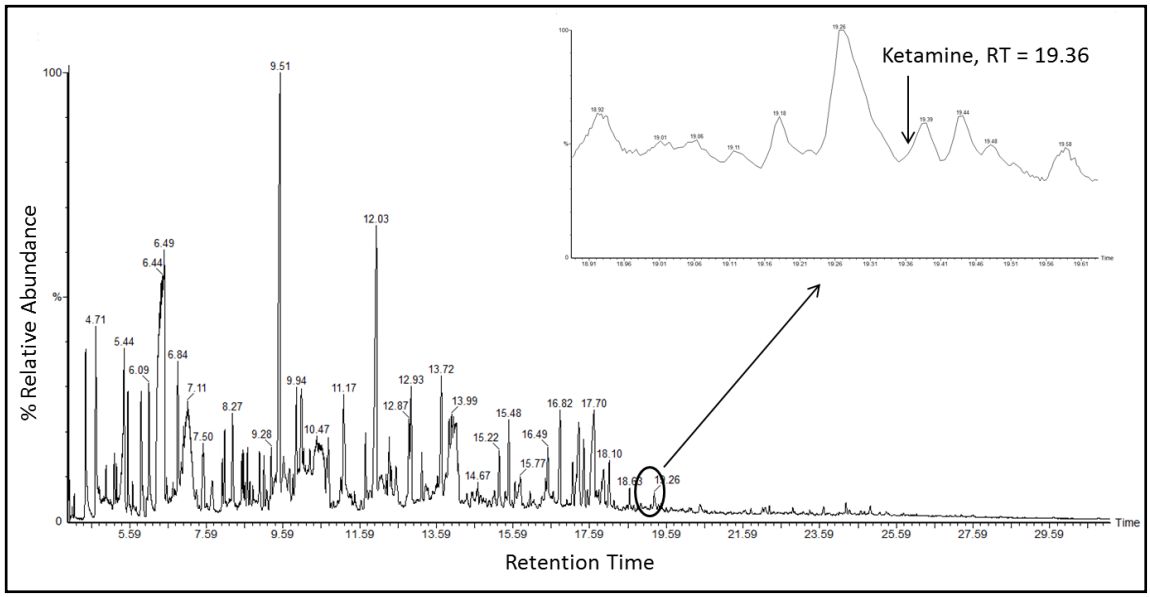


Figure 5.4: TIC of unspiked 72 h composite waste water sample depicting the position of ketamine in relation to other matrix components.

The standard addition method used for quantification also increased the reliability of the method with regards to identification and confirmation of the target analytes. In literature comparisons between standard addition and matrix-matched calibration, higher concentration values were obtained with the former (Furey, et al., 2013). In Furey's (2013) publication, significant errors attributed to ion suppression in the matrix-matched calibration were also observed when compared to standard addition which had fewer errors. With the standard addition method, the same matrix is used for calibration and quantification and hence any matrix effects or interferents will be identical for each injection and will be automatically accounted for. This makes standard addition an effective and reliable method of accurately determining the actual analyte concentration (Gorazda, et al., 2013; Furey, et al., 2013).

Based on the calculated concentration of drugs found in waste water, a back-calculation can be made to determine the estimated usage of these drugs (in milligrams per day) in the community served by the WWTP.

5.3 ESTIMATION OF COMMUNITY USAGE

The information required in order to calculate the estimated community drug usage is discussed in section 1.4.4. The Anglian Water WWTP in Cambridge serves a population of around 145, 310 (2012 figures) comprising domestic waste from the city of

Cambridge and surrounding villages as well as trade effluent. The average daily flow of waste water at the plant ranges from 37, 330 L/day during dry weather (no contribution from storm water run-off) to 109, 987 L/day (with contributions from rainfall and storm run-off). A mid-range of 73, 658 L/day was used in calculations.

5.3.1 Conversion Factors (CF) for Back-calculating Drug Consumption

In order to calculate drug consumption, the urinary excretion rate of major or minor metabolites in humans is required. This allows for the back-calculation to the total drug consumed. Not all drugs of abuse included in this study, especially piperazines and cathinones, have established human urinary excretion profiles, in-sewer stability profiles, as well as commercially available standards of their metabolites (Castiglioni, et al., 2013; Gautam, et al., 2013; Baker, et al., 2014; Chen, et al., 2014; Reid, et al., 2014a). Therefore, back-calculations for drug consumption estimations could not be conducted for them. However, daily loads of the target drug into the WWTP (in mg/day), normalized against the flow at the WWTP and the number of people served by the particular WWTP (Equation 5.1), could be calculated for comparison with published reports, where available, as these do not rely on the urinary excretion profile of the target analyte (Baker, et al., 2014; Khan, et al., 2014). As the composite sample for this research was collected over 72 h, the calculated loads are for a 72 h period.

$$\text{cocaine daily loads} = \frac{\text{EME Concentration (ng/L) x flow rate (L/day)}}{\text{(population served /1000)}}$$

$$= \frac{114100\ \text{ng/L} \times 73658\ \text{(L/day)}}{(145310/1000)} \times \frac{1}{10^6}\left(\frac{\text{mg}}{\text{ng}}\right)$$

$$= 57.8\ \text{mg/day per 1000 people} \qquad \text{Equation 5.1}$$

For drugs with known excretion profiles in humans, back-calculations were normalized against the flow at the WWTP and the number of people served by the particular WWTP and the urinary excretion profile. Normalizing the results in this manner is essential for comparisons of drug consumption estimates obtained from different sources, cities and countries (Castiglioni, et al., 2013). As a result, most recent publications in sewage epidemiology report normalized daily mass loads or

drug consumption as opposed to concentration, especially when comparisons with other studies are being made or when spatial or temporal drug usage trends are being reported (Thomas, et al., 2012; Nefau, et al., 2013; Baker, et al., 2014; Kankaanpaa, et al., 2014; Khan, et al., 2014; Ort, et al., 2014; Ostman, et al., 2014).

Therefore, for the 11 drugs and metabolites detected and quantified in Table 5.1, only those with available human urinary excretion profiles and commercially available standards of the major metabolite, at the time of writing of this thesis, could be used in back-calculations to levels consumed. These were AMP, COC, EME, KET, MAMP and MOR. The urinary excretion rates and CF, as calculated using Equation 1.2, are listed in Table 5.3. A background to the main metabolites for these drugs has been discussed in Appendix II.

Table 5.3: Excretion profile and conversion factors used in back-calculations to estimate community drug consumption.

Drug	Molar Mass of Parent Drug (A)	Molar Mass of Drug Compound Used in Back-Calculations (B)	% Excretion of Drug Dose (C)	CF
AMP	135.2	135.2[pd]	30[1]	3.3
MAMP	149.2	149.2[pd]	43[1]	2.3
COC (using EME)	303.4	199.3	45[2]	3.4
COC	303.4	303.4[pd]	9[2]	11.1
KET	237.7	237.7[pd]	2[2]	50.0
HEROIN (using MOR)	369.4	285.3	42[1]	3.1
MOR	285.3	285.3[pd]	77[3]	1.3

[1] Postigo, et al., 2010; [2]Moffat, et al., 2011b; [3]Baker, et al., 2014; pd = parent drug; CF = conversion factor

Using Table 5.3, the CF is calculated according to Equation 5.2.

$$CF = \frac{A}{B \times (C/100)} \qquad \text{Equation 5.2}$$

An example of how the CF was calculated for COC, using EME as the major excretion product has already been shown in Equation 1.2 (page 27), but repeated here.

$$CF = \frac{\text{Molar mass of cocaine}}{\text{Molar mass of EME x (45/100)}} = \frac{303.4}{199.3 \text{ x } (45/100)} = 3.4 \qquad \text{(Equation 1.2)}$$

The calculations of the CF for COC and heroin could be conducted using their major metabolites (EME and MOR, respectively). The CF for COC was also calculated using the parent drug since it was also detected in waste water. For drugs such as AMP and MAMP, the parent compound is excreted as the major metabolite (30 and 43 %, respectively) and hence was used in the CF calculation. Calculation of CF using the parent drug was conducted for KET because its major metabolites were not monitored for this research. However, using a parent drug as a target analyte and low urinary excretion rates (e.g. 2 % for KET) potentially introduces higher uncertainties for back-calculation estimates since a distinction cannot be made between consumption and direct disposal (Khan and Nicell, 2011; Baker, et al., 2014). Once established, the CF can then be used to back-calculate to the total amount of drug consumed.

5.3.2 Drug Consumption Calculations

Once the CF (of COC) is known, it can be applied in Equation 1.1, resulting in the consumption of COC as depicted in Equation 5.3 (Lai, et al., 2013a):

$$\text{cocaine consumption} = \frac{\text{EME Concentration (ng/L) x flow rate (L/day) x CF}}{1 \text{ x } 10^6}$$

$$= 114100 \text{ ng/L x } 73658 \text{ (L/day) x } 3.4 \text{ x } \frac{1}{10^6}\left(\frac{\text{mg}}{\text{ng}}\right)$$

$$= 28406.8 \text{ mg/day} \qquad \text{Equation 5.3}$$

The COC consumption, in mg/day, is then normalized for the number of people served by the WWTP in Cambridge, i.e. 145,310 people, expressed in mg/day per 1000 people (Equation 5.4).

$$\frac{28406.8 \text{ mg/day}}{(145310/1000)} = 195.5 \text{ mg/day per 1000 people} \qquad \text{Equation 5.4}$$

In like manner, the estimated drug consumption values for the rest of the drugs were calculated. These, in addition to daily loads, are listed in Table 5.4.

From the drug consumption results, KET has the highest consumption figures, followed by total COC and MOR (2463.5, 412 and 399.4 mg/day per 1000 people, respectively). These figures are put in perspective of global and UK drug consumption in section 5.4.

Table 5.4: Estimated 72 h loads and drug consumption levels in Cambridge, UK (mg/day per 1000 people).

DRUG	Conc. ± StdDev (µg/mL)	Estimated 72 h Loads At WWTP (mg/day per 1000 people)	Estimated Consumption in Cambridge , UK (mg/day per 1000 people)
AMP	0.023 ± 0.015	11.7 ± 7.4	38.9 ± 24.8
MAMP	0.072± 0.050	36.2 ± 25.5	84.3 ± 59.1
MCAT	0.253 ± 0.083	128.3 ± 42.1	N/A
MEPH	0.549 ± 0.046	278.0 ± 23.3	N/A
3-TFMPP	0.098 ± 0.004	49.7 ± 2.2	N/A
BUTY	0.004 ± 0.002	1.8 ± 1.2	N/A
4-MEOPP	0.008 ± 0.006	4.3 ± 3.2	N/A
KET	0.097 ± 0.007	49.3 ± 3.7	2463.5 ± 182.5
COC (through EME)	0.114 ± 0.056	57.8 ± 28.2	195.5 ± 95.4
COC	0.038 ± 0.005	19.5 ± 2.7	216.5 ± 29.9
Heroin (through MOR)	0.256 ± 0.058	129.6 ± 29.5	399.4 ± 90.8
MOR	0.256 ± 0.058	129.6 ± 29.5	168.4 ± 38.3

N/A = not applicable due to unavailability of published metabolic excretion rates in humans which is required for drug consumption estimations

5.3.2.1 Assumptions Made in Estimating Drug Consumption Levels

The sewage epidemiological approach is still under development and while many advances have been made since the idea first came to light almost 15 years ago (Daughton, 2001b), several aspects still need further investigation. Therefore, due to the evolving nature of the sewage epidemiological approach, any calculations (daily

mass loads or drug consumption) are based on the current knowledge at the time of sampling.

There are many variables that have an impact on the calculation of drug usage patterns, such as urinary excretion patterns, degradation within the sewer system, attenuation processes, the fluctuating flow rates and population served by a WWTP, and sampling method (Banta-Green, et al., 2009; van Nuijs, et al., 2011b; Baker & Kasprzyk-Hordern, 2011b; Lai, et al., 2011; Castiglioni, et al., 2013). All these variables cannot be determined with great accuracy and give rise to uncertainties with any drug consumption calculations. This has led to a few recent published articles that provide an assessment of the entire sewage epidemiological approach from sampling to the back-calculation, in order to highlight uncertainties/limitations arising from the approach (Lai, et al., 2011; Castiglioni, et al., 2013 & 2014; Baker, et al., 2014).

The key variables and uncertainties/limitations, and recommendations for reducing them have been summarized in Table 5.5 which has been adapted from Castiglioni's (2013 a&b) publication, with contributions from other published articles (EMCDDA, 2008 & 2014b; Ort, et al., 2010a&b; Lai, et al., 2011; van Nuijs, et al., 2011a; Baker, et al., 2014).

Some of the information required under 'recommendations', such as hydrochemical parameters and flow-rate, can be obtained by communication with personnel at the WWTP (van Nuijs, et al., 2011a; Castiglioni, et al., 2013 & 2014).

Table 5.5: Variables, limitations and recommendations for the sewage epidemiological approach.

Variable (uncertainty)	Current Practice	Limitations	Recommendation
Inhabitants Served by the WWTP (7-55 %)[4]	Use of census data or WWTP design capacity.	Fluctuates on a daily, monthly and seasonal basis based on commuters, tourists, special events, etc.[1,2,3]	• Use of different biomarkers, in addition to the target analytes, e.g. PhACs, creatinine, caffeine[4]. • Use of hydrochemical parameters such as BOD, chemical oxygen demand (COD), total phosphorous and total nitrogen[1,5].
Sampling Mode & Flow Rate (5-10 %)[4]	Flow-, volume- or time-proportional composite sampling; grab sampling; passive sampling.	• Flow varies on an hourly, daily, monthly or yearly basis. • An over or under-estimation can have a significant impact on calculated loads[4,6].	• Flow- or volume-proportional sampling with short sampling intervals preferable to time-proportional sampling[4]. • Use as accurate a flow-rate as possible[6].
Sample Analysis (1 - 34 %)[4]	• Mainly LC-MS • Each laboratory has its own analytical protocol (from sample preparation to analysis).	• Matrix effects[4]. • Variable LODs/LOQs and method validation protocols[4].	• Incorporate stable-isotope labelled internal standards and quality control measures.[4] • Participate in interlaboratory studies[1, 4]. • Use guidelines (e.g. EC 2002) for confirmation of positives.

[1]van Nuijs, et al., 2011a; [2]Vuori, et al., 2013; [3]Östman, et al., 2014 ; [4]Castiglioni, et al., 2013b;[5]Chen, et al., 2013;[6]Baker, et al., 2013

Table 5.5 *cont'd*: Variables, limitations and recommendations for the sewage epidemiological approach.

Variable (uncertainty)	Current Practice	Limitations	Recommendation
Analyte stability in waste water & during sampling, storage and analysis (<10 %)[4]	Target analytes are relatively stable.	Underestimation of drug consumption if degradation is not accounted for[6].	• Incorporate stable-isotope labelled internal standards to compensate for any losses during the entire analytical process.[4]. • Conduct stability studies & incorporate degradation into calculations[1,4]. • Avoid using unstable analytes if possible[4]. • Refrigerate samples during composite sampling[4]. • Acidify and/or freeze samples after collection[4].
Pharmacokinetic data e.g. urinary excretion rates (26 % for COC only)[4]	Use of literature-based pharmacokinetic data.	• Highly variable, old data based on few subjects[6]. • Does not take into account individual and ethnic variations[1,7]. • Unavailability of urinary excretion rates for NPS[4].	• Selection of the right metabolite (especially with respect to its stability and 'specificity to the drug')[1]. • Use of proper correction factors based on all known routes of drug administration[4,6]. • Use of homogeneous values for each target analyte across laboratories.
Distinguishing between therapeutic use versus illicit use versus direct disposal of drugs (Not available)	Use of non-specific target analytes for some drugs.	Over estimation of drug consumption if distinction is not done[7,8,9].	• Chiral chromatography where necessary e.g. ATS[1]. • Use of very specific markers where possible e.g. 6-MAM as opposed to MOR for heroin consumption[1].

[1]van Nuijs, et al., 2011a; [2]Vuori, et al., 2014; [3]Östman, et al., 2014 ; [4]Castiglioni, et al., 2013;[5]Chen, et al., 2014;[6]Baker, et al., 2014;[7]Lai, et al., 2013; [8]Bones, et al., 2007;[9]Emke, et al., 2014

During the research discussed in this thesis, the following key assumptions were made which had an impact on the final drug consumption estimations as presented earlier in Table 5.4:

- A static population served by the WWTP i.e. 145, 310 (section 5.3).
- A static flow-rate estimate i.e. average of 73, 658 L/day (section 5.3).
- A constant urinary excretion rate for the target analytes e.g. 9 % for COC (Table 5.3).
- Insignificant degradation of target analytes in waste water (based on Table 4.8).
- The concentration of detected drugs is due to human consumption with no contribution from direct disposal.
- No loss of waste water along the sewage pipe network from source to WWTP.

However, a number of recommendations were incorporated into the method developed during this research in order to reduce some of the uncertainties as presented in Table 5.5. These included the use of stable-isotope labelled internal standards and incorporating quality control measures to reduce uncertainties during sample analysis (section 2.6). In addition, waste water samples were acidified and refrigerated/frozen to minimize analyte degradation. Stability studies were also conducted in order to understand the stability profile of the analytes under different conditions and determine the effect on the final results. As the sewage epidemiological approach is still in its infancy, a lot of the recommendations have been evolving and published when more knowledge was obtained from various research studies in various parts of the world. A number of recommendations were not publicly available during the time of sampling for this research and some were beyond the scope of this research e.g. use of hydrochemical parameters to estimate population and chiral chromatography for differentiating between pharmaceutically active and inactive enantiomers.

Due to the huge potential of the sewage epidemiological approach, research in this area has, therefore, recently turned its focus towards refining the approach in order to minimise these uncertainties and enhance the reliability of the back-calculation method for drug consumption. This includes modification of the formula originally

suggested by Zuccato (2005) to account for the use of therapeutic drugs as well as the stability of the target drugs (Lai, et al., 2011; van Nuijs, et al., 2011 a&b; Baker, et al., 2012 & 2014). Aspects of environmental engineering are also being incorporated into sewage epidemiological studies (Repice, et al., 2013).

Refining the sewage epidemiological approach will also ensure that data can be adequately compared between different sites and countries as well as provide reliable estimates of drug consumption that complement alternative drug monitoring methods. In this regard 'best practice protocol' has been developed by leading researchers in this field based on current learnings with respect to sample collection, storage and analytical procedures (Castiglioni, et al, 2013 a&b; EMCDDA, 2013). The majority of recommendations had been adopted during the research discussed in this thesis. However, the recommendations themselves are dependent on current knowledge and should not be prescriptive as they will undoubtedly also evolve as the sewage epidemiological approach continues to be refined. For instance, while some researchers report the adsorption of drugs onto suspended solids as a possible source of uncertainty (Baker & Kasprzyk-Hordern, 2011b; Verlicchi, et al., 2012; Baker, et al., 2014), it is now widely believed that this factor has a negligible impact on the uncertainty since less than 9 % of target analytes are adsorbed onto suspended solids (Gheorghe, et al., 2008; Gonzalez-Marino, et al., 2009; Metcalfe, et al., 2010; Castiglioni, et al., 2013b; Repice, et al., 2013).

In addition, the recommendations also have their own limitations which need to be taken into consideration when adopting them. For instance, while LC-MS has become the preferred analytical method for the sewage epidemiological approach (Thomas, et al., 2012; Repice, et al., 2013), it is still plagued by limitations such as matrix effects. On the other hand, GC-MS-EI, with PFPA derivatization, produces very distinct mass spectra for the majority of drugs, is not as affected by matrix effects as LC-MS is and is capable of LODs lower than or as good as LC-MS/MS (Heath, et al., 2010; Vazquez-Roig, et al., 2013). Therefore, GC-MS is equally viable for sewage epidemiological studies and its advantages have been listed in Table 1.10.

The main research gaps with the sewage epidemiological approach, as identified by Castiglioni (2014), include the improvement of sample analysis methodology through standardised protocols, the improvement of stability data to further understand contributions due to degradation, conducting additional pharmacokinetic studies on target drugs to provide more accurate human urinary excretion profiles, and developing a way of more accurately estimating the number of people contributing to the waste water sample.

However, despite these uncertainties and research gaps, sewage epidemiological results often correlate well with those from other drug monitoring sources and, therefore, any calculated drug consumption results are a generally good estimation of actual values (Banta-Green, et al., 2009; Thomas, et al., 2012; Lai, et al., 2013c; Khan, et al., 2014).

In this research, since the composite sample used for standard addition was collected over 72 h, there was probably loss of some analytes through degradation or ex-filtration leading to them not being detected or detected below their LOD and LOQ (Lai, et al., 2011; Burgard, et al., 2013). In addition, time-proportional sampling, as has been used by many other research groups, was used (Thomas, et al., 2012; Lai, et al., 2013b; Khan, et al., 2014; Vuori, et al., 2014). However, composite sampling has more advantages that negate the possible loss due to degradation, such as obtaining time-weighted drug concentrations as well as average concentration values based on influent flow rate (Repice, et al., 2013; Boles & Wells, 2014). Corrections for stability were beyond the scope of this research and hence were not performed as part of the main results. In addition, as samples were collected for a specific period only, the results cannot be used as a general pattern of drug consumption in Cambridge or the rest of the UK.

5.4 LINKING DETECTED DRUGS WITH GLOBAL AND UK CONSUMPTION

This section puts into perspective the results from the drug consumption calculations obtained from this research with information obtained from social surveys and other published articles that reflect the types and levels of drugs consumed within the UK and on a more global scale. As far as the researcher is aware, only three other

research groups have included cities in the UK in sewage epidemiological studies (Bones, et al., 2007; Zuccato, et al. 2008 and the group comprising Baker & Kasprzyk-Hordern (2007 - 2013). Comparative UK studies for the past three years can only be made with Baker & Kasprzyk-Hordern's publications. In addition, samples in this thesis were collected over a 72 h period as compared to the 24 h composite samples collected by other researchers, with which comparisons have been made.This allowed more time for any target analyte to be collected, if present, as compared with a 12 or 24 h composite sample, assuming negligible attenuation processes and sample degradation (section 1.3.4.2).

5.4.1 Classic Drugs of Abuse

Classic drugs of abuse such as cannabis, cocaine, amphetamines, ecstasy, and opioids still remain the most abused drugs in Europe (EMCDDA, 2014a). Heroin use has seen a significant downward trend and ecstasy and methamphetamine are showing trends of resurgence after a downward spiral in the past few years (*ibid*). In England and Wales, cannabis, cocaine, ecstasy and amyl nitrate remain the most abused classic drugs (CSEW, 2014) while in the UK overall, crack cocaine and the opioids (heroin and methadone) top the list together with cannabis and NPS [Centre for Social Justice (CSJ), 2013].

It is, therefore, not surprising that AMP, MAMP, EME, COC and MOR were detected and quantified in waste water samples from Cambridge, UK (Tables 5.1 and 5.4). These are discussed in more detail below. Comparisons between data obtained from Cambridge, UK (i.e. this research) with that from published articles have been made based on daily loads (Equation 5.1) and/or consumption data (Equations 5.3 and 5.4).

5.4.1.1 Amphetamine

Sources of AMP in waste water include illicit use and prescription drugs such as Dexedrine and dexamphetamine, used for the treatment of attention deficit hyperactivity disorder (ADHD) (Lai, et al., 2013a). It is also formed as a metabolite of selegiline (for Parkinson's disease) and MAMP (van Nuijs, et al., 2011a; Moffat, et al., 20llb). Therefore, the total estimated daily loads of AMP into the WWTP in

Cambridge, UK, and consumption of AMP can be attributed to both illicit and therapeutic use.

Table 5.6 compares daily loads and consumption levels for AMP as determined in this research with those from published literature. Maximum concentrations are quoted except where indicated.

Table 5.6: Amphetamine daily loads and consumption data from different countries.

AMPHETAMINE				
Daily Load mg/day/ 1000 people ± StdDev	Consumption in mg/day/ 1000 people ± StdDev	Sampling Method	Population per WWTP (thousands, except where indicated)	Reference (Country)
11.7 ± 7.4	**38.9 ± 24.8**	**Raw, 72 h composite**	**145, 310**	**This research (England)**
-	102 ± 3.8	Raw, 24 h composite	3.4 million	Baker, et al., 2014 (England)
41.8[1,2]	-	Raw, 24 h composite	1.5 million	Khan, et al., 2014 (China, 4 cities)
-	400[2] (150 - 400)[3]	Raw, 24 h composite	100, 000	Kankaanpää, et al., 2014 (Finland, 10 cities)
-	93	Raw, 24 h composite	466, 667	France (Nefau, et al., 2013)
140[2] (10 - 140)[3]	610[2] (47 - 610)	Raw, 24 h composite	658, 114	Ostman, et al., 2014 (Sweden, 33 cities)
3040[2] (33 - 3040)[3]	-	Raw, 24 h composite	448, 700	Thomas, et al., 2012 (multicountry, 19 cities)

[1]Average; [2]value from one city; [3]range

At daily loads of 11.7 ± 7.4 mg/day/1000 people, data from Cambridge is similar to that from some cities in Sweden, such as Koping and Trelleborg (10 - 18 mg/day/1000 people), but much lower than that from other cities in Sweden, such as Soderhamn and Gothenburg (100 - 140 mg/day/1000 people) and Eindhoven in The Netherlands (3040 mg/day/1000 people). In like manner, at 38.9 ± 24.8 mg/day/1000 people, consumption data from Cambridge is similar to that from Mora in Sweden (47 mg/day/1000 people) but lower than that from other parts of England (102 ± 3.8 mg/day/1000 people), Finland (150 - 400 mg/day/1000 people) and France (93

mg/day/1000 people). Overall, AMP consumption levels range from 47 - 610 mg/day/1000 people but this range could be higher if the daily load of 3040 mg/day/1000 people (The Netherlands) is normalized to consumption. The daily loads and normalized consumption data from several studies conducted in different countries indicate varied AMP consumption as a result of different drug use preferences and habits, waste water composition and sources, population sizes served by the WWTPs, sampling methods, etc. These factors are expounded upon in section 5.5.1 below.

5.4.1.2 Methamphetamine

Table 5.7 compares consumption levels for MAMP as determined in this research with those from published literature.

Table 5.7: Methamphetamine daily loads and consumption data from different countries.

METHAMPHETAMINE				
Daily Load mg/day/ 1000 people ± StdDev	Consumption in mg/day/ 1000 people ± StdDev	Sampling Method	Population per WWTP (thousands, except where indicated)	Reference (Country)
36.2 ± 25.5	**84.3 ± 59.1**	**Raw, 72 h composite**	**145, 310**	**This research (England)**
-	29.8 ± 0.9	Raw, 24 h composite	3.4 million	Baker, et al., 2014 (England)
121.7[1,2]	-	Raw, 24 h composite	1.5 million	Khan, et al., 2014 (China, 4 cities)
-	190 ± 11	Raw, 24 h composite	3.5 million	Lai, et al, 2013a (Hong Kong)
32[2] (1 - 32)[3]	71[2] (2.3 - 71)[3]	Raw, 24 h composite	21, 700	Ostman, et al., 2014 (Sweden, 33 cities)
117.3	-	Raw, 72 h composite	557, 000	Reid, et al., 2014b (Norway)
376[2] (3 - 376)[3]	-	Raw, 24 h composite	780, 000	Thomas, et al., 2012 (multicountry, 19 cities)

[1]Average; [2]value from one city; [3]range

MAMP, mainly used illicitly, also has limited therapeutic use in the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy and is also a metabolite of selegiline (Lai, et al., 2011). Therefore, the total calculated consumption for MAMP (84.3 ± 59.1 mg/day per 1000 people) also does not differentiate between illicit or therapeutic use.

At 84.3 ± 59.1 mg/day/1000 people, the MAMP consumption result from Cambridge is lower than that from Hong Kong (190 ± 11 mg/day/1000 people) but higher than that from other parts of England (29.8 ± 0.9 mg/day/1000 people) and Sweden (2.3 - 71 mg/day/1000 people). Overall, MAMP consumption levels range from 2.3 - 190 mg/day/1000 people across Europe. However, the daily load for Cambridge (36.2 ± 25.5 mg/day/1000 people) was lower than that from China (121.7 mg/day/1000 people) and Norway (117.3 mg/day/1000 people).

For both AMP and MAMP, direct disposal into the sewage system can also not be ruled out since the parent drugs were used in the back-calculations (Boles & Wells, 2014). In addition, both AMP and MAMP are known to exist as a pair of enantiomers which differ in pharmacological activity and metabolism. The S-(+)-enantiomer is known to be more potent and is present in recreational formulations, while the R-(-)-enantiomer is less potent and often present in pharmaceutical products (Kasprzyk-Hordern, et al., 2009a; Bagnall, et al., 2013; Emke, et al., 2014). Therefore, the only way to truly distinguish between recreational and pharmaceutical AMP and MAMP is by separation and analysis of their enantiomers through derivatization or chiral chromatography, which was beyond the scope of this research (Herraez-Hernandez, et al., 2002; Emke, et al., 2014). However, other research groups have been investigating enantiomeric profiling of drugs in waste water to enable better estimations of illicit use (Kasprzyk-Hordern & Baker, 2012; Emke, et al., 2014).

AMP has been more widely abused than MAMP in Europe over the past few years but MAMP has seen a recent increase in usage (EMCDDA, 2014a). Results from this research, where MAMP was detected at a higher concentration than AMP, therefore, correlates with findings from the EMCDDA (2014a) report.

5.4.1.3 Other Phenylethylamines

Other phenylethylamines analysed in this research but not detected include MBDB, 4-FMA and MDMA. MBDB was also not detected elsewhere in England probably due to low consumption but MDMA has been detected at consumption levels of 148 mg/day/1000 people (Baker & Kasprzyk-Hordern, 2013; Baker, et al., 2014). It is surprising that MDMA was not detected especially since it is a popular 'club drug' among students and 'yuppies' which make up a substantial portion of the demographics of Cambridge, UK, and also as one of the top drugs consumed in England & Wales (CSEW, 2014). In addition, it had a low analytical LOD and LOQ (0.33 and 1.33 pg on column, respectively), was stable and had relatively good recovery of 70 %. This could be due to different drug usage habits within England with a higher preference for KET and MEPH in Cambridge, UK, or that its presence in the particular waste water sample was below the analytical LOD as also reported for Norway (Reid, et al., 2014a) and Hong Kong (Lai, et al., 2013b). As far as the author is aware, this is the first reported method to incorporate 4-FMA in sewage epidemiological studies and hence no comparative figures were available at the time of writing of this thesis. However, it could also have been present below the analytical LOD or not substantially consumed in Cambridge, UK.

5.4.1.4 Cocaine

A comparison of COC consumption estimations from this research with those from published literature are listed in Table 5.8.

The result for Cambridge was much lower than elsewhere in England (Baker, et al., 2014), Portugal (Lopes, et al., 2014) and Belgium (van Nuijs, et al., 2011b) but higher than Norway (Redi, et al., 2014) and on par with Sweden and Norway (Thomas, et al., 2012). The consumption of COC varied widely between countries and within cities/regions within the same country. However, comparisons are made based on different target analytes used (i.e. BZE, COC and EME) which affects results as described in section 5.4.1.4.1 below. In this research, COC consumption estimates were calculated using EME and COC as target drugs and both results were lower than publications that used similar target analytes (van Nuijs, et al., 2011b & Baker, et al, 2014, respectively). Although occurrence and consumption habits differ widely

between countries, COC is second only to cannabis as the most commonly used illicit drug in Europe (EMCDDA, 2014a; Lopes, et al., 2014).

Table 5.8: Cocaine consumption data from different countries.

COCAINE			
Consumption in mg/day/ 1000 people ± StdDev	Sampling Method	Population per WWTP (thousands, except where indicated)	Reference (Country)
195.7 ± 95.4 (EME)	**Raw, 72 h**	**145, 310**	**This research**
216.5 ± 29.9 (COC)	**composite**		**(England)**
12876 ± 47 (COC)	Raw, 24 h	3.4 million	Baker, et al., 2014
1767 ± 45 (BZE)	composite		(England)
1324.6 (BZE)	Raw, 24 h	134, 780	Lopes, et al., 2014
(464 – 1324)[2]	composite		(Portugal)
2434 (BZE)	Raw, 24 h composite	188, 333	Nefau, et al., 2013 (France)
146 (BZE)	Raw, 72 h composite	557, 000	Reid, et al., 2014b (Norway)
1998 (BZE)[1]	Raw, 24 h	117, 200	Thomas, et al., 2012
(2 - 1998)[2]	composite		(multicountry, 19 cities)
523 (EME)	Raw, 24 h	1.1 million	van Nuijs, et al., 2011b
519 (BZE)	composite		(Belgium)

[1]value from one city; [2]range

Results from literature reviews for sewage epidemiology found some of the highest consumption values for illicit drugs across countries were related to COC consumption (Thomas, et al., 2012; Ort, et al., 2014).

5.4.1.4.1 Target Analytes for Estimating Cocaine Consumption

In published literature, the consumption of COC has been estimated in several ways through the use of COC, or one or more of its metabolites, benzoylecgonine (BZE), nor-BZE and EME (Nefau, et al., 2013; Baker, et al., 2014; Ort, et al., 2014). The majority of published reports used BZE rather than EME or COC itself, citing the instability of both EME and COC and the high LOQ of EME as a basis for the selection (van Nuijs, et al., 2009c; Baker, et al., 2014). The reasoning is that since EME and COC

exhibited degradation in stability studies based on sewage conditions, it was most likely to be unstable in the sewage system and hence the amount detected would be an underestimation of the original concentration in waste water samples (van Nuijs, et al., 2012; Thai, et al., 2014). On the other hand, BZE has been found to be stable in waste water stability studies (Baker, et al., 2014). Lai (2011) also targeted EME and BZE but only detected BZE due to instability of EME. This correlates with findings in this thesis where EME was observed to be unstable during matrix-based stability studies conducted at pH 2, although COC was stable (Table 4.8).

It can, therefore, be postulated that due to the observed degradation of EME in waste water, the estimated COC consumption (using EME) reported in this research (195.5 ± 95.4 mg/day per 1000 people) is most likely an underestimation and the result is much higher than that.

The levels for COC consumption can also be calculated using the parent drug (Bones, et al., 2007; Karaolak, et al., 2010). This resulted in an estimated consumption of 216.5 ± 29.9 mg/day per 1000 people in this research. However, this is most likely an overestimation of COC consumption since it does not differentiate between direct disposal of the parent drug into the sewer system and illicit use (van Nuijs, et al., 2011a; Nefau, et al., 2013). In Baker's (2013) study, COC consumption estimations using the parent drug were significantly higher than those when using BZE (12876 ± 47 and 1767 ± 45 mg/day per 1000 people, respectively). However, in this thesis, results for COC estimation using the parent drug and EME were similar (i.e. 216.5 ± 29.9 and 195.5 ± 95.4 mg/day per 1000 people, respectively). van Nuijs (2011b) calculated COC consumption using EME and BZE and found comparable values of 523 and 519 mg/day per 1000 people, respectively.

In this regard, EME can also be effectively used in back-calculating for COC consumption since it is also a significant metabolite of COC (Gheorghe, et al., 2008; Nefau, et al., 2013; Baker, et al., 2014; Khan, et al., 2014). Improving sample preservation practices from the sampling to analysis stage can help in the measurement of significant amounts of EME in waste water in the future. For instance, if the waste water samples are acidified and extracted immediately upon

arrival at the laboratory and analysed within 24 h of extraction, EME can still be detected above the LOQ, if present, as was the case in this thesis. If the sample is to be extracted at a later time then storage at -20 °C is imperative for EME since it degrades when stored at 5 °C (Table 4.8). In addition to having a low LOQ (Table 4.5), EME was moderately stable when derivatized and stored at -20 °C for up to 2 weeks (Table 3.5) and also when stored underivatized at -20 °C for up to 4 weeks (Table 3.6). However, even though EME is a suitable target analyte for COC consumption, the reliability of the result can be improved if its degradation is incorporated in drug consumption calculations and if BZE and nor-BZE can be simultaneously detected for comparison (Baker, et al., 2013; Khan, et al., 2014). It is worth bearing in mind that using BZE for COC estimations also has its own drawbacks. COC is known to hydrolyse to BZE in waste water and hence the amount of BZE quantified maybe an overestimation of the initial concentration which would also give rise to uncertainties with the drug consumption estimations for COC (van Nuijs, et al., 2012; Castiglioni, et al., 2013; Thai, et al., 2014).

5.4.1.5 Opiates

MOR is a major metabolite of heroin but it is also a metabolite of the analgesic codeine and several other therapeutic drugs such as nicomorphine and pholcodine (Levine, 2006; Baker, et al., 2014). It is also a drug in its own right. Table 5.9 compares consumption levels for MOR as determined in this research with those from published literature. In this thesis, as in published literature as well, MOR was used to estimate both heroin and MOR consumption, with results differing based on the conversion factor (CF) used (Table 5.3).

From results presented in Table 5.9, the MOR daily load for Cambridge (129.6 ± 29.5 mg/day/1000 people) was within the range for cities in Sweden (50 - 350 mg/day/1000 people) but higher than that for Finland (50.7 mg/day/1000 people).

Although MOR was not detected in raw waste water samples from a WWTP serving 3.5 million people in an unspecified location in England (Baker, et al, 2014), it was detected and quantified in raw waste water samples from a WWTP in Cambridge, UK, serving a population of 141,310 people. However, this difference is not surprising

considering the temporal and spatial differences in drug consumption values within the same day, country and between countries as documented in several studies (section 5.5.1).

Table 5.9: Heroin and morphine daily loads and consumption data from different countries.

HEROIN/MORPHINE				
Daily Load mg/day/1000 people ± StdDev	Consumption in mg/day/1000 people ± StdDev	Sampling Method	Population per WWTP (thousands, except where indicated)	Reference (Country)
129.6 ± 29.5	**399.4 ± 90.8 (Heroin) 168.4 ± 38.3 (MOR)**	**Raw, 72 h composite**	**145, 310**	**This research (England)**
350 (MOR)[1]	-	Raw, 24 h composite	30, 500	Ostman, et al., 2014 (Sweden, 33 cities)
(50 - 350)[2]				
50.7 (MOR)	-	Raw, 24 h composite	24, 781	Vuori, et al., 2014 (Finland)
-	173 ± 29 (Heroin)	Raw, 24 h composite	5.5 million	Zuccato, et al., 2008 (England)

[1]value from one city; [2]range

In comparing heroin consumption using MOR as the main metabolite, results from Cambridge, UK (399.4 ± 90.8 mg/day/1000 people) are more than twice as high as those from London, UK (173 ± 29 mg/day/1000 people) (Table 5.9). In England, it has been reported that approximately 0.01 % of morphine in wastewater is due to illicit use while the rest can be attributed to the therapeutic use of primarily codeine (Baker, et al., 2014). Therefore, using MOR to calculate the consumption of heroin would lead to an overestimation (*ibid*). As a result, the heroin consumption value of 399.4 ± 90.8 mg/day per 1000 people using MOR as a metabolite, as in this thesis, is most likely an overestimation. When only the consumption of MOR was taken into account, using the parent drug as the main metabolite (Table 5.3), the estimated value for Cambridge, UK, was 168.4 ± 38.3 mg/day/1000 people. However, as far as the author is aware, there was no comparative value found as few researchers investigated opioids in waste water.

During preliminary investigations, a reversible reaction between MOR-2PFP and MOR-PFP was proposed in Figure 3.14, which most probably had an impact on the total MOR concentration since only the diagnostic ions of MOR-2PFP were used for quantification. Therefore, the estimated MOR consumption value is most likely an underestimation of the actual amount present in the waste water sample which in turn affects the calculation of drug consumption using MOR. However, as observed in Figure 3.13, a higher level of MOR-2PFP was present and hence any impact of deacetylation to MOR-PFP on the concentration detected is expected to be minimal. Nontheless, in hindsight, perhaps both MOR-PFP and MOR-2PFP should have been quantified and their results pooled together to give total MOR concentration.

A more suitable target analyte to use for estimating the consumption of heroin would be 6-MAM, since it is an exclusive indicator of heroin use (Karacic & Skender, 2000; Fugelstad, et al., 2003; Khan, et al., 2014). However, heroin was not detected in this research and 6-MAM was detected below its LOQ and therefore could not be used in calculations due to the uncertainties this would introduce into the final result. As heroin quickly hydrolyses to 6-MAM and then MOR, both heroin and 6-MAM are unlikely to be found in any significant amounts in waste water (Khan & Nicell, 2011; Jones, et al., 2013; Yargeau, et al., 2014). In another study in England, while heroin was also not detected in waste water, 6-MAM was detected (Baker, et al., 2014) while both heroin and 6-MAM were detected in France (Nefau, et al., 2013). Although there are lower uncertainties when using 6-MAM, as opposed to MOR, to estimate heroin consumption, these increase significantly with longer in-sewer HRTs due to its instability and high back-calculation correction factors as a result of its low excretion rate of 1.3 % (van Huijs, et al., 2011a; Baker, et al., 2014; Senta, et al., 2014). Since 6-MAM is a minor metabolite of heroin with a low excretion rate, low LODs would be needed for reliable quantification. The degradation reactions, lack of stability in waste water, high LOQs and lack of recovery for 6-MAM has been documented throughout this thesis. Therefore, although its recovery from the sample matrix and detection can be improved with further work, estimating heroin consumption using 6-MAM is still fraught with high uncertainties which still need to be addressed before the results can be reliable (Baker, et al., 2014).

5.4.2 New Psychoactive Substances (NPS)

A background to the NPS, cathinones, piperazine and KET, has been presented in section 1.4.2.1 and Appendix II. The most commonly misused drugs in these groups include MEPH, 3-CPP, BZP, 3-TFMPP and KET (Staack, 2007; Dickson, et al., 2010a; UNODC, 2013). Within Europe, the UK leads in terms of usage reporting of NPS (UNODC, 2013) and MEPH and KET are among the most highly abused drugs in England and Wales (CSEW, 2014). These reports correlate with findings in this research where MEPH, 3-TFMPP and KET were detected and quantified at concentrations of 0.549 ± 0.046, 0.098 ± 0.004 and 0.097 ± 0.007 μg/mL, respectively.

The human urinary excretion profile for NPS is largely unknown and hence pharmacokinetic data is also limited or non-existent (Castiglioni, et al., 2013; Reid, et al., 2014a). The ever changing chemical structure and occurrence of NPS makes it even more difficult to predict which NPS is most prevalently consumed at any given time. Hence, the choice of NPS targeted in various published studies, are based on reports from conventional drug monitoring processes (Table 1.5). In addition, the unavailability of standard analytical references for the majority of these compounds and their metabolites hinders accurate detection, identification, quantification and back-calculation to drug consumption (Favretto, et al., 2013; Helander, et al., 2014). In order to accurately determine the community drug consumption profile for NPS, relatively stable analytical standards of known excretion products are required, which are also specific to the drug and excreted at high enough concentrations, i.e. used by a sufficient number of people in the community, to be detected (Castiglioni, et al., 2013).

However, derivatization with PFPA and analysis with GC-MS, as conducted in this research, has shown to be a suitable method for the accurate detection, identification and quantification of the targeted NPS, when present in high enough quantities (above the LOQ of the method). In addition, some NPS, such as MDPV, have been detected in waste water from different parts of the world, with some results corroborating with those from other drug monitoring sources (Table 1.5). Therefore, the detection and quantification of NPS is currently possible with the sewage epidemiological approach and will no doubt continue to be refined as the approach

continues to be developed, but further work is still required to enable back-calculation to community drug consumption. An alternative matrix for the detection of NPS for the sewage epidemiological approach has been pissoirs as these may contain higher levels of NPS or reflect a different drug use pattern to that of the general population as determined by samples from a WWTP (Reid, et al., 2014a).

Although there has been a steady increase in sewage epidemiological studies targeting NPS in the analytical method, these are only few in comparison with studies targeting classic drugs of abuse (Castiglioni, et al., 2014). In addition, not all studies have been able to detect these NPS in waste water samples.

5.4.2.1 Cathinones

Cathinones detected in this research were MCAT, MEPH and BUTY at concentrations of 0.253 ± 0.083, 0.549 ± 0.046 and 0.004 ± 0.002 µg/mL, respectively. The higher concentration of MEPH suggests a wider usage of this drug in Cambridge, UK, compared to the other cathinones. However, the back-calculation to drug consumption could not be made due to the lack of complete data on the urinary excretion rate in humans (Meyer, et al., 2010; Corkery, et al., 2012; Khan, et al., 2014). Instead, daily loads were used for comparison according to Equation 5.1 (Khan, et al., 2014). Only a handful of publications have included MEPH in sewage epidemiological studies (Table 1.5) and even fewer report detection of MEPH in waste water (Lai, et al., 2013b; Chen, et al., 2014). Maximum loads reported are 3 mg/day/1000 people (Chen, et al., 2014) which is much less than the 278.0 ± 23.3 mg/day/1000 people reported in this thesis (Table 5.4). As mentioned earlier, MEPH is among one of the most highly abused drugs in England and Wales, with usage not seeming to abate and therefore high daily loads in waste water are not surprising (Khreit, et al., 2013; CSEW, 2014). In addition, there have been reported increased usage of MEPH in Australia, and the USA and as more researchers include this NPS in their methods, higher loads may also be detected especially in regions known for high consumption of this drug (Winder, et al., 2013; Chen, et al., 2014). As far as the author is aware, this is the first time BUTY has been quantified and reported in sewage epidemiology and hence comparative values were not available in literature. However, BUTY has been

detected in blood and urine samples during toxicological analyses (Helander, et al., 2014).

Analysis of waste water in Norway, Sweden and Belgium has failed to detect MEPH and CAT (van Nuijs, et al., 2013; Ostman, et al., 2014; Reid, et al., 2014b), while MCAT has not been detected during other studies in England (Baker, et al., 2014). However, MCAT was detected in Australia at maximum daily load of 3.5 mg/day/1000 people (Chen, et al., 2014) in comparison to the daily load of 128.3 ± 42.1 mg/day/1000 people as reported in this thesis (Table 5.4). Therefore use of MCAT so far appears to be higher in Cambridge, UK but there are not many other published reports with which to compare results with.

Other cathinones incorporated in the analytical method but not detected were CAT and 3-FMC. As far as the author is aware, only one other published study included CAT as a target analyte but also did not detect it (Reid, et al., 2014b). With regard to 3-FMC, its in-sewer residence time is short-lived due to its instability in waste water (Table 4.8) and hence it is unlikely to be detected. In order to be detected, it would have to be consumed at levels similar to classic drugs of abuse, and none of the official reports (CSEW, 2014; EMCDDA, 2014a; UNODC, 2014) seem to indicate 3-FMC as an emerging drug that is increasing in usage.

These results reflect the different drug usage habits of people in different countries and within the same country. However, it could also reflect the different analytical methods used, since the other referenced publications used LC-MS/MS and matrix-matched calibration which introduces its own uncertainties, while GC-MS with derivatization and the method of standard addition was used in this research. Therefore, the sewage methodological approach and analytical method as detailed in this research is suitable for the detection and quantification of MCAT, MEPH and BUTY.

5.4.2.2 Piperazines

It has been reported that piperazines are extensively metabolised, therefore one would expect to detect less of the parent drug in waste water (Staack, et al., 2003;

Staack & Maurer, 2003; Staack, et al., 2004). However, 4-MEOPP and 3-TFMPP were all detected in waste water samples from Cambridge, UK. 4-MEOPP was detected at 0.008 ± 0.006 µg/mL while 3-TFMPP was detected at 0.098 ± 0.004 µg/mL.

As far as the author is aware, only 2 other research groups have targeted piperazines using the sewage epidemiological approach. In studies conducted in England, BZP and 3-TFMPP were at times detected (Baker & Kasprzyk-Hordern, 2011a & 2013) and other times not detected at all (Baker, et al., 2014). BZP was also detected in studies conducted in Australia (Lai, et al., 2013c). Back-calculations, however, could not be done due to the lack of urinary excretion data and hence normalized comparisons could not be made. Since BZP is also a metabolite of the antidepressant piberaline, its detection in waste water can be attributed to both therapeutic and illicit use (Staack, et al., 2002; Moffat, et al., 20llb). As far as the author is aware, this is the first reported sewage epidemiological study targeting 11 piperazines and the first time the detection and quantification of 4-MEOPP has been reported in sewage epidemiology. In this regard, comparative values were not available in literature. However, the structural isomer, 2-MEOPP, was detected in Norway but not quantified (Reid, et al., 2014a) showing the importance of incorporating different isomers during method development (section 3.2.5.1). Although BZP and MBZP were targeted in this thesis, they were not detected possibly due to low usage in Cambridge, UK, or that their levels were below the LOD of the method.

5.4.2.3 Ketamine

It is worth noting that although a consumption rate of 2463.5 ± 182.5 mg/day per 1000 people, was estimated for KET (Table 5.4), this is not an indication that the total amount of KET consumed was as a result of misuse. KET is widely abused for its hallucinogenic effects at nightclubs and raves and has also been implicated in drug-facilitated sexual assaults (Lin, et al., 2010; Lian, et al., 2012; Negrusz & Gaensslen, 2013). However, KET is also used as a general anaesthetic and short-acting analgesic in human and veterinary practice (Moffat, et al., 2011b; Lian, et al., 2012; Lin, et al., 2014). Since only the parent drug was quantified, a distinction between direct disposal into the sewage system as opposed to consumption is also unknown. In addition, as ketamine metabolites were not monitored in this research, the urinary

excretion profile for the parent drug as reported in Moffat (2011b) was used in the back-calculations. However, the increased bias in using low values, i.e. 2 % in this case, has been mentioned in section 5.3.1 and therefore daily loads of KET were also included for comparison. Table 5.10 list the daily loads and consumption figures for KET from this research and other publications.

Table 5.10: Ketamine daily loads and consumption data from different countries.

KETAMINE				
Daily Load mg/day/1000 people ± StdDev	Consumption in mg/day/1000 people ± StdDev	Sampling Method	Population per WWTP (thousands, except where indicated)	Reference (Country)
49.3 ± 3.7 (KET)	**2463.5 ± 182.5**	**Raw, 72 h composite**	**145,310**	**This research (Cambridge)**
327 ± 1.4[a]	-	Raw, 24 h composite	3.4 million	Baker, et al., 2014 (England)
47.72[a]	-	Raw, grab	897,962	Baker & Kasprzyk-Hordern, 2013 (England)
229.7[1] (KET)	-	Raw, 24 h composite	1.5 million	Khan, et al., 2014 (China, 4 cities)
290 ± 27 (norKET)	1500 ± 240	composite	3.5 million	Lai, et al, 2013a (Hong Kong)

[a]calculated without normalization to population (g/day); [1]value from one city

Daily KET loads in Cambridge, UK, are lower than those from other countries. However, consumption figures are higher in Cambridge than other countries due to the use of different target analytes for back-calculation. Of the handful of studies that have incorporated KET as a target analyte, not all resulted in its detection. KET and nor-KET were analysed but not detected elsewhere in England and Sweden (Baker, et al., 2014; Ostman, et al., 2014). This further reflects the different spatial patterns in drug use within and between countries.

Although detected at a concentration of 0.097 ± 0.007 µg/mL in Cambridge, UK, KET was detected at concentrations of up to 0.01 µg/mL in hospital effluents from Taiwan (Lin, et al., 2014) reflecting the effect of different usage habits and sources. As a note, even though some compounds occur at low excretion rates, this does not necessarily

indicate that they are present at low levels in waste water influent. High levels of use of these compounds can compensate for the low excretion rates and hence they can still be detected in large quantities, as in the case of KET. The high usage levels of KET found in this study correlates with reported high levels of usage in England and Wales (Baker, et al., 2014; CSEW, 2014; EMCDDA, 2014a; UNODC, 2014) as well as in China, Hong Kong and Taiwan (Lai, et al., 2013b; Lin, et al., 2010 & 2014; Khan, et al., 2014). In fact, it has been reported that in Hong Kong, KET consumption was even higher than MAMP and COC (Lai, et al., 2013b). The CSEW (2014) reports a statistically significant increase in the illicit use of KET in 2013/2014 (0.6 %) than 2012/2013 (0.4%) for adults between 16 and 59 years of age.

Aside from its increased illicit use, the contribution of KET in wastewater from leading research hospitals, drug treatment centres, and other health facilities in Cambridge and surrounding villages cannot be ruled out.

5.4.3 Amitriptyline

AMIT is used to treat depression but has traditionally been abused for its relaxant properties. Although AMIT was not reliably detected in this thesis, its consumption was estimated to be quite high at 2455 ± 197 mg/day/1000 people in a separate published study in England (Baker, et al., 2014). Nortriptyline, one of the major metabolites was detected at a much less value of 117 ± 18 mg/day/1000 people (*ibid*).

5.4.4 Diazepam

Although obtained legitimately, benzodiazepines, especially diazepam, are among the most widely prescribed groups of drugs (Julien, 2005; Drummer & Wong, 2013; King, et al., 2013). However, DIAZ was not detected in waste water samples from Cambridge, UK, as well as from other parts of England (Baker, et al., 2014) but was detected in raw waste water samples from Slovenia (Kosjek, et al., 2012). The metabolite, nor-DIAZ, was detected in Baker's (2013) study, further confirming that careful evaluation needs to be made when selecting target analytes in order to increase the chances of detection.

5.5 VARIABILITY IN DRUG CONSUMPTION RESULTS

From the discussions in sections 5.4.1 to 5.4.4, it is evident that there is a wide variability in the occurrence and concentration data of the target drugs from various sewage epidemiological studies undertaken in different countries and even cities and regions within the same country. The reasons for this variability are further expounded upon in the following section.

5.5.1 Spatial and Temporal Variability

Spatial and temporal variability in drug consumption results can largely be attributed to different sample matrices (no two waste water samples are identical in composition), sampling techniques (type, frequency and duration), sampling site, weather conditions, analytical methods, etc. which all have an effect on the final results (Ort, et al., 2010 a&b; van Nuijs, et al., 2011a; Castiglioni, et al., 2013; Verlicchi, et al., 2014). Most importantly, the occurrence and type of drugs in waste water varies within regions and also with time within a country (Zuccato, et al.,2008; Gerrity, et al., 2011; Irvine, et al., 2011; Reid et al., 2011; Bijlsma, et al., 2012; Lai, et al., 2013a; Thomas, et al., 2012). Rainfall also affects the flow rate of waste water influent and this in turn affects the levels of compounds present. In Kasprzyk-Hordern's (2009c) study, most PPCPs in waste water influent doubled when the flow was halved in dry weather conditions indicating a possible dilution effect. Other attenuation processes have been mentioned in section 1.3.4.2. Therefore, results obtained from one area are not necessarily representative of another area within the same country or the entire country as a whole (EMCDDA, 2014b; Vuori, et al., 2014).

Other additional factors that have a significant influence on the occurrence and concentration of drugs include the different habits of each city and/or country, with regards to consumption and or direct disposal of therapeutic and drugs of abuse (Kümmerer, 2009; Irvine, et al., 2011; Zuccato, et al., 2011; Repice, et al., 2013). The demographics of each city and/or country also plays a role in the types and quantities of drugs detected (i.e. pensioners, students, tourist town). Vuori (2014) observed a higher usage of AMP in larger cities and university cities such as Helsinki and Turku due to a prevalence of night clubs where such drugs are consumed. Lai (2012) found a higher usage of COC and MDMA in an urban area and a popular holiday resort versus

a rural area during a busy holiday period in Australia. The increase in drug usage in the area frequented by holiday makers was attributed to the increase in seasonal visitor numbers to the area (*ibid*).In a separate study, the consumption of MDMA was higher for attendees of a music festival than for a nearby city (Lai, et al., 2013).

Irvine (2011) demonstrated higher COC and MAMP consumption in larger urban cities (150,000 to 800,000 people) than in smaller towns (< 23, 000 people) in Australia but the reverse was true for MDMA consumption. In comparison with results from other cities, the same study found that there was up to 30 times greater use of COC in Milan, Italy and London, UK, than in Adelaide but the latter had a 10 times higher usage of MDMA. Greater drug use in metropolitan versus rural settings within Oregon (USA) has also been reported (Banta-Green, et al., 2009). Thomas (2012) showed higher COC use in European cities located in the west and central than those in the north and east.

The sewage epidemiological approach has also been used to describe temporal trends in drug use. There are several studies that observed higher drug use on weekends than weekdays for most illicit drugs (Reid, et al., 2011; Irvine, et al., 2011; Thomas, et al., 2012; Nefau, et al., 2013; Baker, et al., 2014; Vuori, et al., 2014), with up to a 5-fold increase (Irvine et al, 2011). However, this intra-week variation in drug use was not observed in Hong Kong suggesting more regular than infrequent use of drugs (Lai, et al., 2013b). This is supported by a lack of intra-week variation in methadone use in Finland and Belgium, which is used as an opiate maintenance treatment and for pain management requiring regular use (van Nuijs, et al., 2011a; Kankaanpää, et al., 2014). Similarly, higher drug loads have been detected in urban waste water samples between December and January, due to the Christmas/New Year holiday season when substance use becomes more common (van Nuijs, et al., 2011b; Lai, et al., 2013a). Higher COC use was also observed in the evening than during the day (Reid, et al., 2011) further showing the temporal trend in drug usage and the advantage of time-weighted average sampling (composite and passive) over grab sampling. The latter may show a much lower concentration than the former if the sample was taken only during the day.

Estimations on AMP and MAMP in waste water samples from two cities in Finland differed considerably in two separate studies by different research groups i.e. Thomas (2012) and Kankaanpää (2014). While AMP was not detected in Thomas's (2012) study, it was detected in Kankaanpää's (2014) study. A possible reason for non-detection of AMP was the higher lower limit of quantification (LLOQ) (0.025 µg/mL) used by the former (Kankaanpää, et al., 2014). A similar observation was made when comparing MOR results from two separate areas in England. While MOR was not detected in Baker's (2013) study covering a population of 3.5 million, it was detected in results from this thesis covering a population of 145, 310 people (section 5.4.1.5). This is similar to a study conducted in France where higher BZE and COC concentrations were observed in a WWTP serving a smaller population (40, 000 people) than those serving larger cities (160,000 to 650, 000 people). This further highlights differences in results, not only from different cities/areas within the same country but even from similar areas with studies conducted by different research groups since no two waste water samples (even from the same source) are alike. In a study conducted in ten main cities in Finland (58,000 to 800, 000 people), illicit drug use was higher and more frequent in cities located in the South of Finland while overall COC consumption was observed to be lower in Finland in comparison to other European countries (van Nuijs, et al., 2011b; Thomas, et al., 2012; Kankaanpää, et al., 2014). In another study conducted in 25 WWTP in different towns and cities across France (13, 250 to 650, 000 people), significant geographical differences were observed especially for COC and AMP which occurred in higher frequency and concentration in larger cities than medium or smaller ones (Nefau, et al., 2013). This was attributed to the larger consumer market and access to entertainment venues and events in larger cities (*ibid*). This further indicates the temporal and spatial fluctuation in the consumption of drugs of abuse within the same city or country.

In a study conducted across 19 European cities (including London, UK), different usage patterns of illicit drugs were observed between Western and Central Europe compared with Northern and Eastern Europe (Thomas, et al., 2012). For instance COC use was highest in Antwerp, Belgium and much lower in Finnish cities. Highest levels for MAMP and AMP were detected in Finnish cities and The Netherlands, respectively. High usage of MDMA was also reported for The Netherlands and elsewhere in

England than for other European cities. Vast spatial differences in drug usage patterns across Europe was further confirmed in a recently published study covering 42 cities in 21 European countries (EMCDDA, 2014b; Ort, et al., 2014). For instance, in Ort's (2014) study, COC use was highest in Amsterdam, Antwerp, London and Zurich but lowest in cities located in northern, eastern and southern Europe. Geographical differences in drug usage habits have also been demonstrated in other studies conducted in various cities in China and Sweden (Khan, et al., 2014; Ostman, et al., 2014). Overall, drug usage was higher in larger, more populated urban cities than smaller towns (Ort, et al., 2014).

Such comparisons indicate that normalization of drug consumption results from studies conducted in different countries and/or cities provides a standardised platform to equally gauge international drug use levels (Lai, et al., 2013 a&b). This is another advantage of the sewage epidemiological method versus conventional epidemiological methods as it can be used by law enforcement to estimate the rate of growth of drug markets among various types of communities within a country or around the world (Lai, et al., 2013 a&b). However, inter-city and inter-country drug consumption comparisons based on daily or weekly assessments provide only a snapshot of drug usage trends and cannot be used to generalize for the rest of the year (Thomas, et al., 2013). Any results only represent the period over which samples were collected.

5.5.2. Concentration of Pharmaceutical and Personal Care Products (PPCPs) in Waste Water

In their review of PPCPs in WWTP influent and effluent from several recent studies, Luo (2014) reports significant spatial and temporal variations due to a number of factors, such rate of production, sales and usage, urinary excretion rate, size of WWTPs, environmental persistence and removal rate during waste water treatment processes. Additionally, PPCP concentrations in waste water correlated well with their production amounts and consumption patterns. For instance, in Wales, UK, high concentrations (0.010 µg/mL) of paracetamol, codeine, and atenolol were detected in waste water influent which correlates with their high levels of their dispensal (Kasprzyk-Hordern, et al., 2009b&c). Table 5.11 list some PhACs as detected in waste

water influent. Therapeutic drugs, such as paracetamol, tend to have higher detection frequencies and concentrations in raw waste water than illicit drugs, such as AMP, due to their high levels of consumption (Östman, et al., 2014).

Table 5.11: Concentration of pharmaceutically active compounds found in waste water influent.

PhAC	Maximum Conc. (µg/mL)	Reference
AMP	0.003	Baker & Kasprzyk-Hordern, 2013
BZE	0.003	Nefau, et al., 2013
Caffeine	0.209	Luo, et al., 2014
Codeine	0.032	Ratola, et al., 2012
Diazepam	0.023	Verlicchi, et al., 2012
Diclofenac	0.203	Ratola, et al., 2012
Ibuprofen	0.603	Luo, et al., 2014
Nicotine	0.009	Baker & Kasprzyk-Hordern, 2013
Paracetamol	0.482	Ratola, et al., 2012

For instance paracetamol and ibuprofen have been detected in waste water influent at levels of up to 0.482 µg/mL and 0.603 µg/mL, respectively, while AMP has been detected at levels of up to 0.003 µg/mL. The higher concentration levels of therapeutic drugs are due to their high consumption and ease of accessibility as over-the-counter medication. Caffeine has also been detected at levels of up to 0.05 µg/mL in waste water influent (Zhou, et al., 2010) due to the high consumption of caffeine containing compounds such as coffee, tea, energy and soft drinks (Luo, et al., 2014). Although official figures of the rate of production, sales and usage do not exist for illicit drugs, it can also be postulated that levels found in waste water correlate with their production and consumption patterns i.e. higher concentration levels indicate higher consumption of that drug (Luo, et al., 2014). Therefore, levels of drugs as determined in this research ranging from 0.004 ± 0.002 µg/mL to 0.549 ± 0.046 µg/mL (Table 5.1) are within the concentration range of PhACs as found in untreated waste

water. As mentioned earlier, the sample was collected over 72 h which would allow more time for accumulation of the target analyte, if present, as compared with a 12 or 24 h composite sample collected during the same period and in the same source. An earlier indication of the concentration levels of various PPCPS as found in waste water influent was made in Table 1.2, page 9.

However, as noted in section 5.3.1, a more appropriate way of comparing drug consumption in different cities and countries is to normalize it against the population. Therefore, Table 5.11 provides only an indication of concentration levels of PhACs that are found in untreated waste water with no inference to per capita usage (relative to population).

As discussed in sections 3.4.3 & 5.2.1, due to the nature of waste water, several other unknown and undesired matrix components were present in the samples as indicated by peaks of high relative abundance in the chromatograms. Therapeutic drugs tend to have higher detection frequencies and concentrations in untreated waste water due to their high levels of consumption (Östman, et al., 2014). Although not a target drug and not quantified, Figure 5.5 depicts the presence of caffeine in the 72 h composite waste water sample. In relation to MOR and KET, caffeine appears to occur at high concentration levels in line with published reports (Zhou et al., 2010).

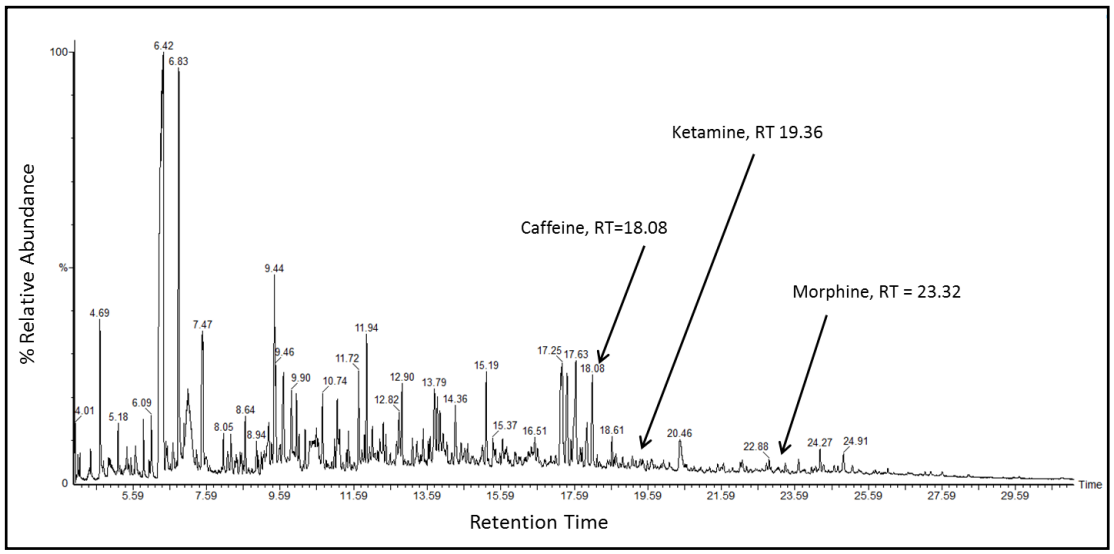


Figure 5.5: TIC of a 72 h composite waste water sample depicting caffeine, ketamine and morphine.

Identification of caffeine in the waste water sample was confirmed by comparison with the NIST database and an analytical standard (Appendix XV).

In spite of the higher concentration of unwanted matrix components, the target analytes could still be reliably detected and confirmed using the GC-MS method with PFPA derivatization developed during this research. In addition, although the matrix components of high intensity were not target drugs, they can provide future insight into the composition of waste water at the time of sampling. Hence, another advantage of using GC-MS in full-scan mode is the ability to do retrospective identification (Farré, M., et al., 2014).

5.5.3 Factors Contributing to Drug Concentrations in Cambridge, UK

Calculated drug consumption levels from data obtained during this research correlate with consumption levels of similar target analytes reported in published literature (sections 5.4.1 to 5.4.4). For drugs where back-calculations to drug consumption could not be conducted i.e. MCAT and MEPH, calculated daily loads (Table 5.4 and section 5.4.2.1) appear to be much higher in Cambridge than reported elsewhere. However, as mentioned in section 5.4.2.1, there is very limited published literature with which to adequately compare results for MCAT and MEPH. In addition, the historic city of Cambridge, UK is an urban city with two main universities, various colleges, hospitals and health centres, drug-rehabilitation and treatment centres, and has an active legal and clandestine night-club and rave scene. These are all enabling variables for illegal and legal drug consumption. Its relatively close proximity to the city of London, UK, also provides seasonal and regular inhabitants of Cambridge access to further entertainment venues. It has been reported in published literature that drugs consumed in one area will inevitably end up in the waste water in the residential areas of the users (Vuori, et al., 2014). In addition, Anglia Ruskin University Forensic Science & Chemistry Unit is working in partnership with Cambridgeshire Constabulary and hence some insight has been gained on the types of drugs being seized in Cambridge. Based on 2009 - 2011 drug seizure data supplied by Parkside Police Station, Cambridge, both KET and MEPH were found in 11 - 29 % and 3 - 24 % of cases, respectively. In 2010, MEPH and piperazines were highest in terms of seizures while in 2011, KET and MEPH were the 3rd and 4th most seized and confirmed drug,

respectively, excluding cannabis. Therefore, the presence of detected illicit drugs (including the NPS, KET and MEPH) in waste water from Cambridge, collected between 2011 and 2012 (section 2.2), is not surprising.

With a huge referral and research hospital in the city, i.e. Addenbrooke's Hospital, that administers and dispenses various pain relief medication, it is not unusual that high levels of MOR and KET were detected in waste water samples from Cambridge throughout the method development (Figures 3.27, 5.2, 5.3 and 5.4; Appendix IX).

Cambridge is also a research and development hub with many science-based companies (including pharmaceutical companies) operating in the city and surrounding villages. Therefore, the contribution of drugs directly disposed into the sewer from research laboratories (universities and companies) cannot be ignored. In addition, the 72 h composite sample was collected from Sunday to Tuesday, incorporating any drugs excreted over the weekend. It has been established in section 5.5.1 above that in many cities globally, higher levels of stimulant drugs were consumed over weekends than weekdays and that certain cities experienced increased drug consumption during holiday periods (i.e. increased tourism) or music festivals. During the Saturday and Sunday of the collection period, there were several events happening in and around Cambridge that brought extra visitors to the city i.e. Bridge the Gap fun walk, Grantchester Fair, Stourbridge Fair. It was also the popular 'Open Weekend' where visitors have a chance to visit the various colleges of the University of Cambridge for free and hence many people take advantage of this opportunity. The Open Weekend also coincides with the beginning of the new university term when many 'fresher's parties' and 'clubbing' occurs. The sample was also collected during the time the Paralympic games were being conducted in London. Cambridge, being a world famous tourist city in relatively close proximity to London, would no doubt have experienced an increase in national and international visitors from those who came to watch the games.

In order to address any uncertainties related to the characteristics of the relevant catchment area, it has been recommended that any known observations, whether speculative or not, be reported with any data as these can result in unusual drug

concentrations in waste water (Castiglioni, et al., 2013). Hence, all the above factors could have contributed to the types and levels of drugs detected in Cambridge, UK. Therefore, the detection of BUTY, MCAT, 4-MEOPP, MEPH, in Cambridge, UK, than other parts of England could indicate a difference in drug usage habits across England and assist law enforcement in determining local hotspots for the emergence of NPS (Kankaanpää, et al., 2014).

Whether the difference in drug consumption is due to the number of people consuming the drug or the daily dosage is difficult to determine without more comprehensive socio-epidemiological and toxicological data. Nonetheless, the ability to assess drug use within a day, during the week or holiday periods across regions is of potential use in allowing law enforcement, social and health agencies to tailor-make drug intervention policies and action plans (Lai, et al., 2013a). In comprehensive published research studies covering 19 to 42 European cities, the results from sewage epidemiological studies largely correlated with data from alternative drug monitoring methods, thereby indicating the reliability of the method (Thomas, et al., 2012; Ort, et al., 2014). Relatively good correlations between estimating drug consumption using the sewage epidemiological approach and comparison with National Health Service (NHS) prescription statistics in England have also been reported for certain drugs such as methadone and dihydrocodeine (Baker, et al., 2014). However, for other drugs such as amitriptyline and codeine, results differed significantly between the two methods *(ibid)*. A recent report by the EMCDDA (2014b) acknowledges that although results from the sewage epidemiological approach and conventional drug monitoring studies correlate to a large extent, some differences can still be found with regard to certain drugs in some cities. For instance, while cannabis use is amongst the highest levels in Italy and the Czech Republic, results from sewage epidemiology did not reflect this (EMCDDA, 2014b). Hence, the sewage epidemiological approach is a complementary, rather than alternative, method for drug monitoring. Comparisons between the sewage epidemiological approach and conventional drug monitoring methods are discussed in the next section.

5.6 THE COMPLEMENTARY ASPECTS OF SEWAGE EPIDEMIOLOGY AND CONVENTIONAL DRUG MONITORING METHODS

Tables 5.12 and 5.13 list some of the main advantages and disadvantages of the sewage epidemiological approach and conventional drug monitoring, respectively. The sewage epidemiological approach is not aiming to become a replacement for conventional drug monitoring methods such as hospital and police records, and population surveys (Castiglioni, et al., 2013). Instead, the various approaches to drug monitoring complement and enhance each other since all have strengths and weaknesses with respect to the data obtained from them (EMCDDA, 2008 & 2014b; Ort, et al., 2014). In a published study, three complementary drug monitoring methods, i.e. waste water analysis, questionnaire survey, and oral-fluid samples from drivers, were used for the estimation of community COC consumption (Reid, et al., 2012). While each method had significant limitations when used alone, the complementary data obtained from an evaluation of all three methods together was useful in providing a balanced overview of COC usage within a given community in Norway (*ibid*).

Table 5.12: Advantages and disadvantages of the sewage epidemiological approach.

SEWAGE EPIDEMIOLOGICAL APPROACH
Advantages
Provides objective near real-time information on which drugs are presently being consumed by a community (Castiglioni, et al., 2013; Baker, et al., 2014).
Provides daily, weekly, monthly or yearly trends of drug usage as well as comparisons before and after events, holidays or weekends (Burgard, et al., 2013).
Relatively quick turn-around of results (Östman, et al., 2014; Prichard, et al., 2014).
Non-intrusive, not subject to third party information or reliance on biased self-reporting (Östman, et al., 2014; Prichard, et al., 2014).
Adaptable to small scale or large scale monitoring (Castiglioni, et al., 2014).
Results can be used retrospectively to establish when and where NPS emerged (Castiglioni, et al., 2014).
Can be used to quickly confirm the trend in the use of emerging drugs or determine if social or police intervention programs have worked (Reid, et al., 2014b)
Disadvantages
An emerging method and needs further optimisation before it can become a standard method (Castiglioni, et al., 2014).
Can be expensive depending on the study design (Castiglioni, et al., 2014).
Limited to 'population' data and not demographics, frequency and mode of use, etc.
Still a challenge for compounds which exhibit significant degradation, e.g. 6-MAM and 3-FMC, and for those not used in significant quantities.

Table 5.13: Advantages and disadvantages of conventional drug monitoring.

CONVENTIONAL DRUG MONITORING e.g. HOSPITAL AND POLICE RECORDS, SOCIAL SURVEYS
Advantages
Provide an idea of the demographics consuming the drugs (e.g. age, sex, income group), health risk behaviour, attitudes towards drugs, frequency and mode of use, etc. (Castiglioni, et al., 2013).
Provides information on purity and prices of drugs (Castiglioni, et al., 2014)
Drug seizures provide an idea of the current consumer preferences and supply chain.
Disadvantages
Can be time consuming, complicated and expensive. Results can be outdated by the time they are released (Castiglioni, et al., 2013; Prichard, et al., 2014).
Surveys are subject to response and non-response biases and tend to under-represent usage (Castiglioni, et al., 2013; Prichard, et al., 2014).
Data from law enforcement, customs agencies and hospitals are subject to various data management limitations (Prichard, et al., 2014).
Can be intrusive

CHAPTER 6

SUMMARY, CONCLUSION AND FURTHER WORK

This chapter provides an overall summary of the conclusions arising from the various studies conducted in chapters 3 (preliminary), 4 (method validation) and 5 (application of the method).

6.1 SUMMARY AND CONCLUSION

The presence of chemical pollutants in water has led to increasing public awareness and concern, as well as scientific interest regarding their effects on the environment. In recent years scientific interest in pharmaceutical products and drugs of abuse as emerging pollutants has steadily increased. These pollutants enter the aquatic environment mainly through the discharge of treated and untreated waste water. Understanding the chemical behaviour and fate of emerging pollutants in surface water mainly relies on the development of analytical methods for their extraction and detection from which particular epidemiological information can be obtained. In addition, drugs of abuse detected in waste water can be used to determine the drugs prevalent in a given geographical area as well as estimate drug consumption patterns. Thus, sewage epidemiology has gained momentum globally owing to the non-intrusive nature of the method as well as the near real-time information obtained. With the constant emergence of NPS such as cathinones and piperazines, quick, reliable, specific and sensitive analytical methods are needed for their detection in complex matrices such as waste water.

This research, therefore, set out to develop and validate an analytical method based on gas chromatography-mass spectrometry (GC-MS) for the simultaneous detection of drugs of abuse in waste water samples from Cambridge, UK. Aside from knowing what drugs are present in the sewer system, the detected and quantified drugs would then be used to estimate drug consumption levels.

A series of preliminary methods were conducted in order to understand the chemical behaviour of the drugs under investigation. These included chemical derivatization, stability studies as well as extraction and recovery studies. In addition, an in-house

database of chromatographic and mass spectral information was developed during preliminary studies, which would be later used to reliably quantify the target drugs in waste water samples.

During chemical derivatization studies, 5 derivatizing reagents based on silylation and acylation were compared on a mixed drug standard (BSTFA, MSTFA, PFPA, HFBA and AcAn:Pyr). Ultimately, derivatization with PFPA: ethyl acetate (2:1, v/v), was selected based on higher peak areas obtained, a cleaner chromatogram (one derivative per added drug), and distinctive mass spectral patterns with ions of high m/z values and abundances greater than 50 %. The last point was critical because in order to reliably detect, identify and quantify the target drugs in a complex waste water sample, the mass spectral patterns needed to be distinctive enough to enable adequate selectivity and sensitivity.

The derivatization process also brought to light possible acetylation and hydrolysis reactions of opioids which highlighted the presence of underivatized 6-MAM and partially derivatized MOR occurring with the fully derivatized analogues. These reactions were thought to be responsible for the low stability and low to no recovery of heroin and 6-MAM during recovery studies. However, the level of derivatized MOR was much higher than that of the partially derivatized analogue confirming that the reaction favours the fully derivatized product.

Various stability studies were conducted to determine adequate sample pre-treatment and storage conditions of the target drugs. Autosampler stability (27 h) was conducted on a derivatized mixed drug standard in ethyl acetate, while 4 week storage stability studies, at 5 and -20 °C, were conducted on both derivatized and underivatized mixed drug standards in ethyl acetate and methanol, respectively. The latter was derivatized on the day of analysis. The majority of drugs were found to be stable to moderately stable in all stability tests especially when stored at -20 °C. The exceptions were EME, 6-MAM, heroin and 3-FMC which were generally unstable. As a result of stability tests, it was recommended to store derivatized mixed standards in ethyl acetate for no more than two weeks and to store the underivatized mixed drug standard in methanol for no more than 4 weeks.

In order to determine which sample isolation and concentration method would be most appropriate for extracting trace levels of drugs from a complex waste water matrix, LLE and SPE were compared. SPE was selected based on higher recoveries and ease of conducting multiple analyses at the same time. Oasis MCX and HLB sorbents were further compared at different sample pH values. Based on higher recovery values, the optimised method resulted in the use of the MCX (mixed mode cation exchange) cartridge at a sample pH of 2.

The challenges associated with multianalyte methods containing drugs with different pK_a and functional groups were highlighted by the different stability and recovery results. Compromises were made to find the most suitable conditions for 29 drugs and metabolites and hence the conditions favoured certain drugs over others.

A number of instrumental parameters such as the GC oven program, SIM analysis, photomultiplier voltage were also optimised during preliminary studies. Ultimately, a PMT voltage of 600 V, SIM analysis with 87 diagnostic ions in 14 RT windows and splitless injection mode were used during method validation and standard addition.

Following on from preliminary studies, method validation was conducted with mixed drug standards and waste water samples. The method had good linearity for all target drugs at $R^2 > 0.9900$. LODs and LOQs for some drugs were lower than with GC-MS/MS and LC-MS/MS and recoveries were > 70 % for the majority of drugs.

As a result of preliminary and validation studies, the method was successfully applied to waste water samples using standard addition to compensate for any matrix interference.

Eleven drugs were detected above their LOQ and within their linear range i.e. amphetamine, methamphetamine, 4-fluoromethamphetamine, methcathinone, 4-methoxyphenylpiperazine, cocaine, ephedrone, 3-trifluoromethylphenylpiperazine, butylone, ketamine and morphine. Two other drugs, benzylpiperazine and methylbenzylpiperazine were detected outwith their linear range, while, amitriptyline and 6-monoacetylmorphine were detected below their LOQ. As far as

the author is aware, this is the first time that butylone and 4-fluoromethamphetamine has been detected in waste water samples.

In addition, for 4-fluoromethamphetamine, cathinone, 3-fluoromethcathinone, 2- and 4-fluorophenylpiperazine, 4-trifluoromethylphenylpiperazine, butylone, methylbenzodioxolylbutanamine, 4-methylphenylpiperazine, methylbenzylpiperazine, 2- and 4- methoxyphenylpiperazine, and 3-chlorophenylpiperazine, this is the first reported analytical method in sewage epidemiology and the first time PFPA mass spectra and diagnostic ions have been reported for the aforementioned drugs including methcathinone, mephedrone and ketamine.

Out of the eleven detected and quantifiable drugs and metabolites, five could be used to estimate drug consumption levels in Cambridge, UK, which were found to be heroin (399.4 ± 90.8), ketamine (2463.5 ± 182.5), cocaine (195.7 ± 95.4), methamphetamine (84.3 ± 59.1) and amphetamine (38.9 ± 24.8), all in mg/day per 1000 people. These estimated values correlate with published socio epidemiological surveys on the prevalent use of the respective drugs but incorporate both illicit and therapeutic use. This highlights the ability of sewage epidemiology to monitor drug use patterns and corroborate findings from other methods.

However, the capability of utilising GC-MS, with derivatization, as a cheaper and effective alternative to GC-MS/MS, LC-MS and LC-MS/MS in the analysis of trace analytes from complex matrices such as waste water has been demonstrated through comparable and lower LODs and LOQs, good selectivity and sensitivity for target analytes in the presence of other undesired matrix components, and high sample throughput. As the first reported sewage epidemiological method to use derivatization with PFPA, it is hoped that this method will gain widespread use especially with laboratories that do not have LC-MS but would still like to conduct similar research.

In addition, the developed method, based on matrix-matched standard addition quantification, use of isotopically-labelled internal standards and dilution of the

sample matrix, as well as carefully monitored quality control parameters (section 2.6), was suitable and effective in quantifying the detected target drugs in waste water.

6.2. SUGGESTIONS FOR FURTHER WORK

The degradation reactions observed for opioids, especially 6-MAM and heroin, are thought to have contributed to their lack of detection in waste water. For further investigations into methods involving these drugs, the use of an alternative solvent to ethyl acetate (e.g. acetonitrile and dichloromethane) for reconstitution and stability studies would be worth investigating. In addition, alternative derivatization methods (e.g. silylation) to avoid degradation reactions of opioids will be worth investigating in future (Guillot, et al., 1997).

During recovery studies, the lower peak areas of the unextracted or post-extracted drug standard compared with the extracted standard was an enigma and worth exploring further. The effect of various evaporation methods (vacuum concentrator and nitrogen gas) on analyte loss or preservation also warrants further investigation.

Although the extraction method was reproducible for the majority of drugs, further work will be needed to improve the reproducibility in recovery for some of the drugs that had high RSDs such as 2- and 4-MEOPP, MCAT and 3-FMC. Coupled to this would be incorporating 'green chemistry' into the research by investigating the possible use of SPME (Mills & Walker, 2000; Östman, et al., 2014) and supercritical fluid extraction (Scott & Oliver, 2001; Kalikova, et al., 2014) to enhance the sensitivity of the method while restricting the amount of sample, solvents and steps needed during sample extraction (Sheldon, 2005).

In addition, alternative longer-term sampling techniques using passive samplers, such as POCIS or Chemcatcher®, can be investigated to facilitate the detection of some of the drugs such as BZP, MBZP, 6-MAM and AMIT (Mills, et al., 2007; Wille, et al., 2012; Boles & Wells, 2014).

Since the majority of PhACs investigated have one or more chiral centres, it could be worth investigating the presence of the relevant potent enatiomers found in

recreatonal drugs to more accurately differentiate between illicit and therapeutic drug consumption (Emke, et al., 2014; Kalikova, et al., 2014).

The developed method was applied on a sample collected during one period in Cambridge, UK, but longer term sampling campaigns to determine weekly, monthly or seasonal variations in drug consumption can further enhance the research outcomes. In addition, sampling in other cities within the UK or being involved in inter-laboratory studies will also enhance the reliability of normalized spatial and temporal comparisons.

Although GC-MS was used as an alternative to LC-MS, it would still be worth conducting comparative studies with LC-MS to determine if in-house results would mirror those made when comparing with other research groups i.e. lower LODs and LOQs with GC-MS.

Therefore, although detection of drugs of abuse in waste water can be linked to consumption levels, there are still many uncertainties and factors that need to be taken into account before sewage epidemiology can become a routine, universal method for drug monitoring (Lai, et al., 2011; van Nuijs, et al., 2011a; Castiglioni, et al., 2013). As this is a relatively new area of research (since 2005), it is expected that the method will continue to be refined as sample collection, preparation and preservation, as well as analytical methods and protocols continue to improve. However, in spite of this, the sewage epidemiological approach has even been adopted by the EMCDDA as a new and complementary method for estimating community drug consumption and has even been included in the 2014 European Drug Report (EMCDDA, 2014a&b). In this regard, research studies have been conducted with the aim of standardizing the approach, from sampling to analysis (Thomas, et al., 2012; Castiglioni, et al., 2014; Ort, et al., 2014).

The analytical method based on GC-MS and derivatization with PFPA, as documented in this thesis, can be applied to almost any polar, derivatizable compound from any water source and as GC-MS is widely known to be more cost-effective than LC-MS/MS, it could have much wider usage especially in developing nations where more

cost-effective yet sensitive methods still need to be available for these studies. With further optimisation, it could also be included as an alternative method for sewage epidemiology thereby becoming an excellent tool for identifying and quantifying PhACs in various environmental matrices. Therefore, it is the hope of the author that GC-MS methods will continue to be optimized for sewage epidemiological studies just as much as LC-MS methods have been.

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Zuccato, E., et al., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. [Online] Available at: http://www.ehjournal.net/content/4/1/14. [Accessed 05 August 2014].

Zuccato, E., et al., 2008. Estimating Community Drug Abuse by Wastewater Analysis. *Environmental Health Perspectives*, 116 (8), pp. 1027-1032.

Zuccato, E., et al., 2011. Changes in illicit drug consumption patterns in 2009 detected by waste water analysis. *Drug and Alcohol Dependence*, 118, pp. 464-469.

APPENDIX I a: Publications in support of this thesis.

Mwenesongole, E.M., Gautam, L., Hall, S.W., Waterhouse, J.S., 2013. Simultaneous detection of controlled substances in waste water. *Analytical Methods*, 2013, 5, pp. 3248-3254.

APPENDIX I b: Publications in support of this thesis.

Mwenesongole, E., Gautam, L., Hall, S.W., Emmet, T., 2012. Estimating Community Drug Usage Patterns by the Analysis of Waste Water. *Salford Postgraduate Annual Research Conference, 2011 Proceedings, pp. 153-169.* [Online] Available at: *http://usir.salford.ac.uk/23158/1/2011proceedingsAug2012_V2.pdf* [Accessed 05 August 2014].

APPENDIX II: Background to drug classes/groups investigated in this thesis.

1. Cocainics

Cocaine is a naturally occurring alkaloid extracted mostly from the leaves of the *Erythroxylon coca* bush found mainly in South America (Julien, 2005; King, et al., 2013). Although less harmful derivatives of cocaine, such as procaine and lidocaine, are therapeutically used as anaesthetics, cocaine is more popularly used as an illicit drug (Moffat, et al., 2011b; King, et al., 2013). Figure 2.3 shows the structures of cocaine and one of its main metabolites, ecgonine methyl ester.

Main metabolites of cocaine

The main metabolites of cocaine excreted in urine are benzoylecgonine and ecgonine methyl ester (Ambre, et al., 1982). They account for 35-55 % and 30-60 %, respectively, of the administered cocaine dose in urine (Moffat, et al., 2011b). A small percentage of cocaine (1-9 %) is excreted as the unchanged drug in urine (Julien, 2005; Melis, et al., 2011). Therefore cocaine and ecgonine methyl ester were chosen as target analytes for the estimation of cocaine consumption in waste water for this research (van Nuijs, et al., 2009c).

2. Phenylethylamines

Phenylethylamines comprise the amphetamine-group substances, such as amphetamine, methamphetamine and 4-fluoromethamphetamine (4-FMA) as well as their ring-substituted analogues, such as methylbenzodioxolylbutanamine (MBDB) and methylenedioxymethamphetamine (MDMA) (Melis, et al., 2011; UNODC, 2013). These are depicted in Figure 2.4.

Phenylethylamines are among the most widely abused classes of psychotropic drugs globally. They are mainly excreted as the parent drug in urine i.e. 30 - 90 % of a dosage for amphetamine, 40 - 50 % for methamphetamine and 65 % for MDMA (Moffat, et al., 2011b). In addition, methamphetamine is also eliminated as amphetamine (4 - 7 %) (Levine, 2006; Moffat, et al., 2011b). Since they are emerging drugs of abuse and as far as the author is aware, the toxicological profiles of 4-FMA and MBDB were not available at the time of writing of this thesis.

Since the majority of the phenylethylamines are excreted in a relatively large percentage as the unchanged drug (in comparison with their other metabolites), the parent drugs were used as target analytes for waste water analysis (Melis, et al., 2011).

3. Piperazines and Cathinones

As mentioned in section 2.1.2, piperazines and cathinones are NPS that have become prevalent over the last few years. Most of the commonly abused piperazines are aryl substituted derivatives of piperazine such as benzylpiperazine (BZP) and 3-trifluoromethyl phenylpiperazine (3-TFMPP). Figure 2.5 shows the piperazines targeted in this research.

Cathinones are semi-synthetic or wholly synthetic derivatives of the extract from the *Catha edulis* plant found mainly in East Africa and the Arabian Peninsula (Gautam, et al., 2013). The leaves and fresh shoots are referred to as 'khat' and can be chewed or brewed as a tea (King, et al., 2013). The main psychotropic and regulated components of khat are cathinone and cathine (Cole, 2003). Both are chemically and pharmacologically similar to synthetically manufactured amphetamine and methcathinone (King, et al., 2013).

Semi-synthetic analogues of cathinone, referred to as beta-keto amphetamines, have recently gained popularity among the same demographic population that uses ATS and piperazines (Measham, et al., 2010). These include mephedrone, methylone and butylone (Meyer, et al., 2010; Gautam, et al., 2013). Figure 2.6 depicts structures of cathinones relevant to this research.

In contrast to the traditional drugs of abuse, the metabolic pathways of piperazines and cathinones in humans have not been as extensively studied. However, both groups are known to mainly form hydroxyl- and 4-hydroxy-3-methoxy- metabolites in rat and/or human urine (Staack, et al., 2002; Staack & Maurer, 2004; Gautam, et al., 2013). Data on the urinary excretion profile in humans for piperazines and cathinones was not readily available at the time of writing of this thesis (Castiglioni, et al., 2014). However, the main metabolites of mephedrone as determined in rat urine were normephedrone 4-(hydroxymethyl) methcathinone and 4-(carboxy)methcathinone (Khreit, et al., 2013).

4. Opiates and Opioids

Opiates are a group of more than 20 active alkaloids extracted from the juice of the opium poppy, *Papaver somniferum* (Levine, 2006). Morphine, a principal alkaloid, is used to make synthetic (e.g. methadone) and semi-synthetic (e.g. heroin) opioids (Cole, 2003; Melis, et al., 2011). The term 'opioid' therefore refers to natural and semisynthetic alkaloids derived from opium as well as synthetic analogues with similar pharmacological activity to morphine (Levine, 2006). Opioids targeted in this research are depicted in Figure 2.7.

Main metabolites of opioids

The main metabolites of morphine are morphine-3-glucuronide and morphine-6-glucuronide, excreted as 65-70 % of a dosage (Moffat, et al., 2011b). However, these are quickly hydrolysed to morphine in waste water (section 2.3.2). In addition, around 3-10 % of a dosage of morphine is excreted as the parent drug in urine (Moffat, et al., 2011b). In aqueous solution, heroin is known to hydrolyse to 3- and 6-monoacetylmorphine and morphine (Moffat, et al., 2011b). Therefore, morphine, 6-monoacetylmorphine and heroin were targeted for analysis (Boleda, et al., 2009; Melis, et al., 2011).

5. Benzodiazepines, Tricyclic Antidepressants and Dissociatives

These drugs are legally manufactured for psychotherapeutic use but are increasingly being abused due to their relaxant properties (Figure 2.8). In contrast to ATS, illicit manufacture of benzodiazepines, tricyclic antidepressants and sedatives is rare. Commonly abused members of these groups are obtained through legitimate (prescription) or illegal (forged prescriptions, stolen from pharmaceutical supplies) means (King, et al., 2013).

Benzodiazepines are among the most widely prescribed groups of drugs (Julien, 2005; Drummer & Wong, 2013). Structurally, they all have a common tricyclic nucleus differing only in the functional groups attached at different positions (Julien, 2005; Levine, 2006). Commonly used members of this group include diazepam (Valium) and lorazepam (King, et al., 2013). Although mainly used in the management of depression, anxiety, insomnia and related conditions (Julien, 2005; Östman, et al., 2014), they are also used in combination with some illicit drugs, such as heroin, to enhance the effects (Scott & Oliver,

2001; UNODC, 2013). Benzodiazepines are extensively metabolised, often with very little of the parent drug present in urine (Drummer & Wong, 2013). Only diazepam was targeted in this research and its key metabolites excreted in urine are desmethyldiazepam, oxazepam and temazepam conjugates, collectively excreted as 70 % of a dose (Scott & Oliver, 2001; Moffat, et al., 2011b).

Tricyclic antidepressants share a three-ring molecular core with different functional groups. A common member of this group is amitriptyline used to treat depression, migraines and pain (Julien, 2005). The main metabolites of amitriptyline in urine are nortriptyline and free and conjugated 10-hydroxynortriptyline and 10-hydroxyamitriptyline, excreted as 35 % of a dose (Julien, 2005; Moffat, et al., 2011b; Drummer & Wong, 2013). The parent drug is excreted at less than 5 % in urine and 8 % in faeces (Moffat, et al., 2011b).

As a tranquiliser, ketamine is mainly used as an anaesthetic for human and veterinary procedures (Levine, 2006; UNODC, 2013). Ketamine is not completely metabolized in humans and other organisms. Ketamine is metabolized primarily as conjugates of hydroxylated metabolites (80 %), norketamine (2 %) and unchanged drug (2 %) (Levine, 2006; Moffat, et al., 2011b*;* Lin, et al., 2014)*.*

According to the World Drug Report (UNODC, 2013), sedatives and tranquilisers, such as benzodiazepines and barbiturates, were reported as the top three misused substances by more than 60 % of the countries assessed.

APPENDIX III: Acid-base equilibria.

In order to understand how drugs of abuse are isolated from complex sample matrices such as waste water by SPE and LLE, the relationship between pH and dissociation constants K_a needs to be explored.

Most drugs will ionise in solution based on their pKa and the pH of the solution in which they are dissolved. The relationship between the pK_a and the pH is represented by the Henderson-Hasselbalch Equation (Harris, 2010) which for acids is;

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

Equation A-1

Where $[A^-]$ is conjugate base and [HA] is the acid,

and for bases is;

$$pH = pK_a + \log \frac{[B]}{[BH^+]}$$

Equation A-2

Where [B] is base and $[BH^+]$ is the conjugate acid.

The pK_a expresses the pH at which 50 % of the analyte molecules in solution are ionised (Levine, 2006; Stimpfl, 2011).

During solid or liquid phase extractions, the aim is to get 100 % of ions either in an ionised or unionised state. This is achieved by either lowering or increasing the pH with respect to the pK_a of the ionisable group in solution (Hendriks, et al., 2007). A rule of thumb when adjusting pH for SPE and LLE is to modify it at least 2 pH units below or above the pK_a of the target analytes (Telepchak, et al., 2004; Flanagan, et al., 2007). This has been referred to as the pK_a ± 2 rule and enables the analytes to either be in an ionised or unionised state depending on the pH adjustment (Hendriks, et al., 2007). Neutral drugs, on the other hand, do not contain ionisable functional groups and hence can be extracted at all pH values between 0 and 14 (Levine, 2006). By taking advantage of the physico-chemical

properties of the analytes (e.g. pK_a) a suitable sample extraction procedure can be developed.

APPENDIX IV: Selection of internal standards used in this thesis.

During method development, internal standards are recommended additions to analytical processes as they help compensate for variability during sample preparation and instrumental analysis (e.g. injection volume, flow rate, operating pressure) (Furey, et al., 2013).

Although various internal standards can be used, stable isotope-labelled internal standards (deuterated, ^{13}C, ^{15}N or ^{17}O) with similar chemical properties to the compound under analysis are more commonly used (Flanagan, et al., 2007; Cooper, et al., 2010). In this research, deuterated internal standards were used. Since many of the NPS under investigation did not have commercially available deuterated analogues, 4 internal standards were used to represent the 29 drugs under investigation. These were AMP-d_6, MDMA-d_5, COC-d_3 and MOR-d_3. However, MOR-d_3 was eliminated as an internal standard during preliminary investigations due to its instability (section 3.3.1, pages 108-109, Figure 3.22). Assigning of the internal standard was based on one or more of the following criteria: similarity in structure to the target drugs, the one closest in retention time (RT) to the analyte and the most stable internal standard for the method (Pedrouzo, et al., 2011; Gago-Ferrero, et al., 2013). The drug standards and corresponding internal standards are listed in Table 3.3 on page 102. Using a few representative internal standards during multianalyte methods is acceptable practice especially when investigating newer drugs with no commercially available deuterated analogues or when the cost of obtaining a deuterated analogue for each drug under analysis becomes too high (Couchman & Morgan, 2011; Pedrouzo, et al., 2011).

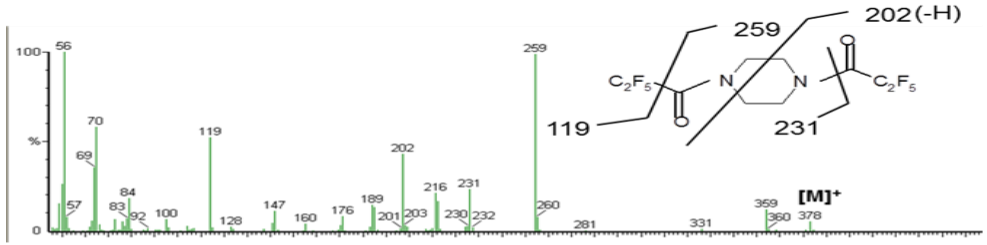
APPENDIX V: Kovat's retention index (RI) formula for linear temperature programing.

$$RI_{analyte} = 100\,n + 100\,\frac{RT_{analyte} - RT_{n}}{RT_{n+1} - RT_{n}}$$

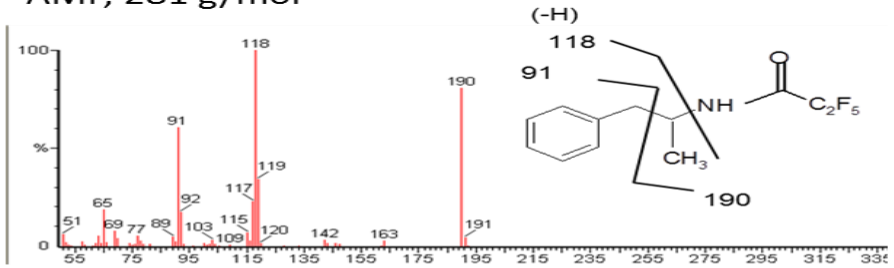
Where RI is the retention index, RT is the retention time, *n* and *n+1* are the smaller and larger n-alkanes, respectively, which bracket the drug (Newton & Foery, 1984). n-alkanes used ranged from n=10 to n=28 as listed in Table 2.2.

APPENDIX VI-a: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

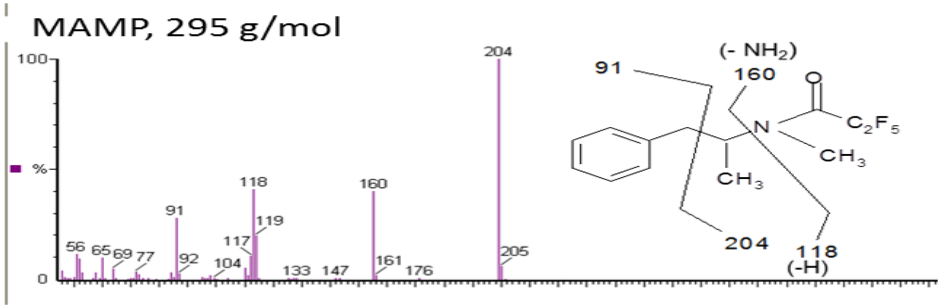
PIP, 378 g/mol



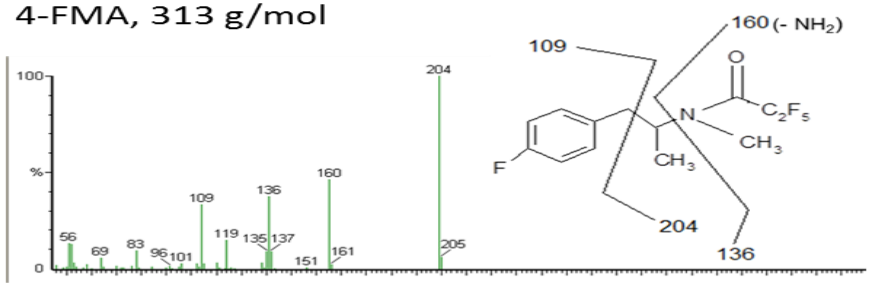
AMP, 281 g/mol



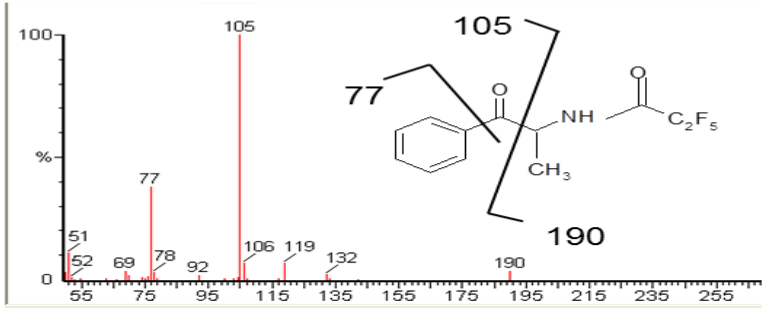
MAMP, 295 g/mol



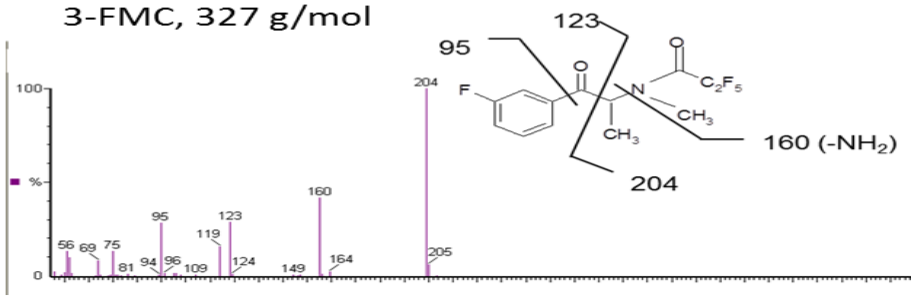
4-FMA, 313 g/mol



CAT, 296 g/mol

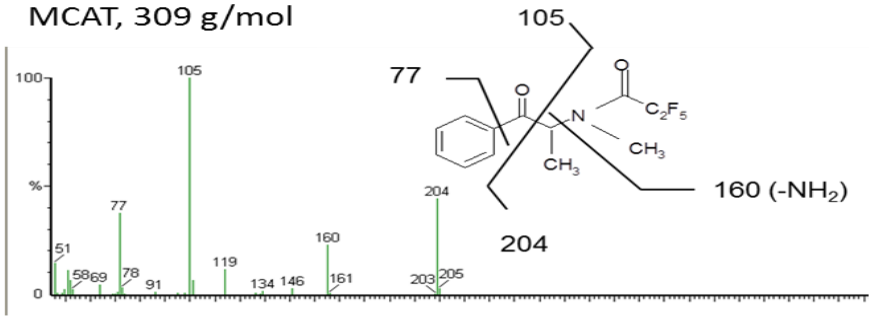


3-FMC, 327 g/mol

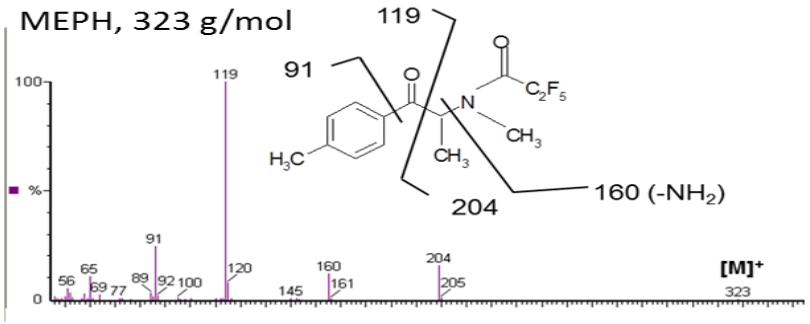


APPENDIX VI-b: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

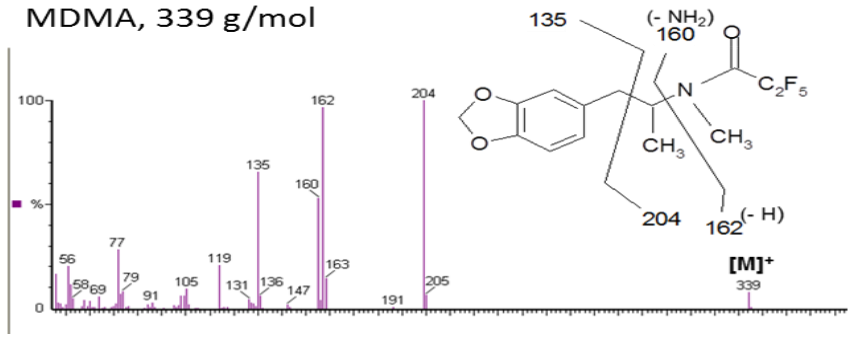
MCAT, 309 g/mol



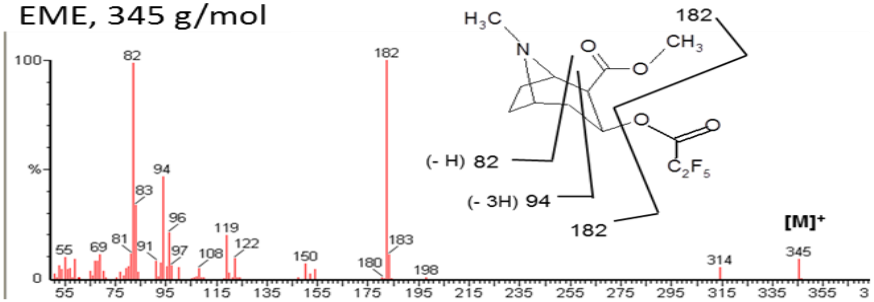
MEPH, 323 g/mol



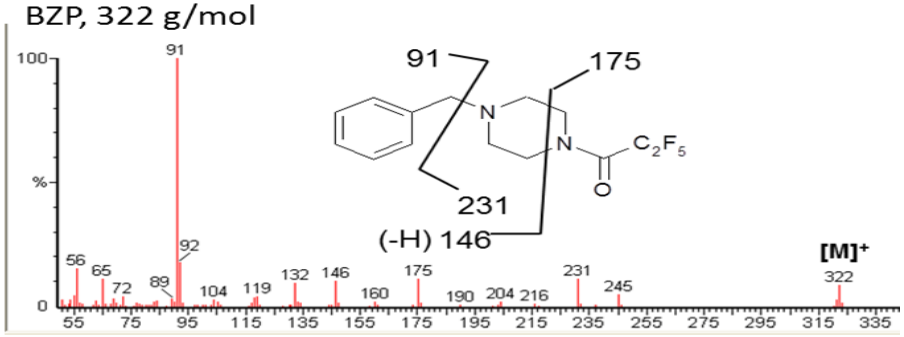
MDMA, 339 g/mol



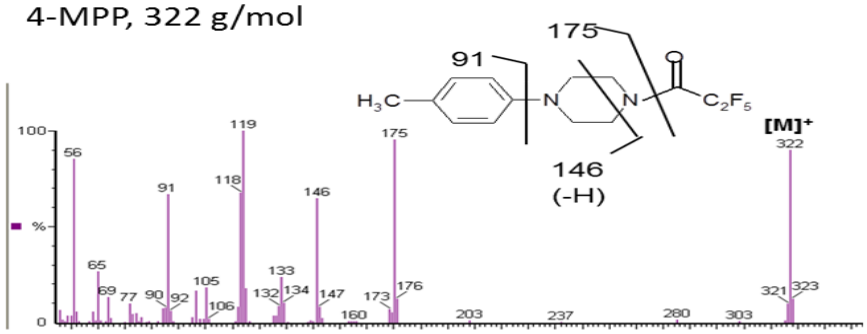
EME, 345 g/mol



BZP, 322 g/mol

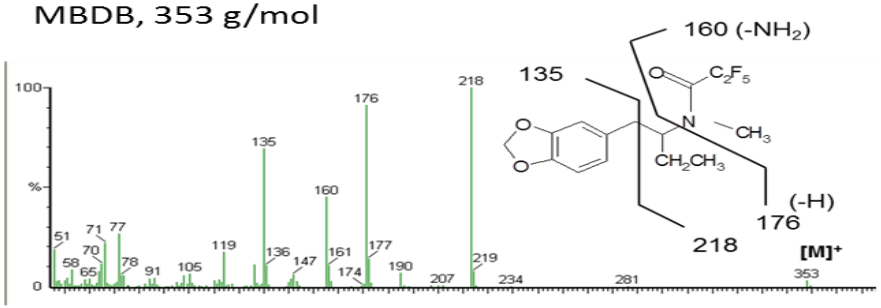


4-MPP, 322 g/mol

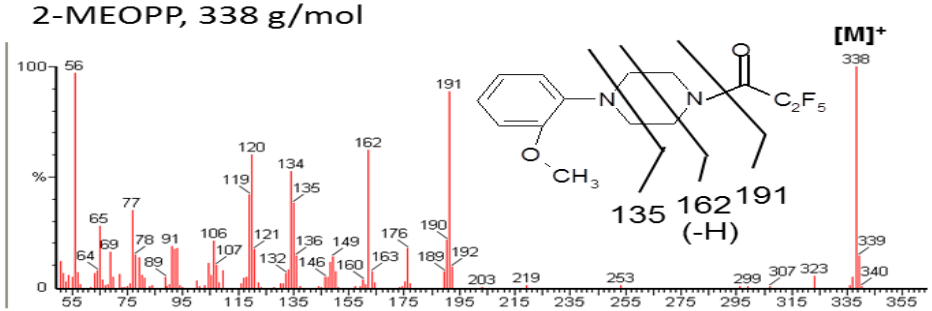


APPENDIX VI-c: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

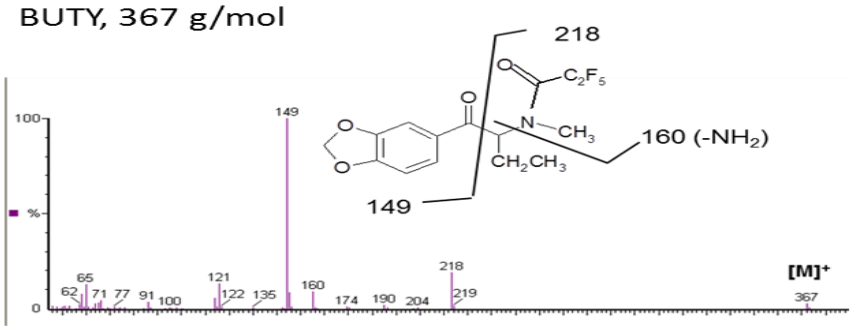
MBDB, 353 g/mol



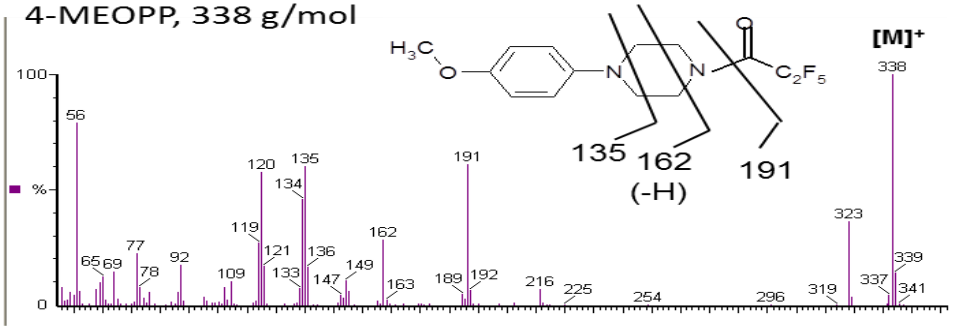
2-MEOPP, 338 g/mol



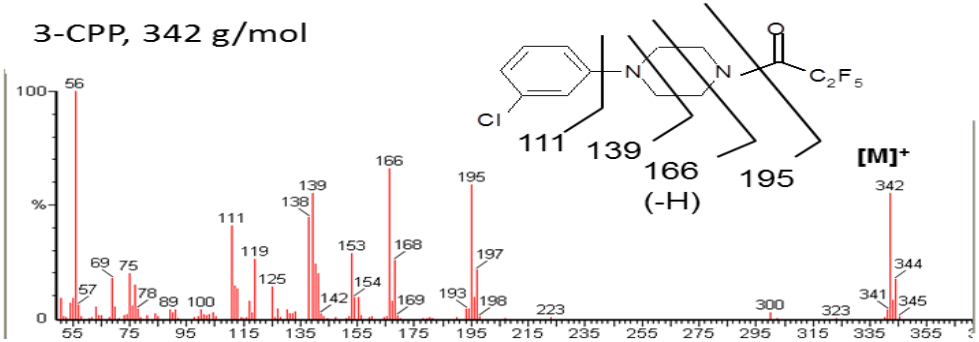
BUTY, 367 g/mol



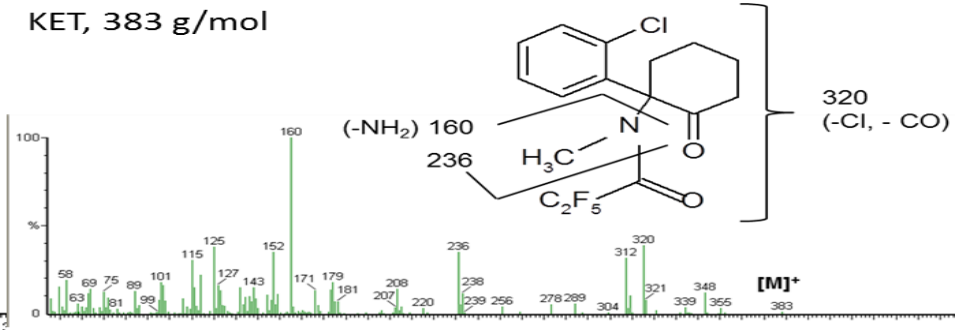
4-MEOPP, 338 g/mol



3-CPP, 342 g/mol

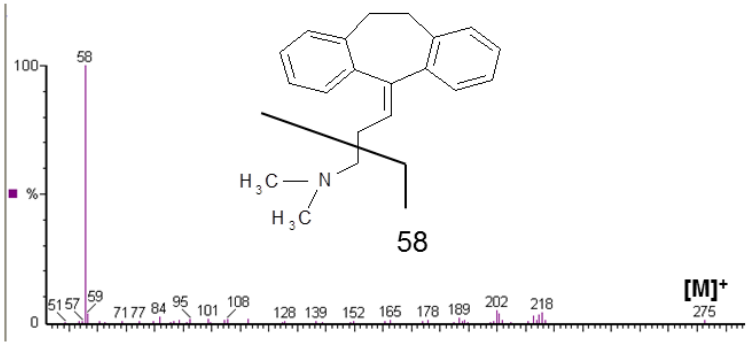


KET, 383 g/mol

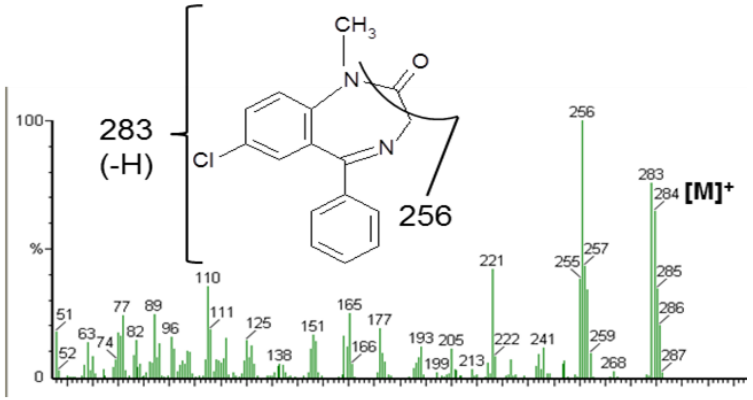


APPENDIX VI-d: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

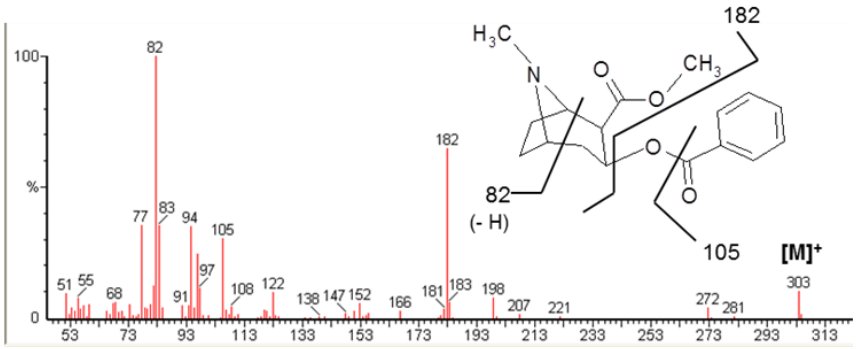
AMIT, 276 g/mol



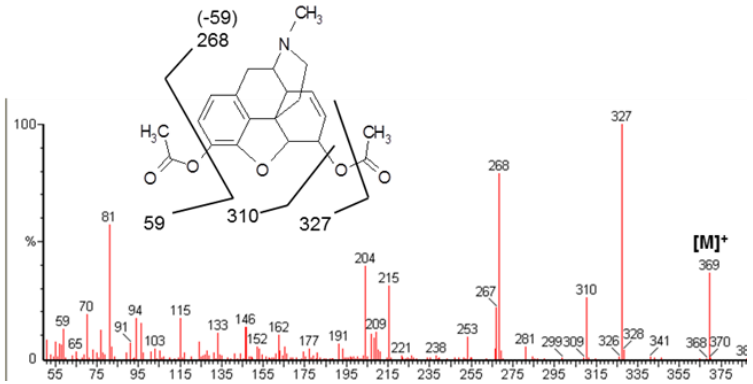
DIAZ, 283 g/mol



COC, 303 g/mol

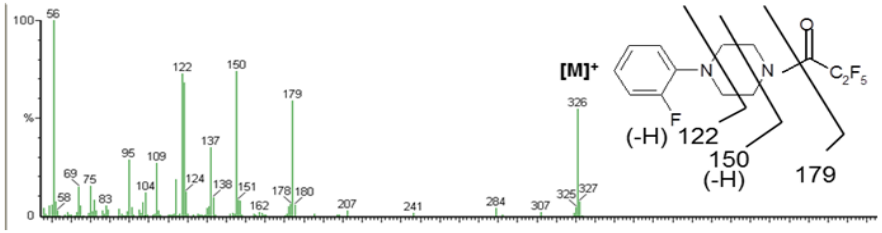


Heroin, 369 g/mol

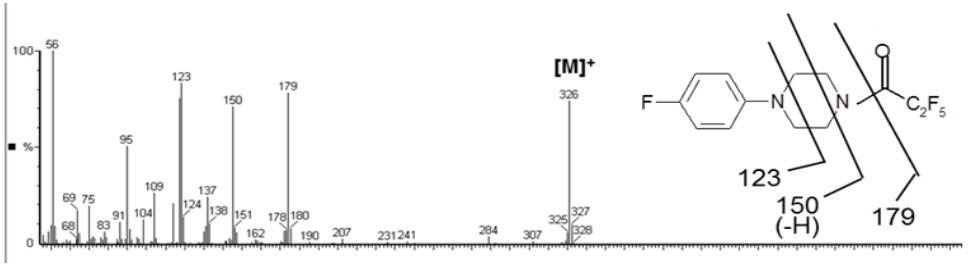


APPENDIX VI-e: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

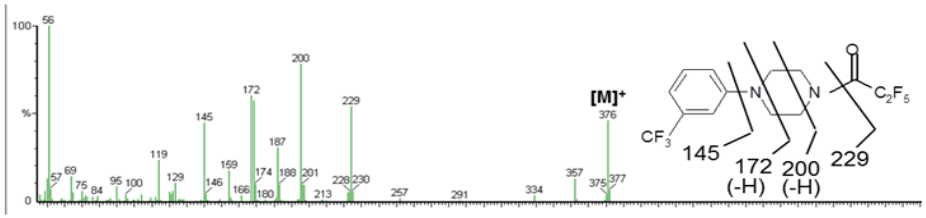
2-FPP, 326 g/mol



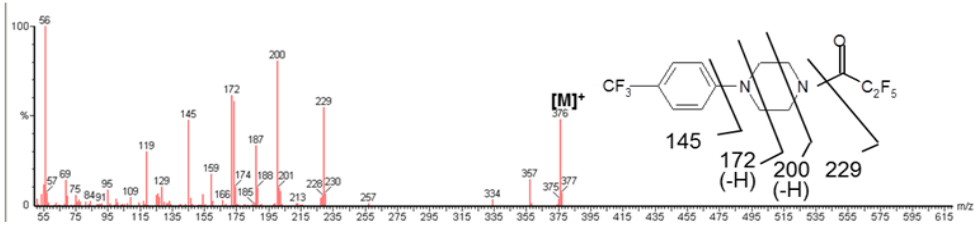
4-FPP, 326 g/mol



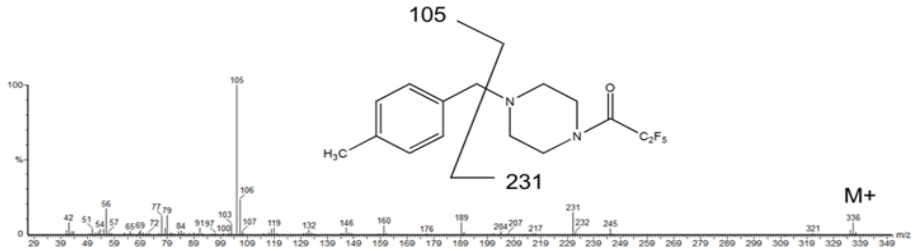
3-TFMPP, 376 g/mol



4-TFMPP, 376 g/mol

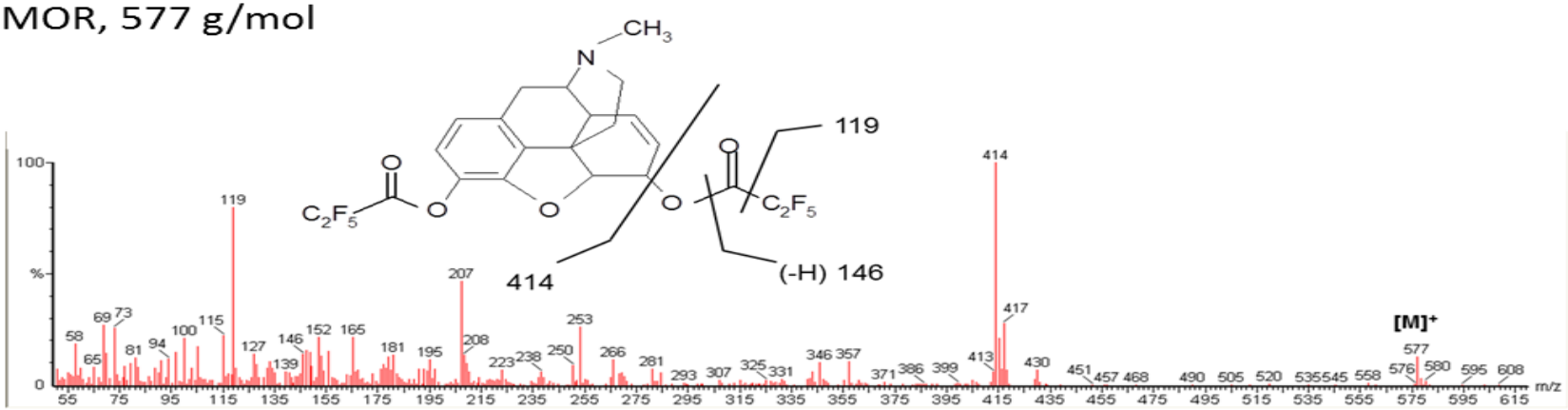


MBZP, 336 g/mol

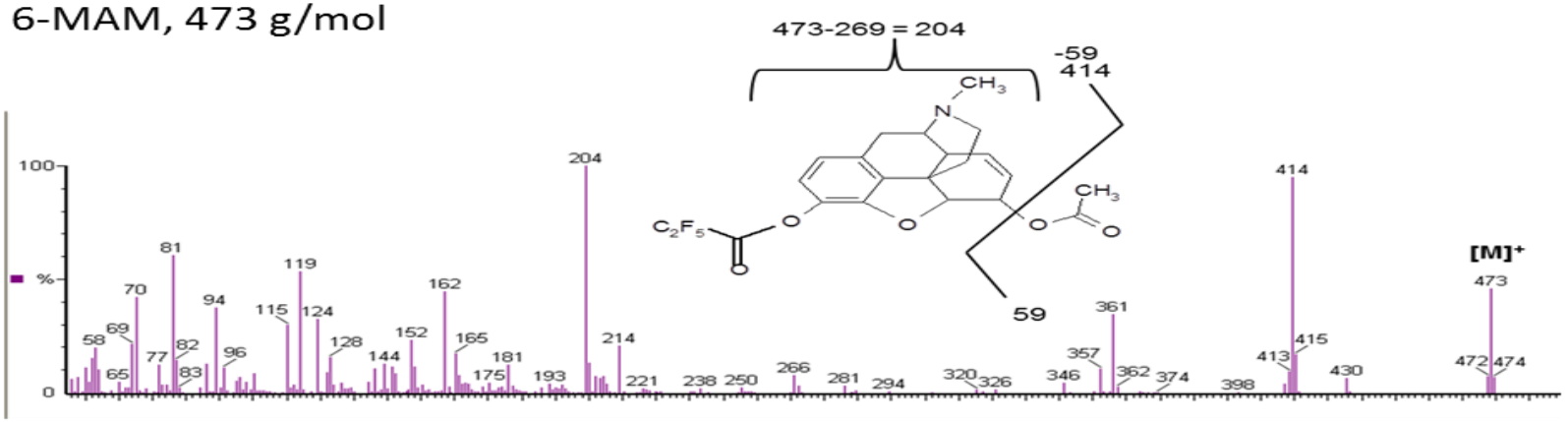


APPENDIX VI-f: Mass spectra, molar mass and proposed fragmentation patterns for PFPA derivatized target drugs.

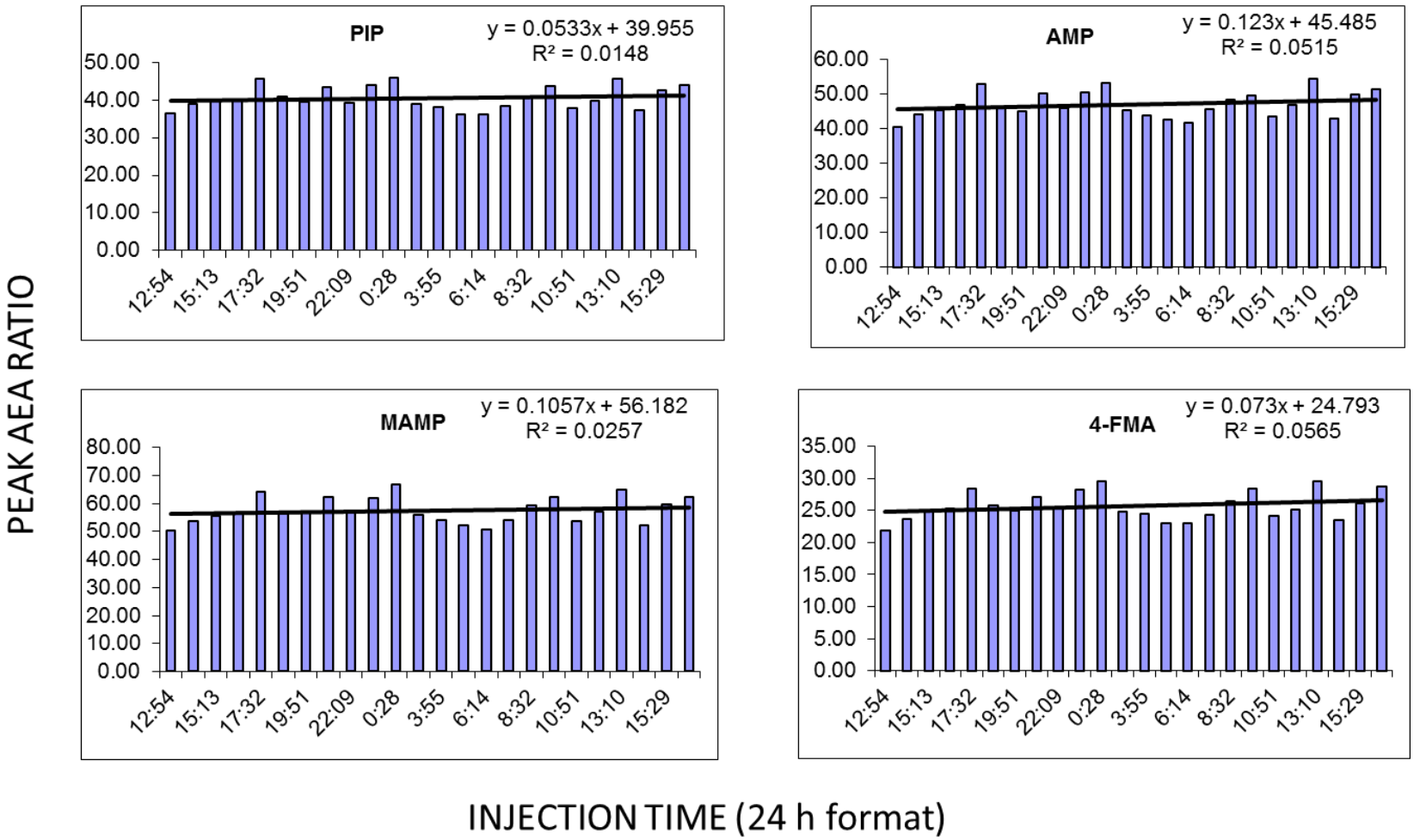
MOR, 577 g/mol



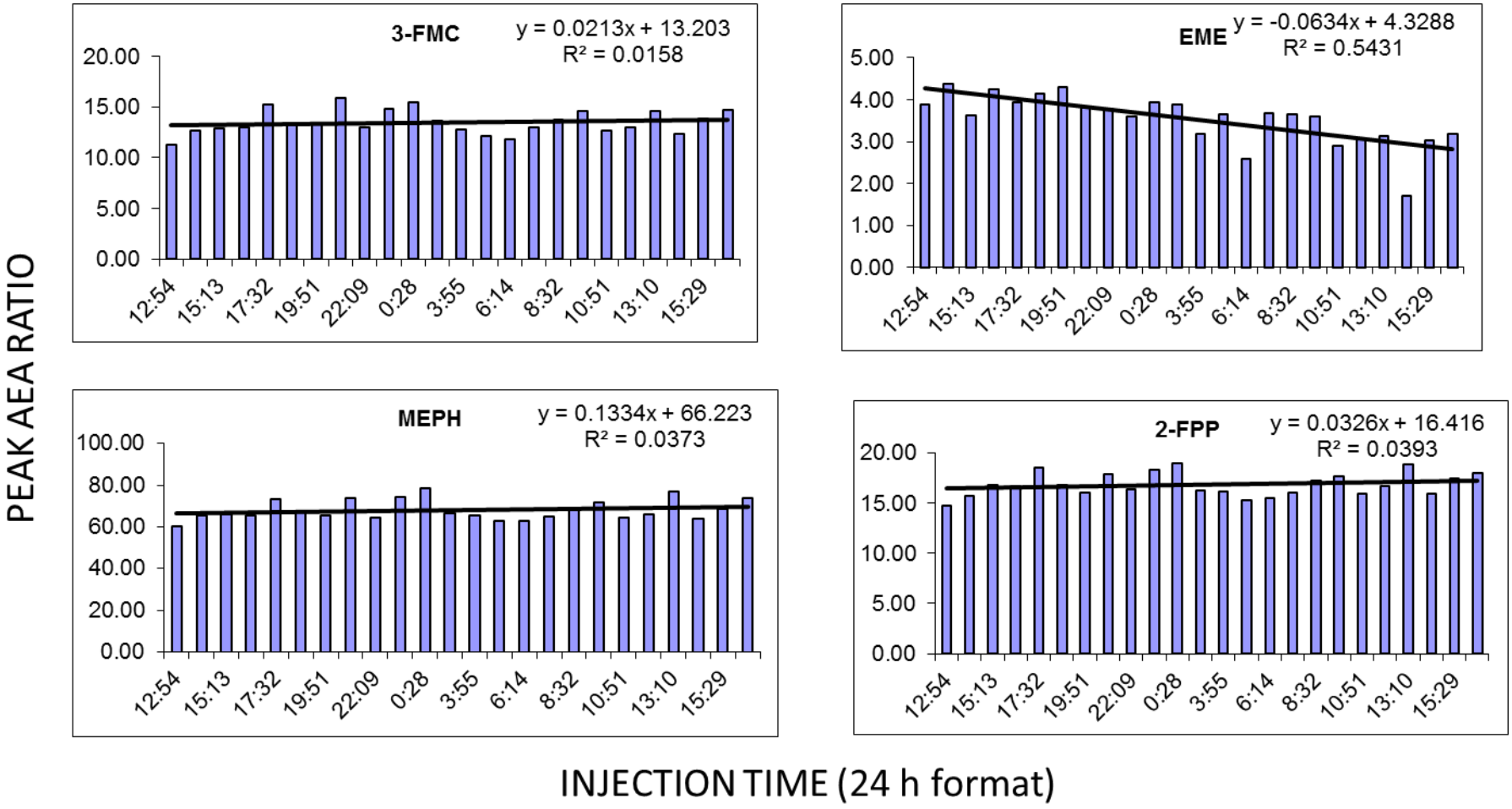
6-MAM, 473 g/mol



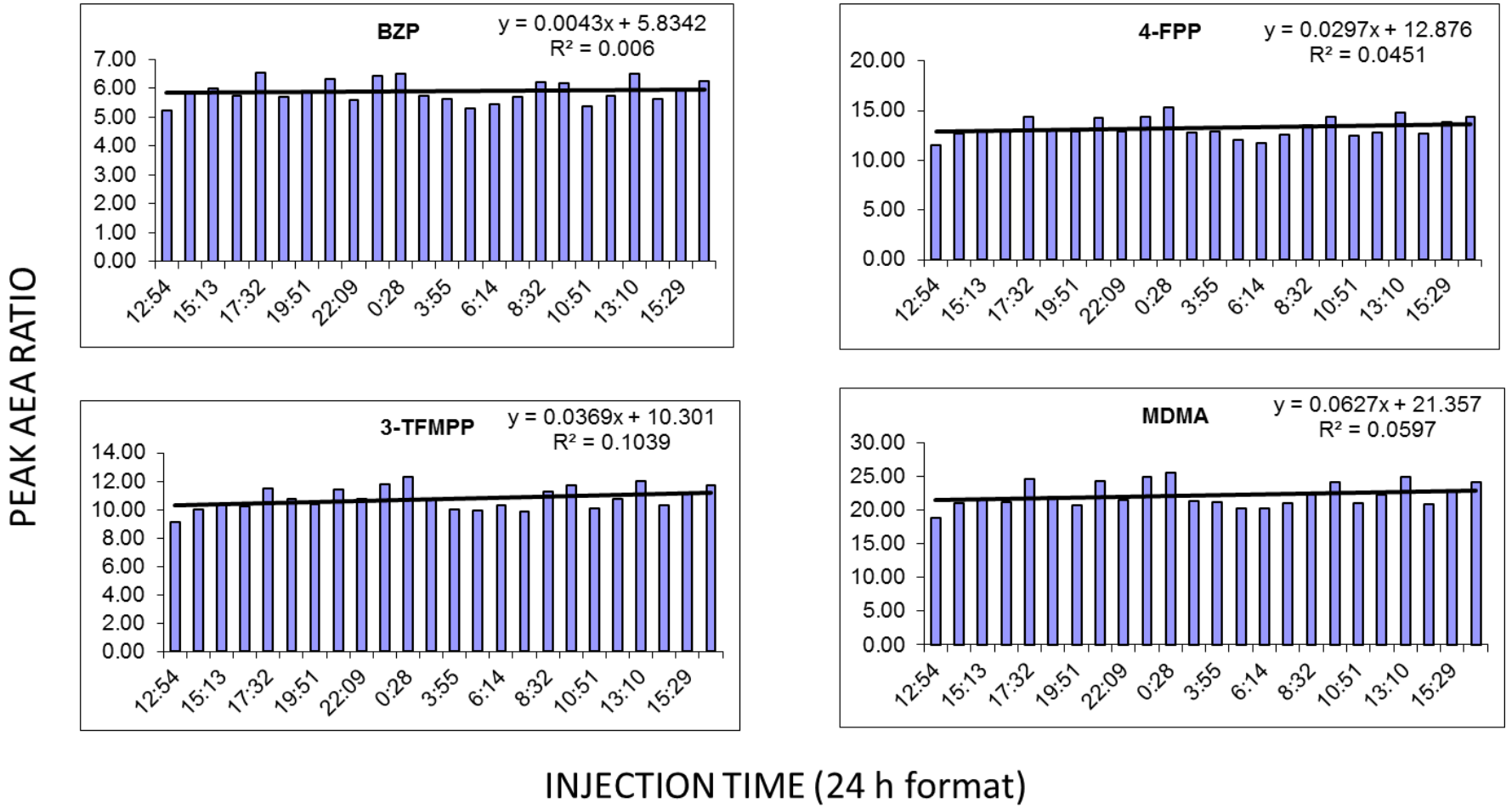
APPENDIX VII-a: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.



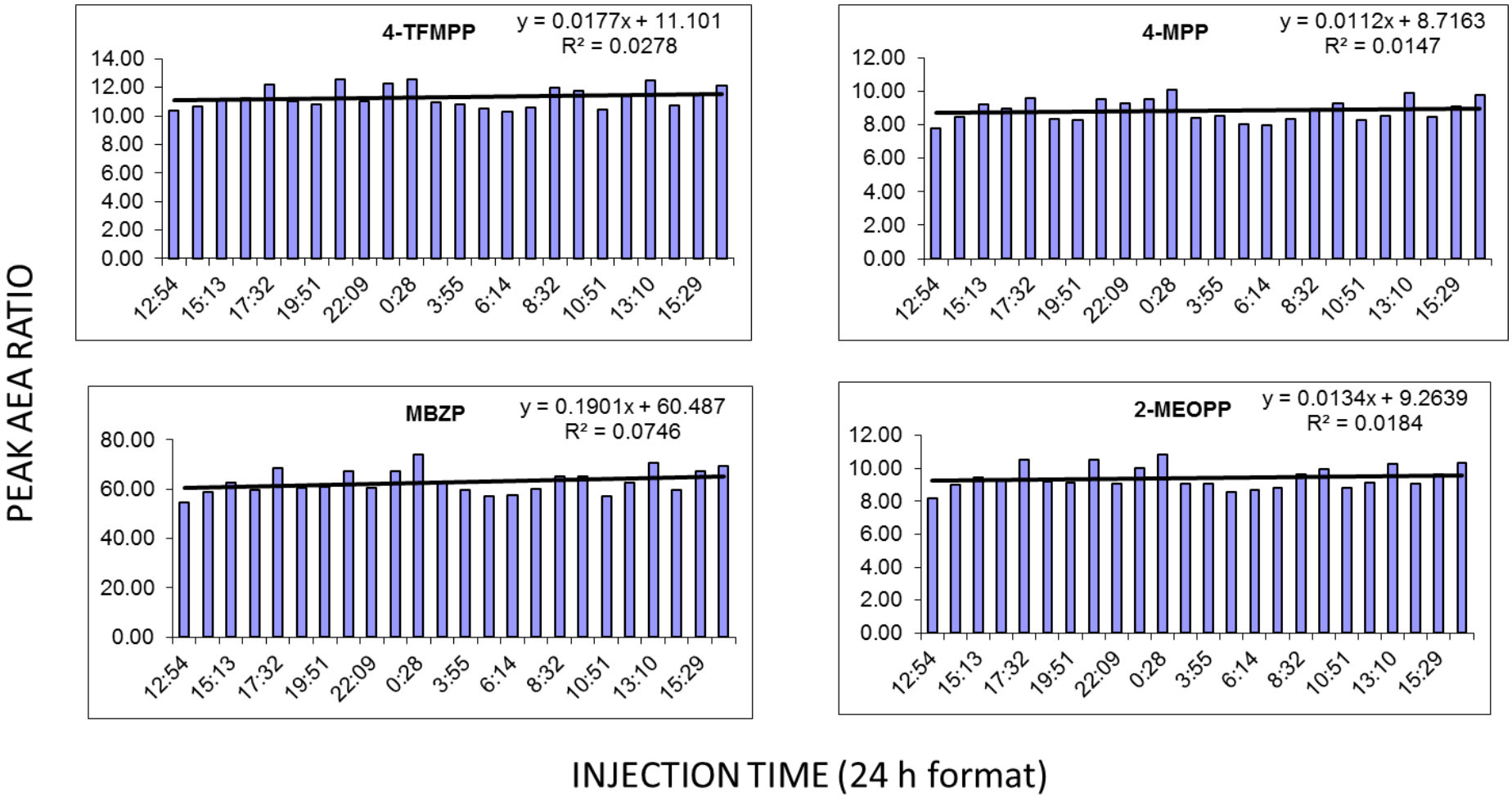
APPENDIX VII-b: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.



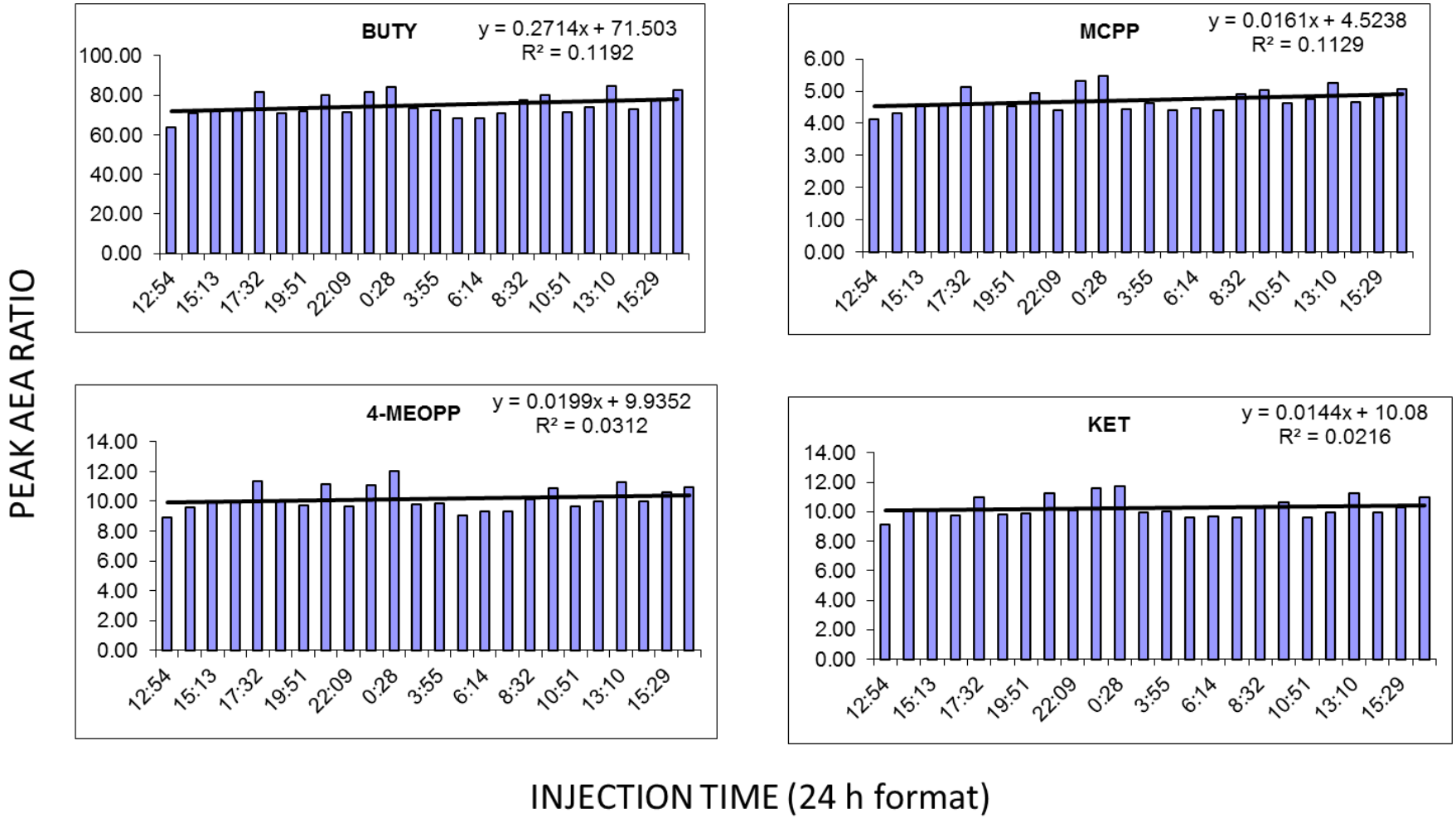
APPENDIX VII-c: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation=24.



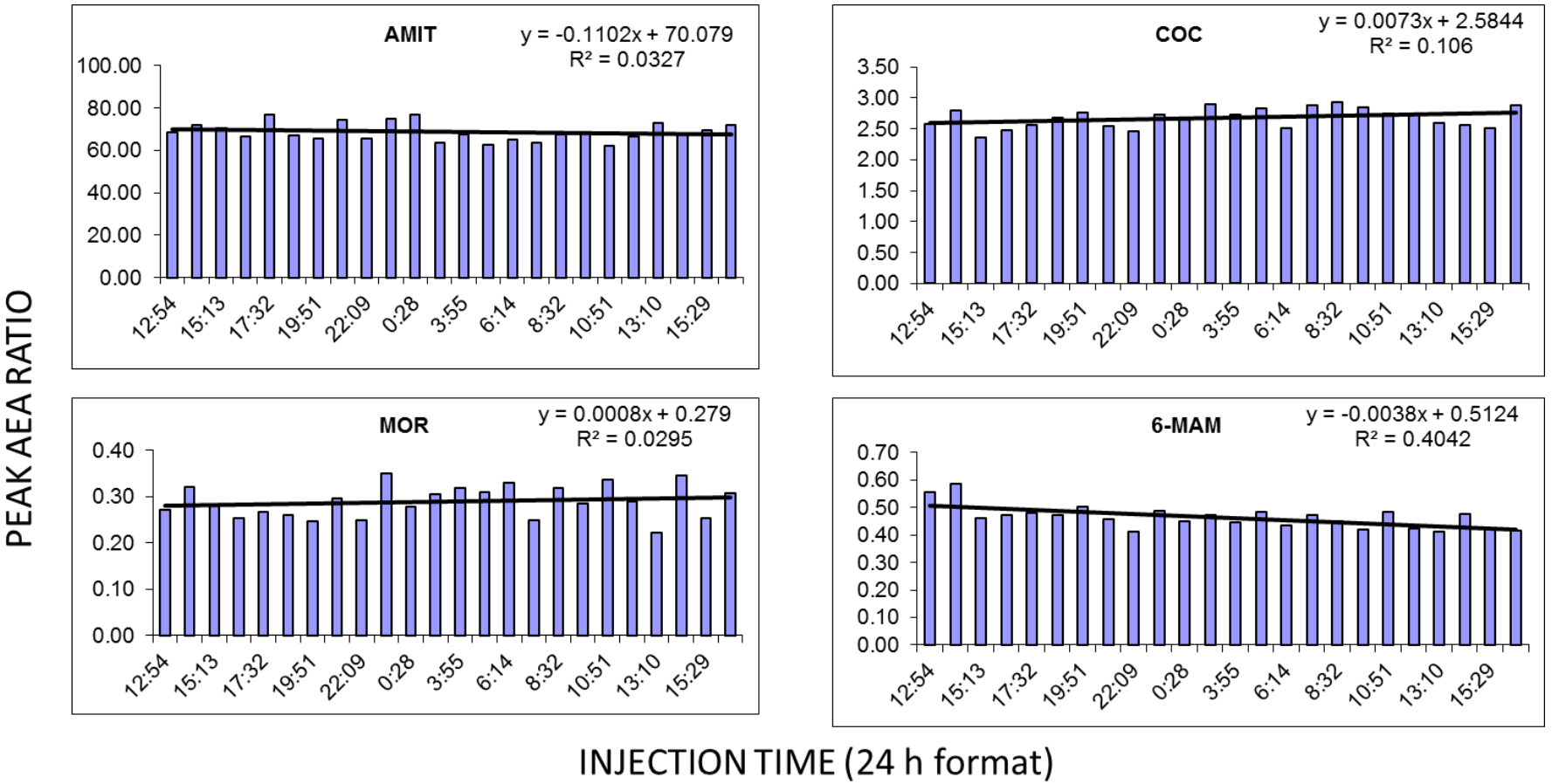
APPENDIX VII-d: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.



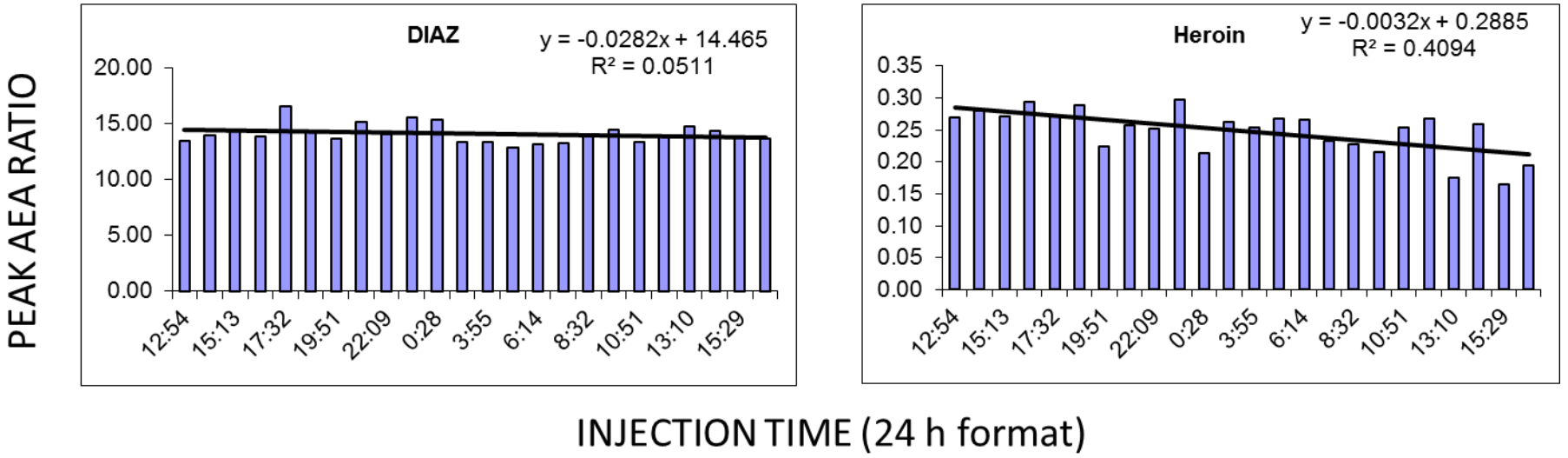
APPENDIX VII-e: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.



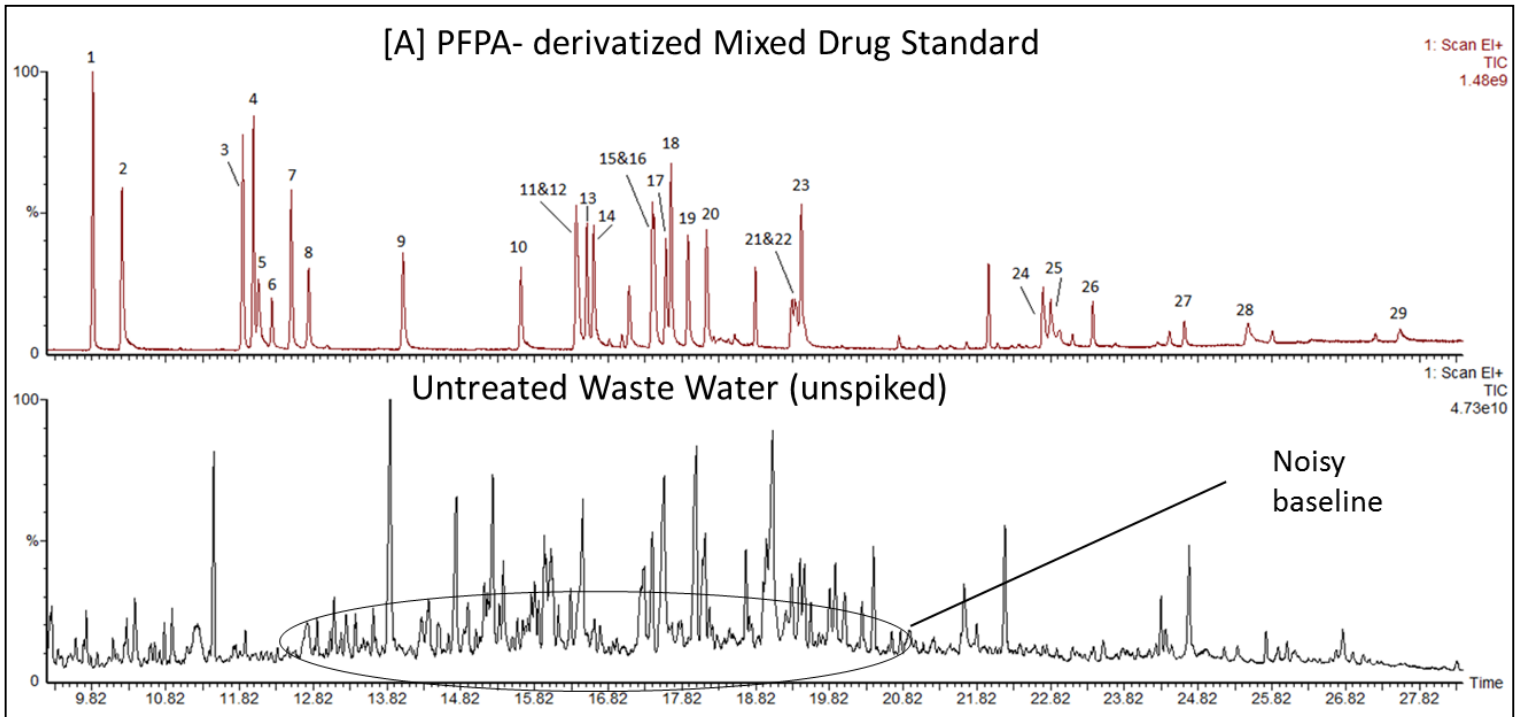
APPENDIX VII-f: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.



APPENDIX VII-g: Graphs of PAR versus injection time for 27 h autosampler stability study, including R^2 and regression equation, n=24.

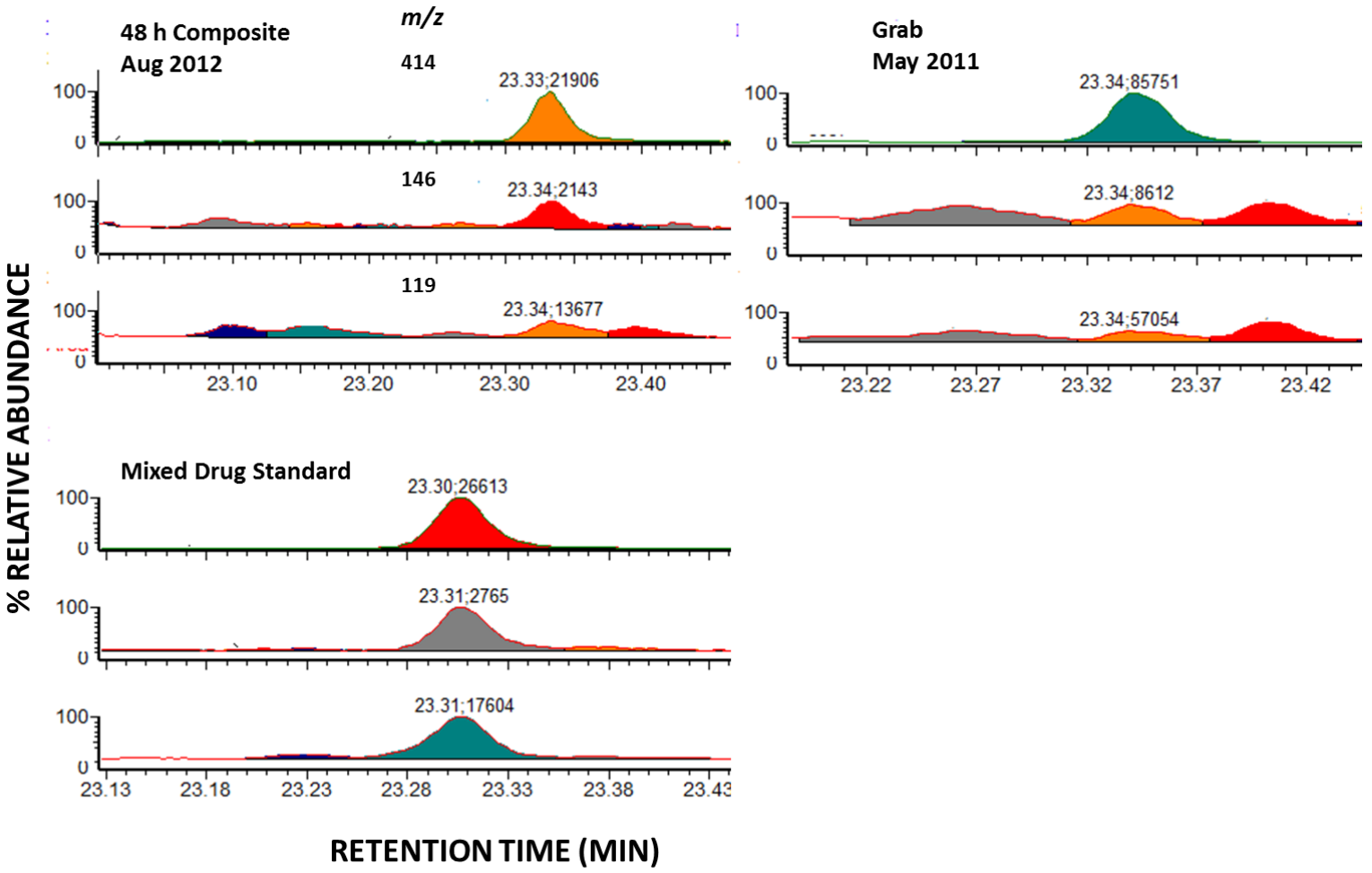


APPENDIX VIII: Comparison of chromatograms of a mixed drug standard and untreated waste water sample.



[A] (1) Piperazine, (2) Amphetamine (3) Methamphetamine, (4) 4-Fluoromethamphetamine, (5) Cathinone (6) 3-Fluoromethcathinone, (7) Methcathinone (8) Ecgonine methylester, (9) Mephedrone,(10) 2-(Fluorophenyl)piperazine, (11&12) 1-Benzylpiperazine & 4-(Fluorophenyl)piperazine, (13) 1-(3-Trifluoromethylphenyl) piperazine, (14) 3,4-Methylenedioxymethamphetamine, (15&16) Methylbenzodioxolylbutanamine & 1-(4-Trifluoromethylphenyl) piperazine, (17) 1-(4-Methylphenyl)piperazine, (18) 1-Methyl-4-benzylpiperazine, (19) 1-(2-Methoxyphenyl) piperazine (20) Butylone, (21&22) 1-(3-Chlorophenyl) piperazine & 1-(4-Methoxyphenyl) piperazine, (23) Ketamine, (24) Amitriptyline,(25) Cocaine (26) Morphine, (27) Diazepam, (28) 6-Monoacetylmorphine (29) Heroin.

APPENDIX IX-a: Detection of morphine in different untreated waste water samples.



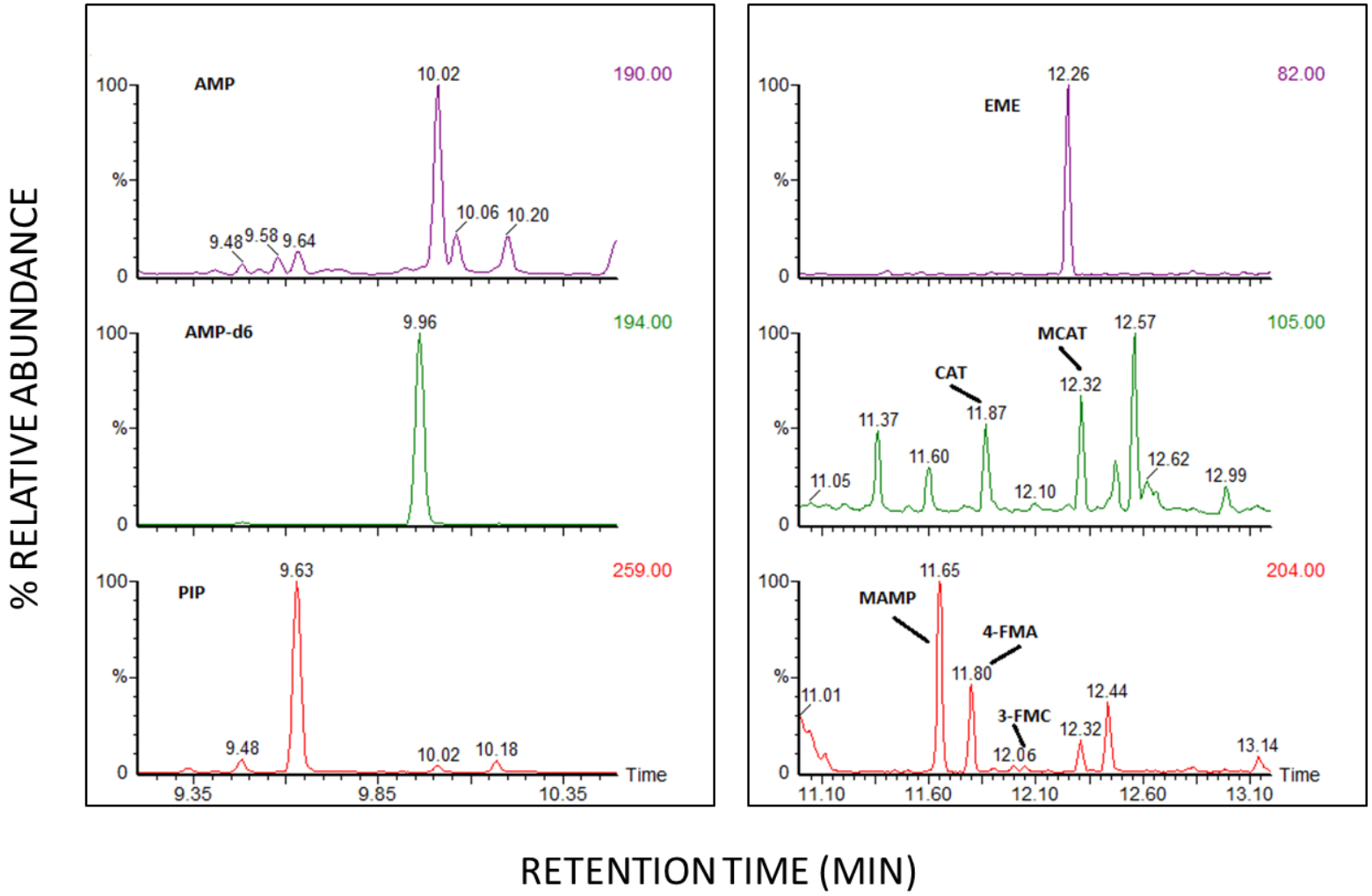
APPENDIX IX-b: Ion ratios for confirmation of morphine in different untreated waste water samples.

Peak Area				Ion Ratios					
								% Difference[1]	
m/z	48 h	Grab	Drug Std		48 h	Grab	Drug Std	48 h/Drug Std	Grab/Drug Std
414, Q	21906	85751	26613	C1/Q	0.10	0.10	0.10	6	3
146, C1	2143	8612	2765	C2/Q	0.62	0.67	0.66	6	-1
119, C2	13677	57054	17604	C1/C2	0.16	0.15	0.16	0	4

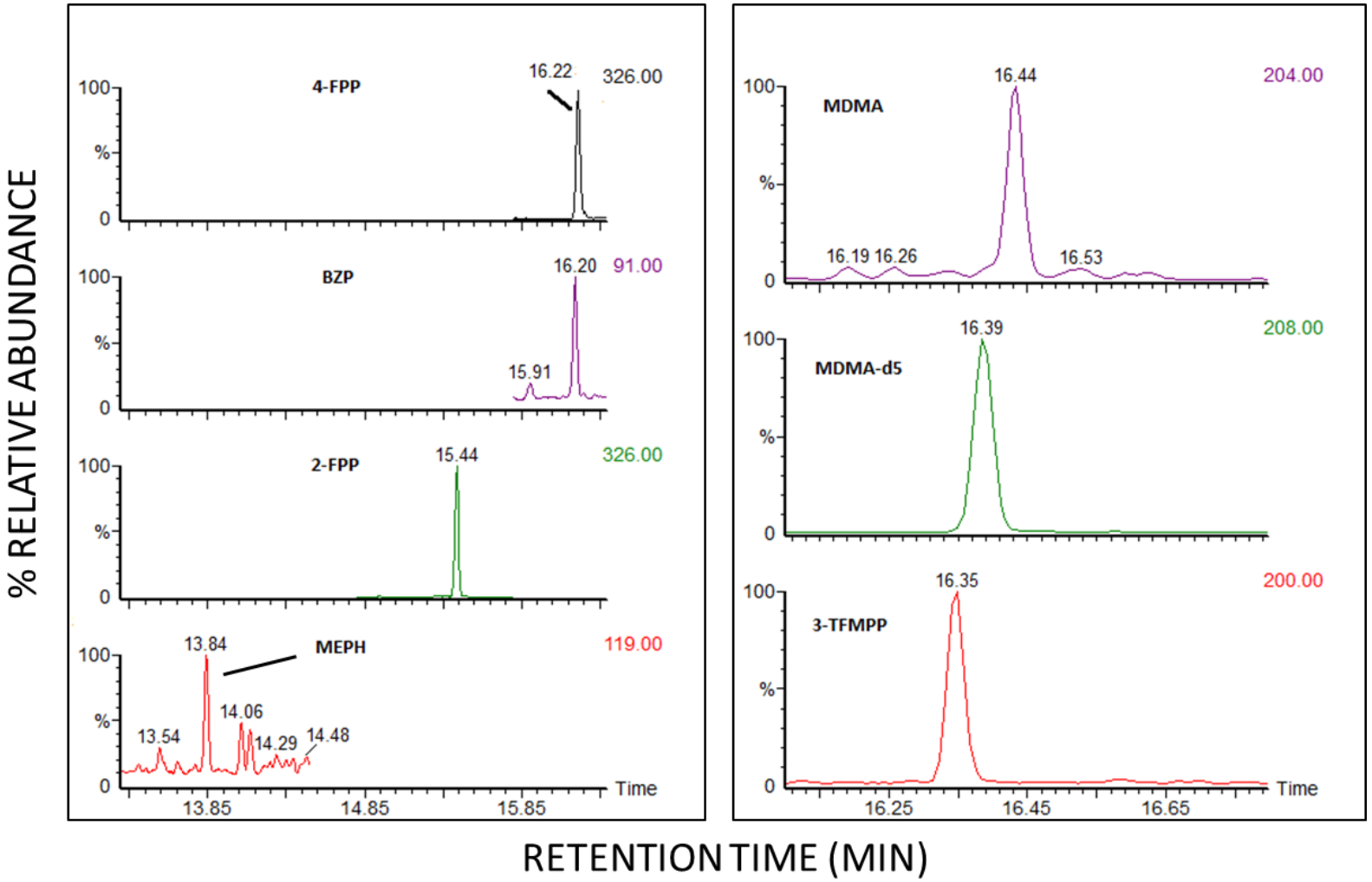
Q=quantifier ion, C=confirmation ion, Std=standard

[1]Based on the commonly used acceptance criteria for ion ratios of ± 20 % relative to that of the corresponding drug standard (Cooper, et al., 2010).

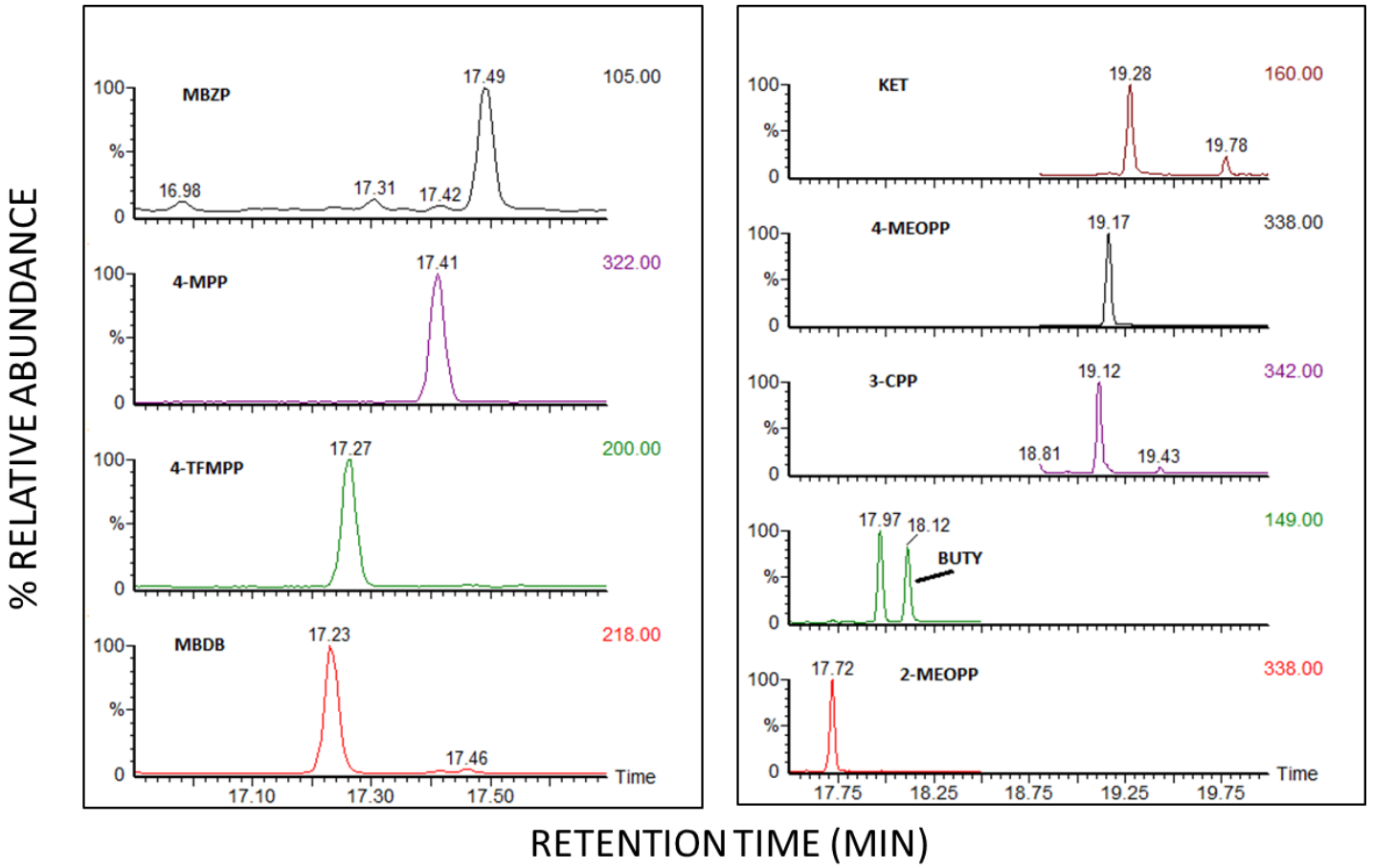
APPENDIX X-a: SIM spectra of the quantifier ions for the target drugs.



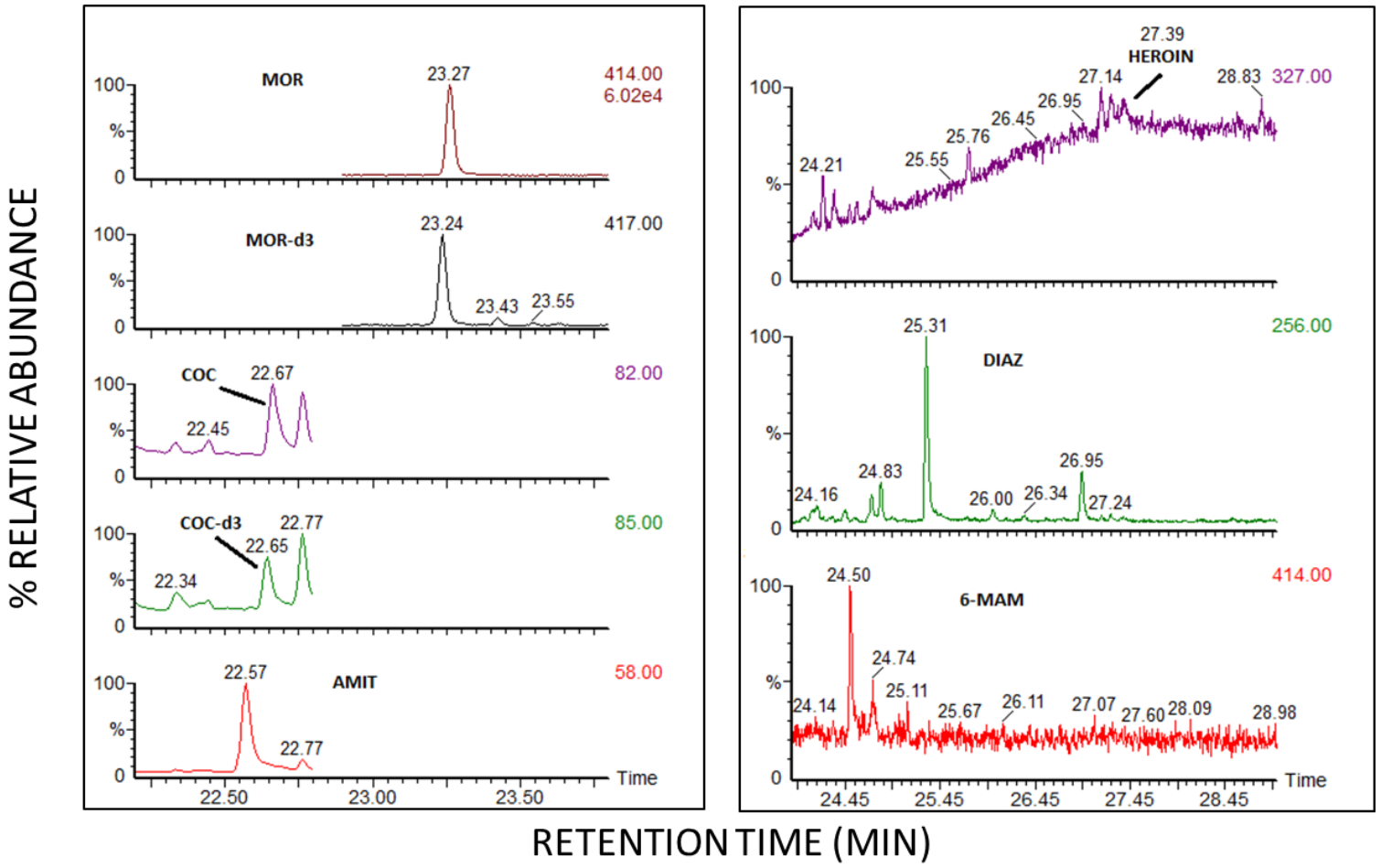
APPENDIX X-b: SIM spectra of the quantifier ions for the target drugs.



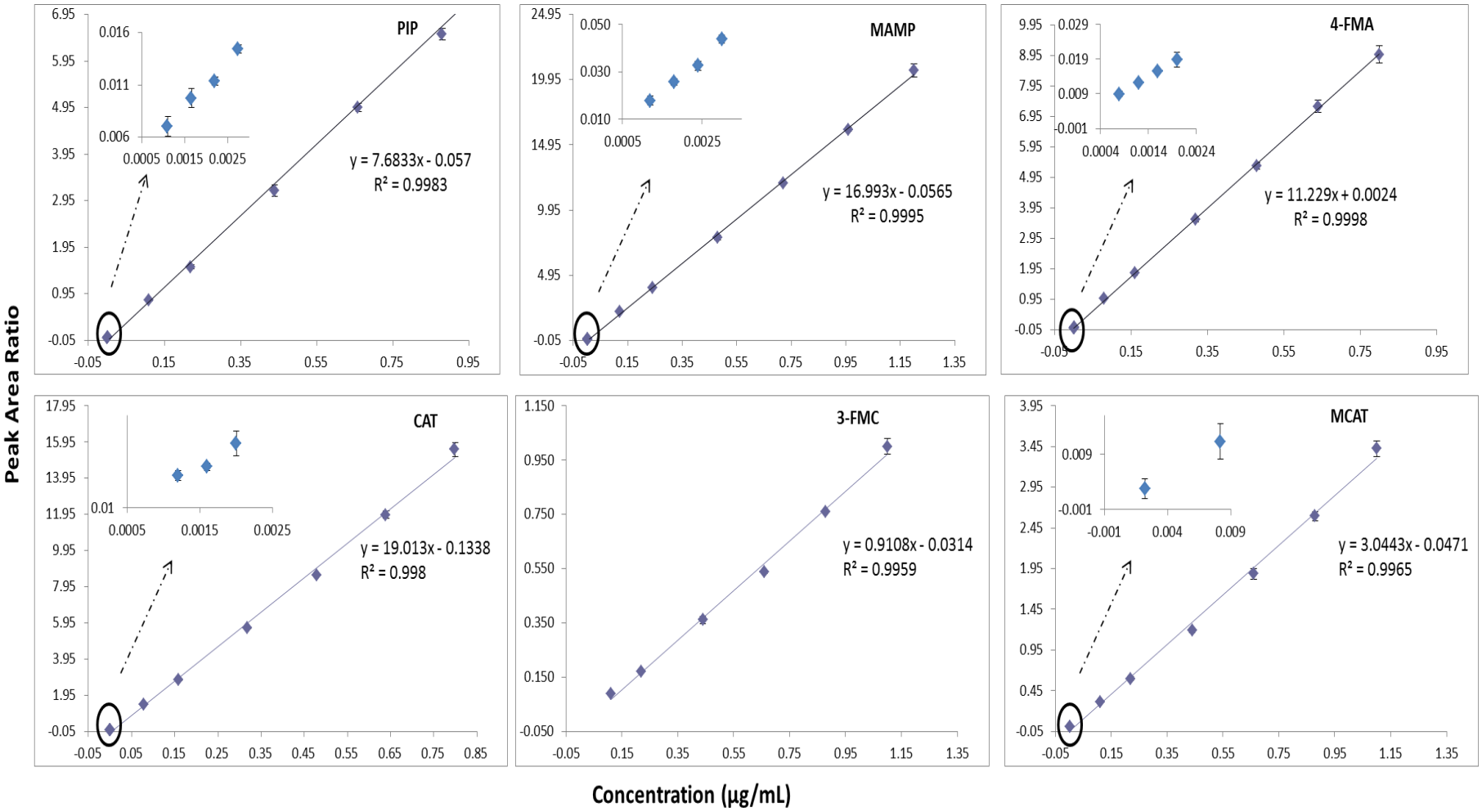
APPENDIX X-c: SIM spectra of the quantifier ions for the target drugs.



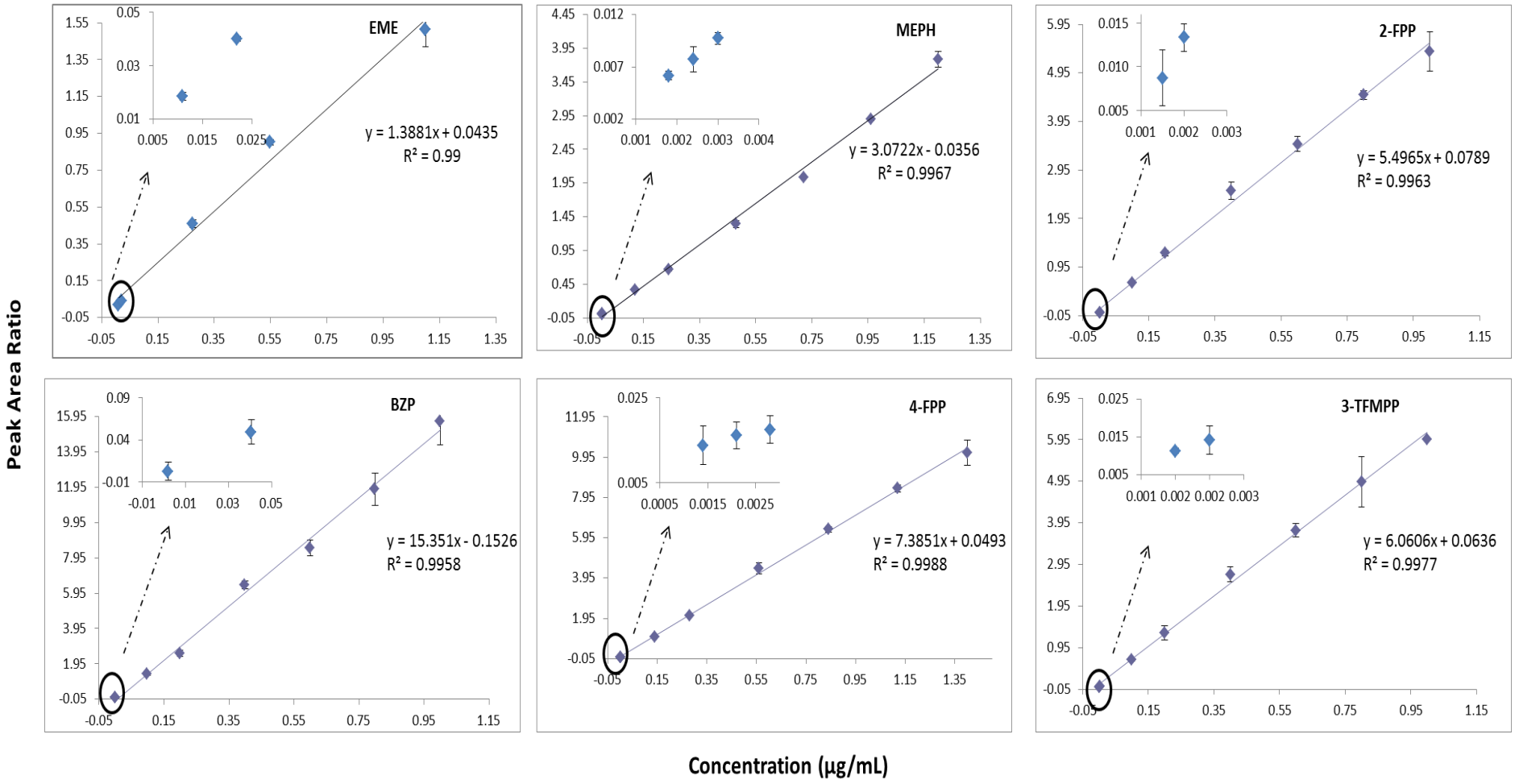
APPENDIX X-d: SIM spectra of the quantifier ions for the target drugs.



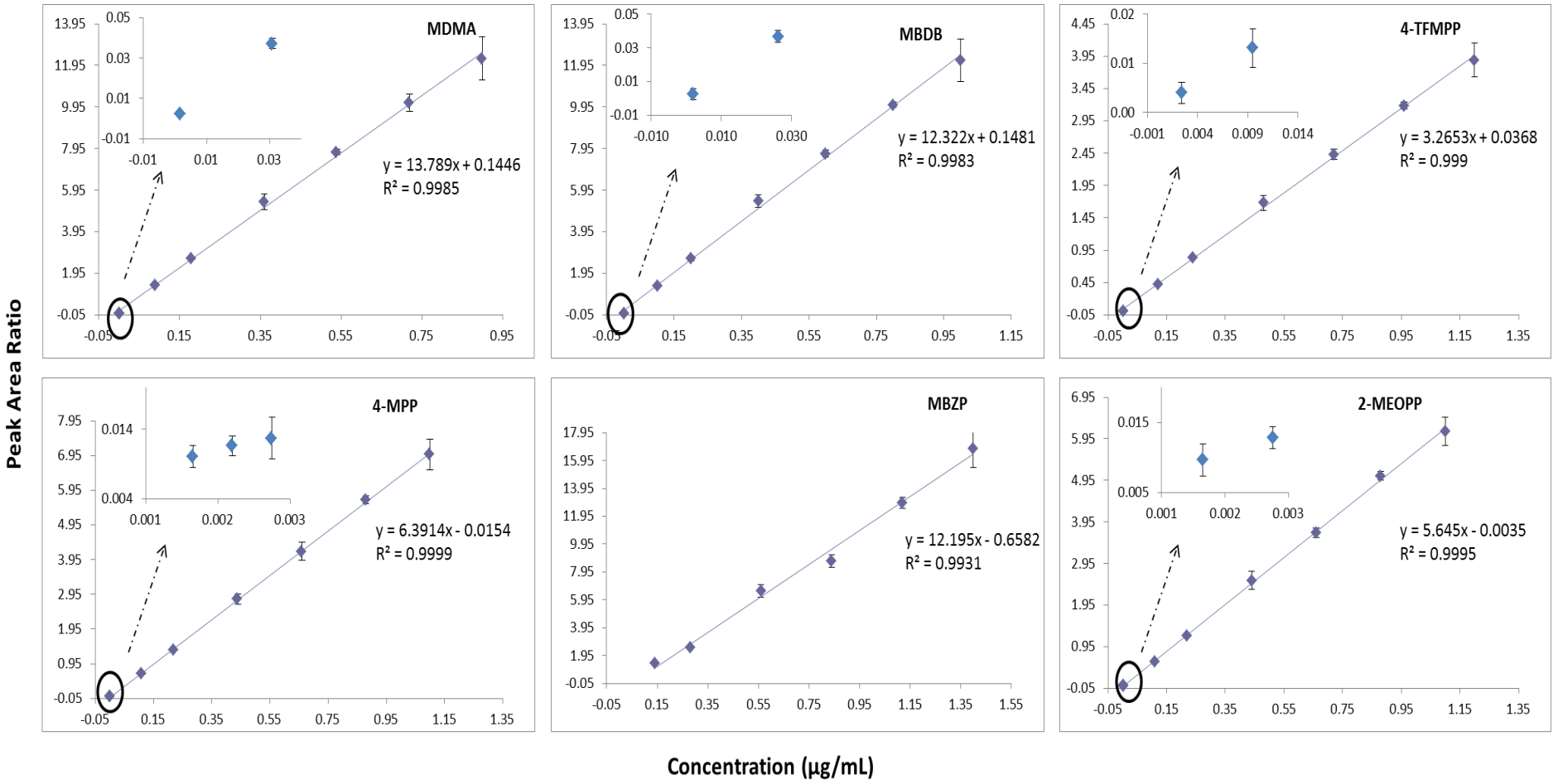
APPENDIX XI-a: Linear regression plots for determination of linear range, n = 3.



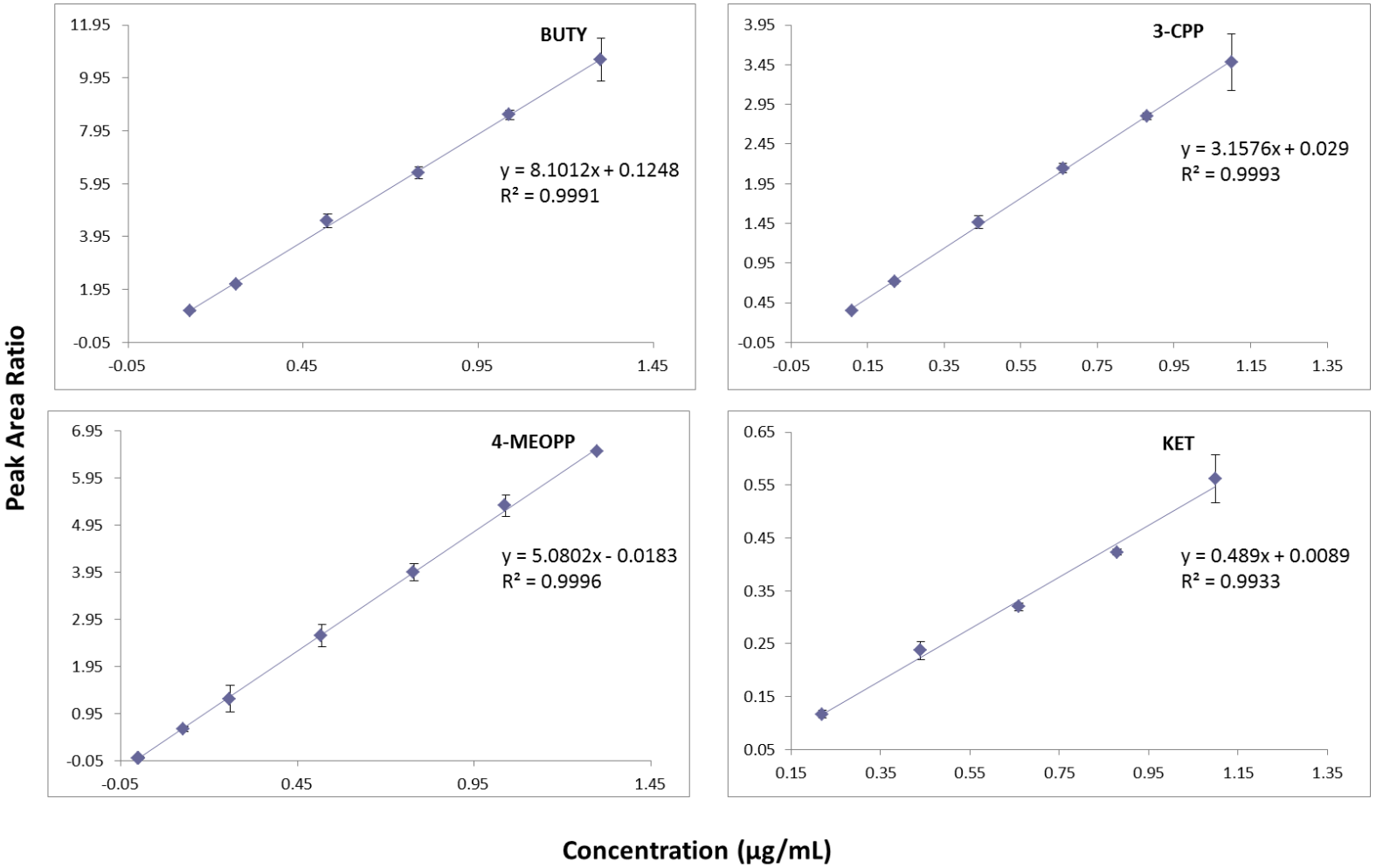
APPENDIX XI-b: Linear regression plots for determination of linear range, n = 3.



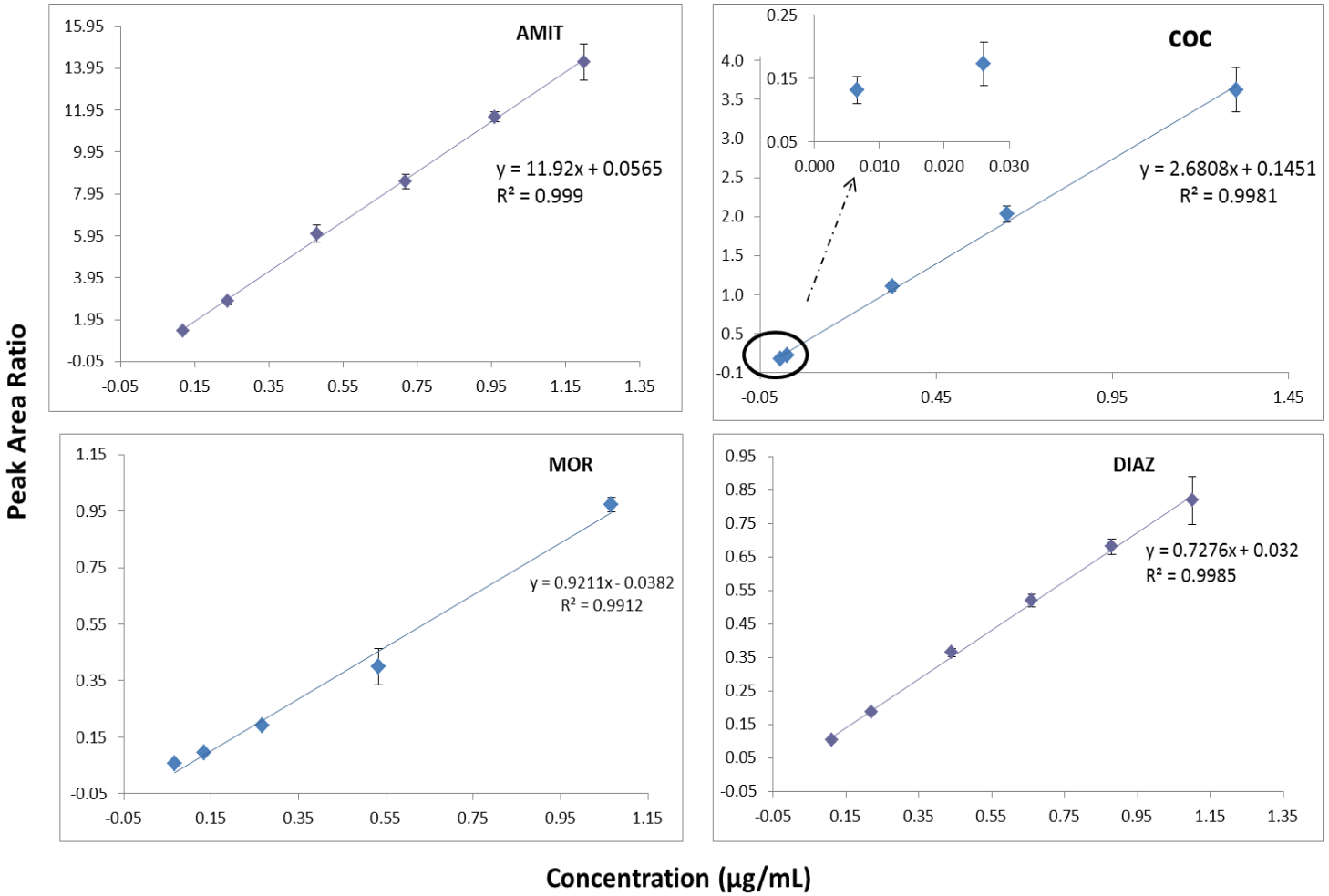
APPENDIX XI-c: Linear regression plots for determination of linear range, n = 3.



APPENDIX XI-d: Linear regression plots for determination of linear range, n = 3.



APPENDIX XI-e: Linear regression plots for determination of linear range, n = 3.

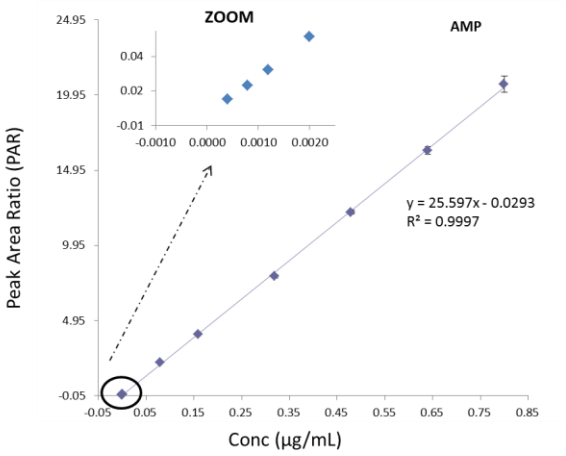


APPENDIX XII: Establishment of linearity for amphetamine

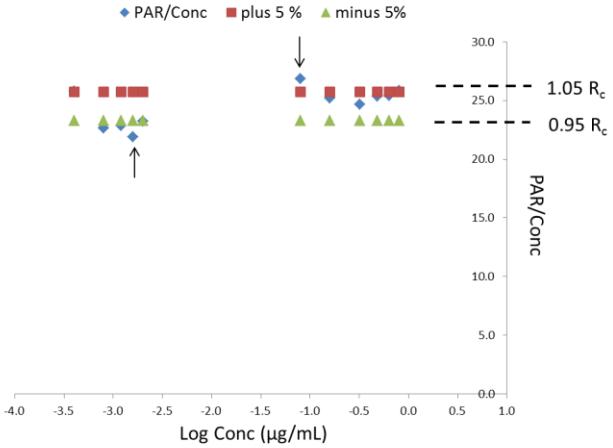
Table of concentration and response for amphetamine

Conc	PAR	log Conc	PAR/Conc
0.0004	0.010	-3.40	25.77
0.0008	0.018	-3.10	22.65
0.0012	0.027	-2.92	22.87
0.0016	0.035	-2.80	21.88
0.0020	0.046	-2.70	23.23
0.0800	2.148	-1.10	26.85
0.1600	4.034	-0.80	25.22
0.3200	7.885	-0.49	24.64
0.4800	12.162	-0.32	25.34
0.6400	16.267	-0.19	25.42
0.8000	20.678	-0.10	25.85

PAR/Conc	
Average	24.52
Average - 5 %	23.29
Average + 5 %	25.75

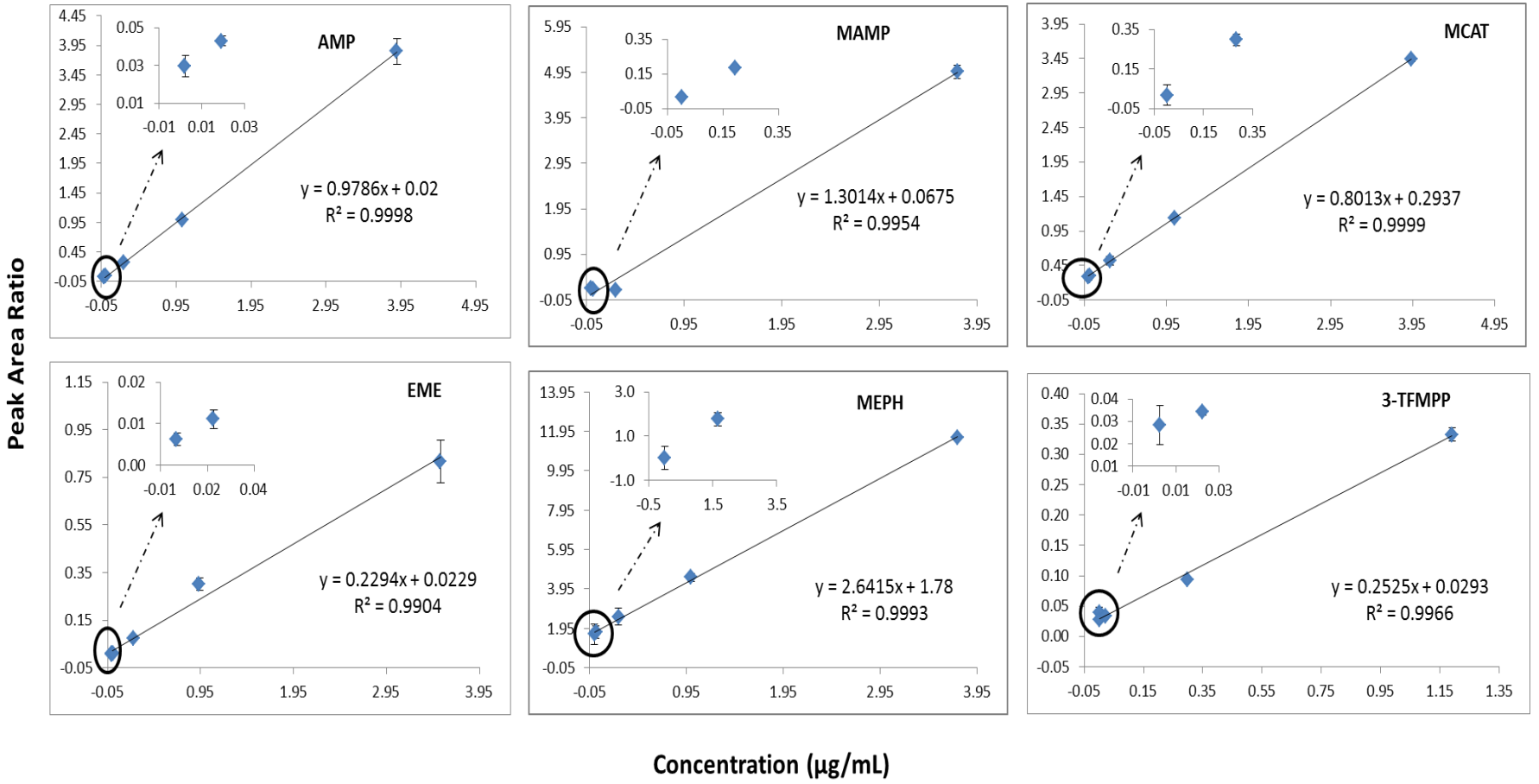


Linear regression plot of concentration vs relative response for amphetamine

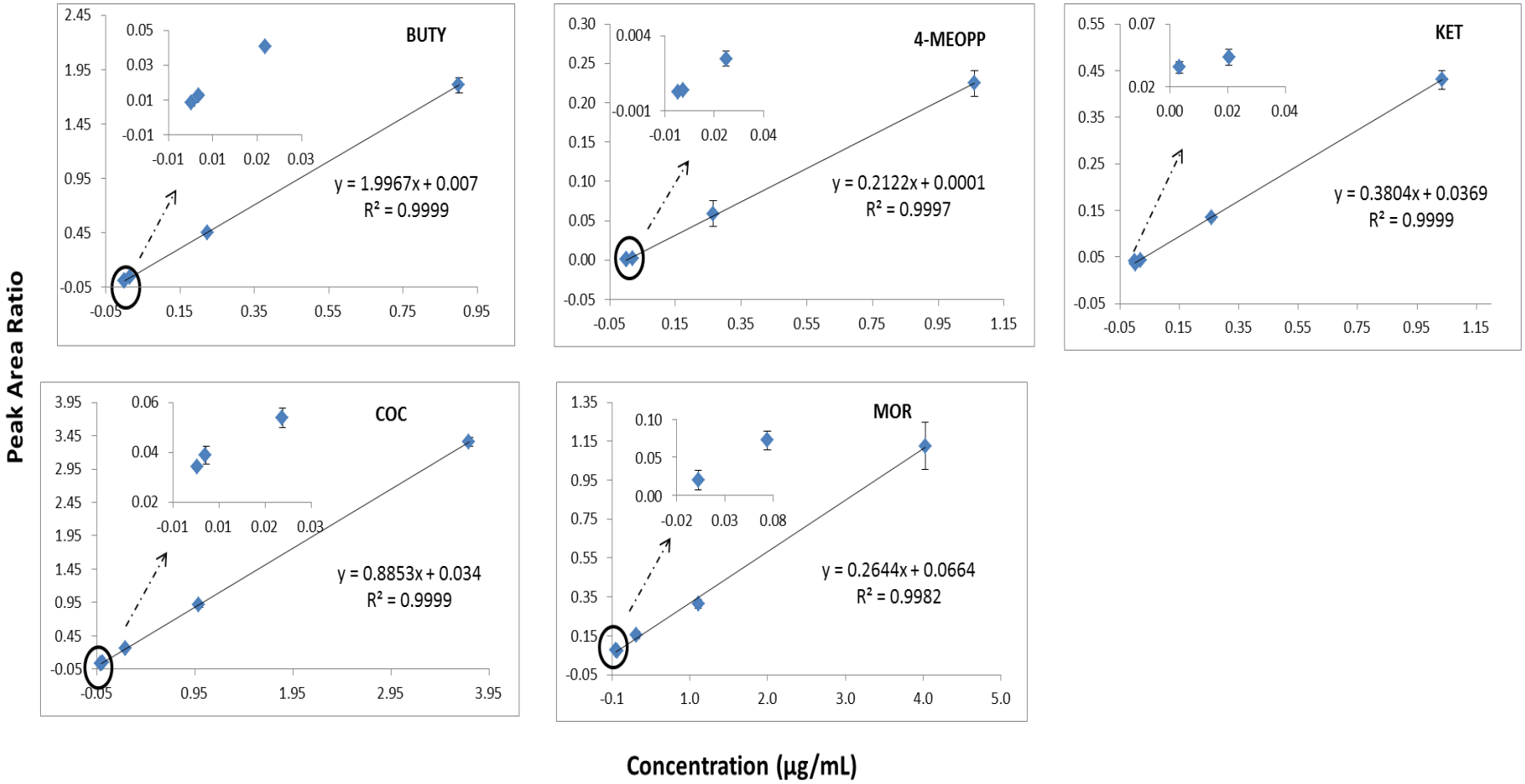


Plot of log of concentration vs relative response/concentration for amphetamine. Arrows indicate outliers outside the 95 -105 % limit. Rc = line of constant response.

APPENDIX XIII-a: Standard addition plots of detected drugs, n = 3.



APPENDIX XIII-b: Standard addition plots of detected drugs, n = 3.

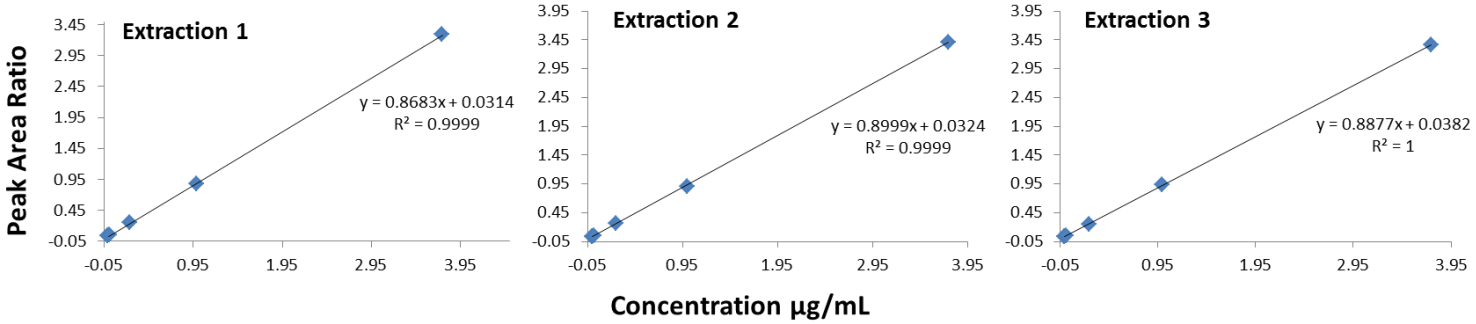


APPENDIX XIV-a: Calculation of the concentration of cocaine using standard addition.

Table of concentration , peak area and peak area ratio for cocaine, n = 3

Extraction 1				**Extraction 2**			**Extraction 3**		
Conc	Peak Area		PAR	Peak Area		PAR	Peak Area		PAR
	COC	COC-d3		COC	COC-d3		COC	COC-d3	
0.0003	4446	132613	0.034	3953	113508	0.035	4072	119759	0.034
0.0022	4085	110542	0.037	4731	110515	0.043	3895	106777	0.036
0.0188	5198	96101	0.054	6146	106285	0.058	5663	113313	0.050
0.2500	31540	125906	0.251	32002	122524	0.261	30045	115830	0.259
1.0000	88259	100171	0.881	83464	92602	0.901	88920	94145	0.945
3.7500	390633	118655	3.292	418207	122460	3.415	425361	126515	3.362

Conc = concentration; COC = cocaine; PAR = peak area ratio



Graphs of peak area ratio vs concentration for cocaine, n = 3

APPENDIX XIV-b: Calculation of the concentration of cocaine (COC) using standard addition.

	Gradient (m)	Intercept (c)	Conc of COC in waste water (x)	
Extraction 1	0.8683	0.0314	0.036	
Extraction 2	0.8999	0.0324	0.036	
Extraction 3	0.8877	0.0382	0.043	
			0.038	Average Conc
			0.005	Std Dev
			10.50	% RSD

The concentration of cocaine in waste water was calculated using the linear equation for standard addition, $y = mx + c$, where:

$y = 0$; m = gradient; c = intercept; x = Conc of COC in waste water

For standard addition, when $y = 0$ (i.e. no drug standard added) the positive intercept is due to the response of the analytes already present in the sample.

APPENDIX XV: Mass spectra of caffeine in drug standard, waste water sample and NIST [Version 2.0(2)] database.