THE ANALYSIS AND DETECTION OF NEW PSYCHOACTIVE SUBSTANCES IN BIOLOGICAL MATRICES

Thesis Submitted in Accordance with the Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

By

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Dedicated to Gran and Gramps

Sadly Gramps was only able to witness the start of this great journey and never saw my return from America. I have little doubt however that if he had witnessed this achievement he would be howling with laughter, smiling from ear to ear and jokingly questioning if I really am his granddaughter. The answer is yes.

And yes Gran …… it was a lot of hard work!

Acknowledgements

So many people have played a vital part in this work, supporting me and guiding me along the way, and in writing these acknowledgements it has dawned on me how privileged I have been to have met so many wonderful people.

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Abstract

New psychoactive substances (NPSs) have appeared on the recreational drug market at an unprecedented rate in recent years. Many are not new drugs but failed products of the pharmaceutical industry. The speed and variety of drugs entering the market poses a new complex challenge for the forensic toxicology community. The detection of these substances in biological matrices can be difficult as the exact compounds of interest may not be known. Many NPS are sold under the same brand name and therefore users themselves may not know what substances they have ingested.

The majority of analytical methods for the detection of NPSs tend to focus on a specific class of compounds rather than a wide variety. In response to this, a robust and sensitive method was developed for the analysis of various NPS by solid phase extraction (SPE) with gas chromatography mass spectrometry (GC-MS). Sample preparation and derivatisation were optimised testing a range of SPE cartridges and derivatising agents, as well as derivatisation incubation time and temperature. The final gas chromatography mass spectrometry method was validated in accordance with SWGTOX 2013 guidelines over a wide concentration range for both blood and urine for 23 and 25 analytes respectively. This included the validation of 8 NBOMe compounds in blood and 10 NBOMe compounds in urine. This GC-MS method was then applied to 8 authentic samples with concentrations compared to those originally identified by NMS laboratories.

The rapid influx of NPSs has resulted in the re-analysis of samples and thus, the stability of these substances is crucial information. The stability of mephedrone was investigated, examining the effect that storage temperatures and preservatives had on analyte stability daily for 1 week and then weekly for 10 weeks.

Several laboratories identified NPSs use through the cross-reactivity of these substances with existing screening protocols such as ELISA. The application of Immunalysis ketamine, methamphetamine and amphetamine ELISA kits for the detection of NPS was evaluated. The aim of this work was to determine if any cross-reactivity from NPS substances was observed, and to determine whether these existing kits would identify NPS use within biological samples. The cross-

reactivity of methoxetamine, 3-MeO-PCE and 3-MeO-PCP for different commercially point of care test (POCT) was also assessed for urine.

One of the newest groups of compounds to appear on the NPS market is the NBOMe series. These drugs pose a serious threat to public health due to their high potency, with fatalities already reported in the literature. These compounds are falsely marketed as LSD which increases the chance of adverse effects due to the potency differences between these 2 substances. A liquid chromatography tandem mass spectrometry (LC-MS/MS) method was validated in accordance with SWGTOX 2013 guidelines for the detection for 25B, 25C and 25I-NBOMe in urine and hair.

Long-Evans rats were administered 25B-, 25C- and 25I-NBOMe at doses ranging from 30-300 µg/kg over a period of 10 days. Tail flick tests were then carried out on the rats in order to determine whether any analgesic effects were observed as a result of dosing. Rats were also shaved prior to their first dose and re-shaved after the 10-day period. Hair was separated by colour (black and white) and analysed using the validated LC-MS/MS method, assessing the impact hair colour has on the incorporation of these drugs. Urine was collected from the rats, analysed using the validated LC-MS/MS method and screened for potential metabolites using both LC-MS/MS and quadrupole time of flight (QToF) instrumentation.

List of Abbreviations

%CV	Coefficient of variation percentage
µg	microgram
µl	Microliter
µm	Micrometre
^{0}C	Degrees Celsius
25B-NBOMe	4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine
25C-NBOMe	2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
25D-NBOMe	2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine
25E-NBOMe	2-(4-ethyl-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethan-1-amine
25H-NBOMe	2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
25I-NBOMe	4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine
25N-NBOMe	2-(2,5-dimethoxy-4-nitrophenyl)-N-(2-methoxybenzyl)ethanamine
25P-NBOMe	2-(4-Propyl-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]
25T2-NBOMe	2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-(methylthio)-benzeneethanamine
25T4-NBOMe	2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-[(1-methylethyl)thio]-benzeneethanamine
25T7-NBOMe	2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-4-(propylthio)-benzeneethanamine
2-DPMP	2-Desoxypipradol
3-MeO-PCE	3-Methoxyeticyclidine
3-MeO-PCP	3-Methoxyphencyclidine
5-APB	5-(2-aminopropyl)benzofuran
5HT	5-hydroxytryptamine
5-IAI	5-Iodo-2-aminoindane
6-APB	6-(2-aminopropyl)benzofuran
A&E	Accident and Emergency
AAPCC	American Association of Poison Control Centres
ADHD	Attention Deficit Hyperactivity Disorder
CH_2Cl_2	Dichloromethane
CI	Chemical ionisation
CO_2	Carbon dioxide
Conc.	Concentration
CYP	Cytochrome
DC	Direct current
DFC	Drug facilitated crime

DFSA	Drug Facilitate Sexual Assault
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
EI	Electron ionisation
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ENU	Europol National Units
ESI	Electrospray ionisation
EtOAc	Ethylacetate
EU	European Union
EUROPOL	European Police Office
EWS	Early warning system
FMS	Forensic Medicine & Science
FRFRF	Fredric Rieders Family Renaissance Foundation
g	Gram
GC-MS	Gas chromatography mass spectrometry
HCOOH	Formic Acid
i.d.	Internal diameter
I.S.	Internal standard
IEC	International Electrotechnical Commission
IP	Intraperitoneal
IPA	Isopropanol
ISO	International Organisation for standardisation
IV	Intravenous
kg	Kilogram
kV	Kilovolt
L	Litre
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LLE	Liqiud liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylaminde
M	molar
m	Metre
m/z	Mass to charge ratio
MDAI	5,6-Methylenedioxy-2-aminoindane
MDMA	3,4-methylenedioxy-methamphetamine
MDPV	Methylenedioxypyrovalerone
MeOH	Methanol
Mescaline-NBOMe	3,4,5-trimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine
mg	Milligram
min	Minute

mL	Millilitre
mm	Millimetre
MoDA	Misuse of Drugs Act
MRM	Multiple reaction monitoring
MSTFA	Methyl-N-(trimethylsilyl) trifluoroacetamide
MXE	Methoxetamine
Na_2HPO_4	Sodium phosphate dibasic anhydrous
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic monohydrate
NaOH	Sodium hydroxide
NFP	National Focal Points
ng	Nanogram
$NH_4CH_3CO_2$	Ammonium acetate
NH_4OH	Ammonium hydroxide
NMDA	N-methyl-D-aspartate
NPS	New psychoactive substances
OSAC	Organisation of Scientific Area Committee
PAR	Peak area ratio
PBS	Phosphate buffered saline
PCP	Phencyclidine
PFPA	Pentafluoropropionic anhydride
pg	Picogram
POCT	Point of case testing
psi	Pound per square inch
Q	Qualifier
QC	Quality control
QC	Quality Control
QToF	Quadrupole time of flight
R^2	Correlation coefficient
ReiTox	Réseau Européend´Information sur les Drogues et les Toxicomanies
ROI	Republic of Ireland
rpm	Revolutions per minute
S/N	Signal to noise ratio
SIM	Selected ion monitoring
SLE	Supported liquid extraction
SMART	Synthetic Monitoring: Analyses, Reporting and Trend programme
SoHT	Society of Hair Testing
SPE	Solid phase extraction
Std Dev	Standard Deviation
SWGTOX	Scientific Working Group of Forensic Toxicology
TFAA	Trifluroracetic anhydride
TFMPP	3-Trifluoromethylphenylpiperazine
TGT	Target
THC	Tetrahydrocannabinol
TIC	Total ion chromatogram

TMSI	N-trimethylsilylimidazole
t-MTBE	Methyl tert-butyl ether
Tris	Tris(hydroxymethyl)aminomethane
UK	United Kingdom
UKAS	UK Accreditation Service
UN	United Nations
UNODC	United Office On Drug and Crime
UPLC	Ultra-performance liquid chromatography
USA	United States of America
V	Volt

Chapter 1: New Psychoactive Substances

1.1 Introduction

New psychoactive substances (NPSs) are defined by the United Nations (UN) as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Convention on Narcotic Drugs or the 1971 Convention of Psychotropic Substances, but which may pose a public health threat".(*1*) The majority of these drugs are not new but simply newly abused with many being failed by-products of the pharmaceutical industry such as 2-desoxypipradol (2-DPMP) and methylone.(*2*) Other NPSs are manufactured solely with the intent to provide users with similar effects to those felt when taking already banned substances such as 3,4-methylenedioxy-methamphetamine (MDMA). These NPSs, however, chemically differ sufficiently from their predecessors to avoid control via the Medicines Act or the Misuse of Drugs Act (MoDA).(*3*)

NPSs pose an unprecedented threat by the speed at which they are launched onto the recreational drugs market. In the time taken for a substance to be detected, for reference standards to become available and legislation to be passed, a new compound will have already taken its place. This means that toxicologists and law enforcement agencies are constantly one step behind in this "cat and mouse" chase.(*4*)

The 2013 United Nations Office on Drugs and Crime (UNODC) Global Synthetic Monitoring: Analyses, Reporting and Trend (SMART) programme report titled the "The Challenge of new psychoactive substances" found that 87% of 80 countries and territories had noticed the appearance of NPSs on the recreational drugs market.(*5*) This report also stated that the appearance of NPSs was most prevalent in Europe which accounted for 44% of countries reporting NPS use within the population. This is in part due to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) early warning system (EWS), an information sharing network between European Union (EU) member states. When a NPS is detected in Europe information is sent to the EMCDDA or the European Police Office (EUROPOL). This information is then disseminated throughout Europe via Europol National Units (ENU), ReiTox (Réseau Européend´Information sur les

Drogues et les Toxicomanies), the European Commission and the European Medicines Agency (EMA).(*6*) As a result of this framework EU members are rapidly alerted to NPSs, increasing their chance of detection within each country. The number of NPSs detected by the EMCDDA continues to grow yearly as shown in Figure 1-1.(*7*)

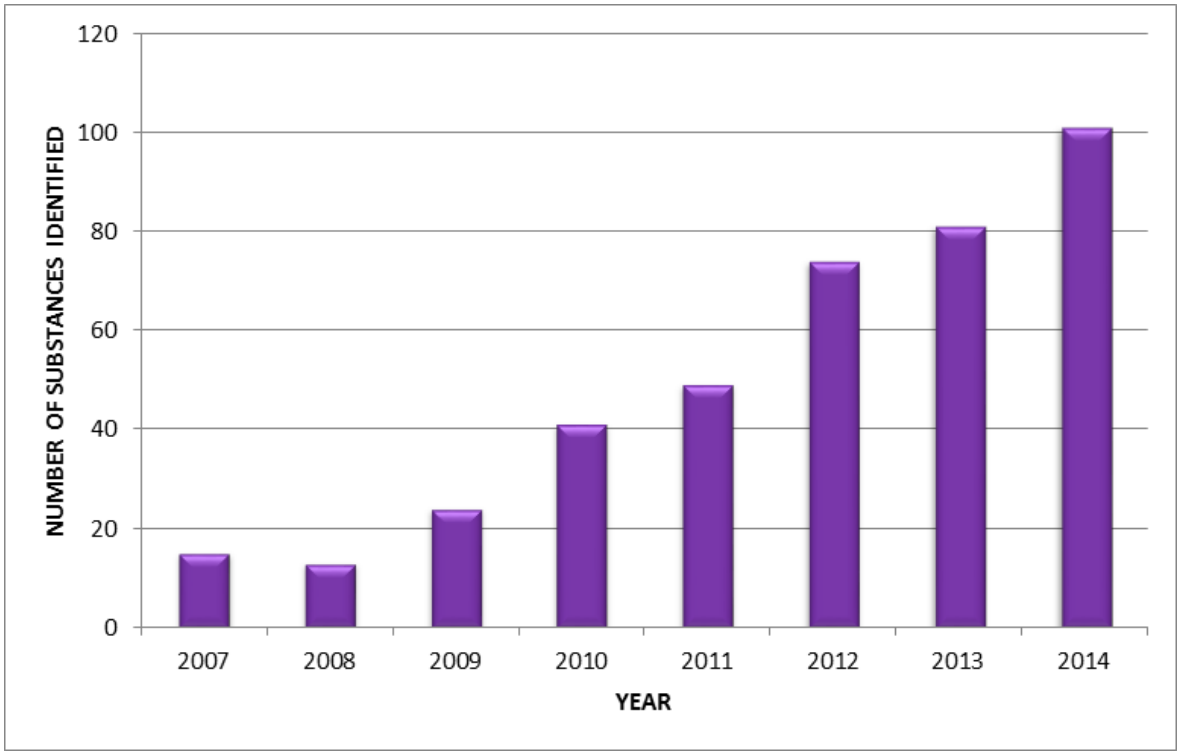


Figure 1-1: Number of NPSs identified by the EMCDDA 2007-2014(*7*)

NPS use has become an integral part of the UK club scene as their effects are similar to those of amphetamines, cocaine and MDMA.(*8, 9*) MDMA and cocaine purities were low in 2009 having reportedly decreased to 22%, whereas mephedrone purities were reported as high as 87%.(*10*) This in itself may be an important factor in the acceleration and decline of these substances respectively in the recreational drug market as shown in Figure 1-2.(*11*) In 2009 a survey of MixMag (a British electronic dance and clubbing magazine) readers found that mephedrone was the sixth most frequently used drug that year, following alcohol, tobacco, cannabis, cocaine and MDMA, again highlighting how quickly this substance infiltrated this demographic.(*12*)

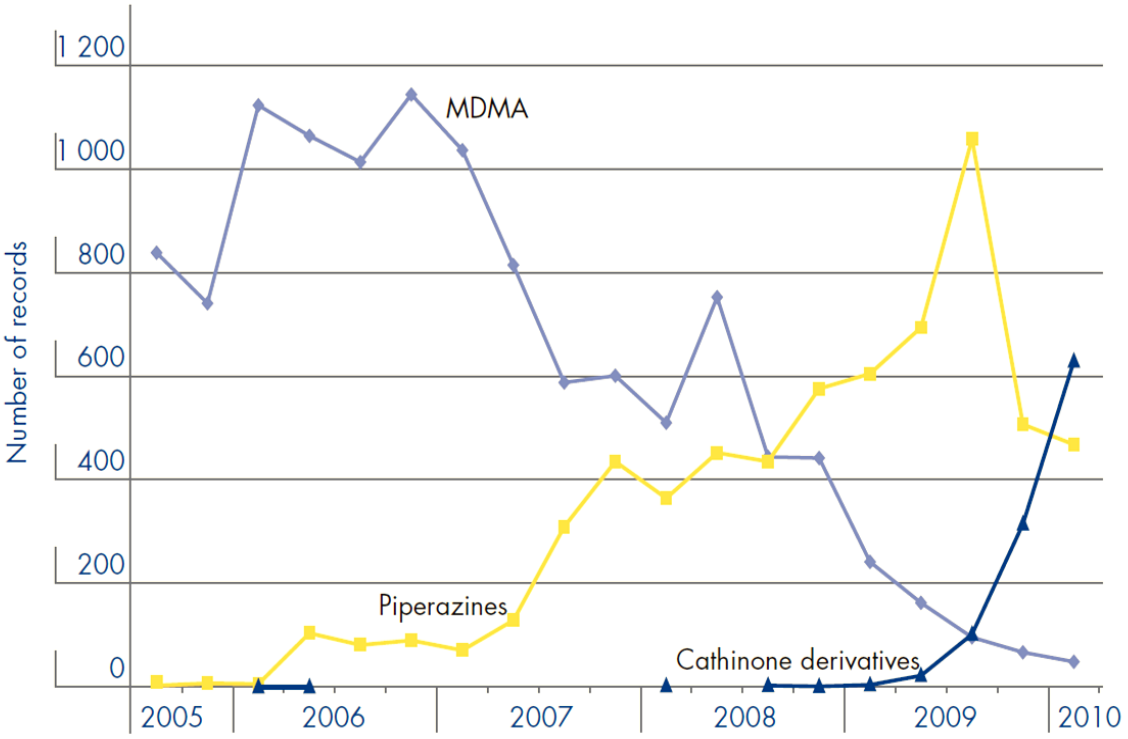


Figure 1-2: MDMA, piperazines and cathinone derivative seizures: July 2005- March 2010.(*11*)

NPSs can be classified into 7 categories as shown in Figure 1-3. This thesis focuses mainly on synthetic cathinone's and substituted phenethylamines although does cover all classifications with the exception of synthetic cannabinoids.

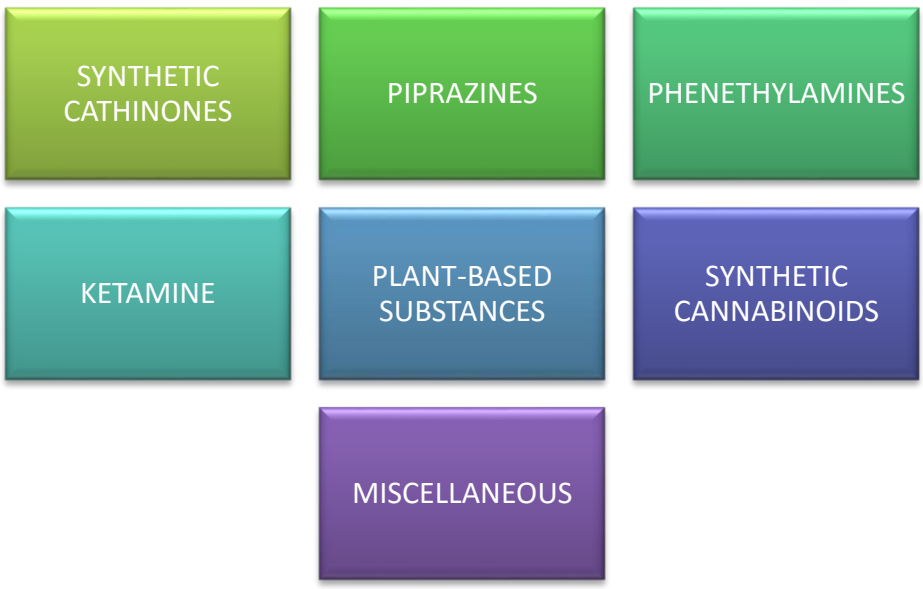


Figure 1-3: UN categorisation of NPSs

1.2 Synthetic Cathinone's

1.2.1 Background

Synthetic cathinones are monoamine alkaloids derived from the psychoactive substance found in the young leaves of the *Catha edulis* plant, commonly known as khat. This plant is native to East Africa and the Arab peninsula, and its use is deeply rooted in everyday life and folklore in these areas; in the Harar region of Ethiopia, khat is included in 501 different medical treatments.(*13*) The use of khat leaves is now illegal in the UK as it is a Class C drug under the MoDA (1971) and licensed as a medicinal product under the Medicines Act 1968.(*14*) Khat use is rare in the UK and its use is mainly restricted to migrant communities, although there is documentation of its use amongst the student population.(*15, 16*)

Cathinone levels in fresh leaves range from 0.3 to 2.1%, although these rapidly decline after harvest, thus khat must be consumed quickly.(*17*) It is thought that this has contributed to its lack of popularity amongst the wider UK population.(*15*)

Recently, synthetic variations of cathinone have appeared in the UK with the most well-known being mephedrone and methylone. Methylone, a patented anti-depressant and anti-Parkinson's medication was the first synthetic cathinone to be identified by the EMCDDA in 2005 followed by mephedrone in 2007.(*18-20*). The only synthetic cathinone currently to have any legitimate use is bupropion which is prescribed as an anti-smoking aide and for treatment of depression.(*11*) Many of the current synthetic cathinones have been produced in response to this legal action and are marketed online as "next generation" mephedrone as was the case for naphyrone. These alternatives are then banned and so the cycle of new legal alternatives began.

Currently synthetic cathinones are the second biggest drug group (after synthetic cannabinoids) monitored by the EMCDDA with over 70 substances identified to date.(*21*)

1.2.2 Chemical Structures

The chemical structures and street names associated with the synthetic cathinones discussed in this thesis are shown in Table 1-1.

Table 1-1: Chemical structures and street names of synthetic cathinones.

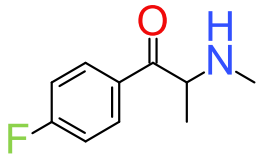
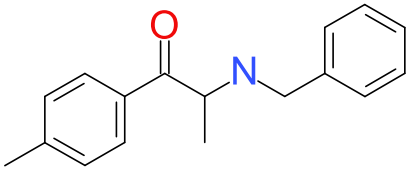
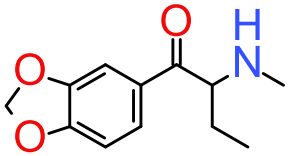
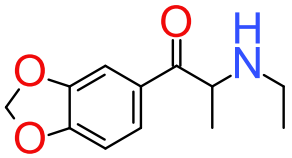
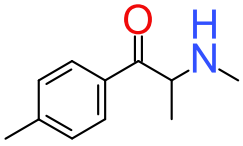
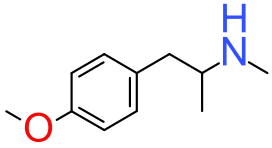
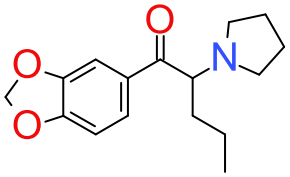
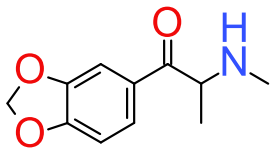
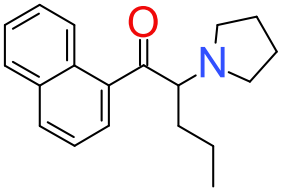
Name	Street Names	Chemical Structure
4-FLUOROEPHEDRONE (FLEPHEDRONE)	4-FMC	
BENZEDRONE	4-MBC	
BUTYLONE	Explosion Ease Arlone	
ETHYLONE (βk-MDEA)	(βk-MDEA)	
MEPHEDRONE	M-Cat Meow Meow White Magic Drone Bubble	
METHEDRONE	Bubbles Bristol Meth Dolley	
METHYLENEDIOXY-PYROVALERONE (MDPV)	Bath salts Magic Super Coke Lunar Wave Vanilla Sky	

Table 1:1 Chemical structures and street names of synthetic cathinones (cont.)

Name	Street Names	Chemical Structure
METHYLONE	Explosion Ease Arlone	
NAPHYRONE	NRG-1 Energy-1 Rave	

1.2.3 Administration

Ingestion of cathinones is typically via oral administration or inhalation, with different preferred routes dependant on the cathinone being administered, *e.g.* methylone is typically taken orally, allowing users to avoid its strong nasal irritancy.(*22*) Reports by users suggest that the majority of these drugs taste unpleasant and if administered orally are done so as "bombs"; small amounts of drug wrapped in paper to avoid the unpleasant taste.(*23*) Other routes such as rectal administration and injection have been noted through a survey on MDPV users showing that the majority chose to inject this substance.(*24, 25*)

Van Hout's study into the injection of mephedrone head shop products found that all participants interviewed had prior experience of intravenous (IV) drug use and that 81% of injecting users had injected mephedrone on first use.(*25*) Injection of synthetic cathinones has also been reported in Hungary and Romania. Hungary reported that up to 58% of individuals enrolled in needle and syringe programmes were injecting synthetic cathinones as their main drug. Opioid substitution programmes in Hungary also reported 10-50% of those enrolled were injecting synthetic cathinones. It should be noted that the synthetic cathinones being injected did change over time in line with changes to availability as substances were controlled as shown in Figure 1-4. Mephedrone was legal in Hungary in 2010, and controlled in Jan 2011. This explains the large decrease in seized paraphernalia associated with this substance and its replacement with 4-MEC and MDPV in 2011.

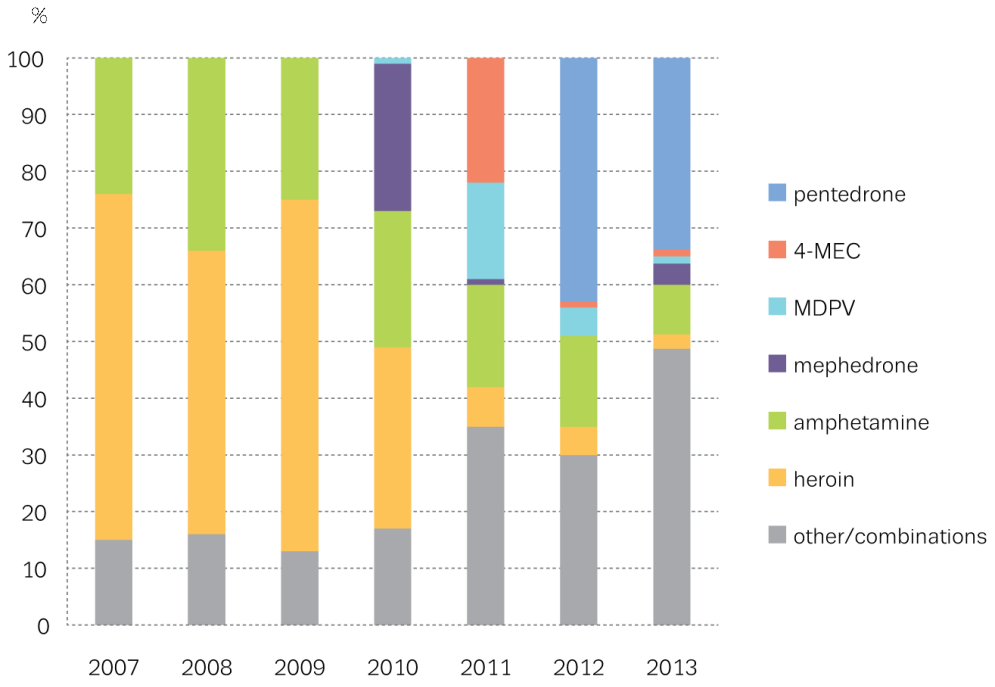


Figure 1-4: Analysis of seized injecting equipment (needle, syringe, filter, and spoon) in Hungary

Oral synthetic cathinones doses are typically in the range of 15-250 mg, although doses of over 1 g have been reported in forums, with lower nasal doses reported of 5-125 mg.(*26*) Due to the nature of the product, users cannot be sure of purity or contents, which may affect dosing.(*27*)

1.2.4 Effects

Effects of synthetic cathinones are thought to be similar to those of amphetamines, particularly MDMA. Euphoria, empathy, increased sex drive, alertness and becoming more talkative are all positive effects commonly mentioned by users. (*28*) The oral onset and duration of effects for mephedrone, methylone and MDPV are shown in Table 1-2. The onset of nasal doses is much quicker, typically taking 2-3 minutes, and the effects for injecting users last anywhere up to 30 minutes.

Table 1-2: Duration of oral effects for mephedrone, methylone and MDPV.

	MEPHEDRONE	METHYLONE	MDPV
TOTAL DURATION	2-5 hours	3-5 hours	2-7 hours
ONSET	15-45 mins	20-60 mins	15-30 mins
COMING UP	15-30 mins	30-45 mins	30-60 mins
COMING DOWN	30-90 mins	60-120 mins	30-120 mins

As expected with any drug, there are also unwanted side effects, most commonly agitation and combative behaviour. Figure 1-5 shows a full list of synthetic cathinone adverse effects as well as their prevalence in Kentucky and Louisiana patients (Aug 2010 – Feb 2011).(*29*) These reported side effects are consistent with other accident and emergency (A&E) reports. MDPV is thought to have slightly more severe side effects than other synthetic cathinones with patients reporting "flash backs" serotonin syndrome and hallucinatory delirium.(*30-32*)

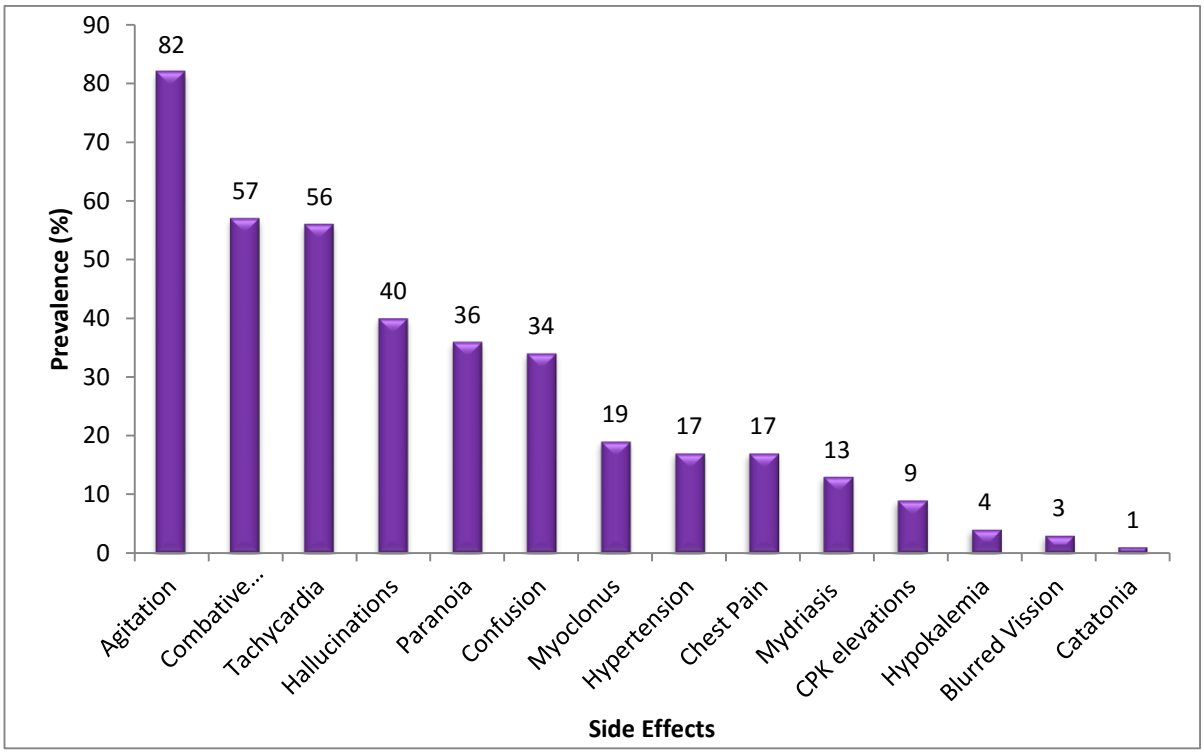


Figure 1-5: Prevalence (%) of adverse synthetic cathinone effects seen in 236 patients in Louisiana and Kentucky, USA (Aug 2010 -Feb 2011).

Four synthetic cathinone IV users admitted to A&E in Dublin showed added soft tissue complications, presenting with cellulitis, thrombophlebitis, and abscesses. Of these four cases, 75% had Hepatitis C, with one individual also having HIV.

These individuals would therefore have had already weakened immune systems which may have exacerbated their symptoms. The use of contaminated injection equipment may have also contributed. No toxicological findings were presented in this paper to confirm that these adverse effects were the result of synthetic cathinone abuse or which synthetic cathinone was responsible. Additional soft tissue problems have also been reported such as rhabdomyolysis and acute compartment syndrome.(*33, 34*)

Synthetic cathinones should not be ingested with other compounds such as alcohol, which may enhance their effects. Recently, there have been reports linking these substances to drug related crime (DFC), in particular drug- related sexual assault (DFSA).(*35, 36*) A study analysed 45 urine samples from DFSA cases and found 13% were positive for methylone. Two cases contained this substance only, whereas, the remaining cases all additionally contained THC or alcohol.(*35*) As these drugs are particularly popular with younger people, it may be that their drug taking behaviour makes them more at risk to DFSA, rather than perpetrators of DFSA selecting these substances specifically in order to carry out these crimes.

Dependency has been reported with synthetic cathinone use, with users needing to re-dose on a frequent basis.(*37, 38*) This has also been demonstrated using animal models. Sprague-dawley female rats trained to self-administer mephedrone did so more than those trained to self-administer methylone and MDMA.(*39*) This highlights that the synthetic cathinone being used will also affect the level to which dependency is shown.

1.2.5 Toxicity

The toxicity of NPSs are not fully understood as the majority of post mortem cases involve polydrug use; therefore death cannot be attributed to one drug alone. In the case of acute toxicity where patients attend A&E, treating the patient is the number one priority for medical staff, and therefore identification of the exact substance taken may never happen. This is also in part due to budget constraints which are affecting hospital priorities. As synthetic

cathinones have been around for the longest period of time, they are the most commonly encountered in A&E and therefore the most understood.

60 mephedrone related UK fatalities were reported which had confirmed toxicology, and concluded inquests. Of the 60 cases, 16 individuals terminated their own lives through hanging or other violent means. The coroner ruled cause of death for these 60 cases is shown in Figure 1-6. In the majority of these cases mephedrone use was thought to have played a role, and therefore its use may have the ability to intensify psychosis and depression. Between March and July 2010 ROAR Forensics, (UK) received 16 urine and/or blood samples which tested positive for mephedrone. Of these, 6 (37.5%) were from suicide cases (5 due to hanging, 1 due to gunshot wound).(*20*) A report into mephedrone-related fatalities in the UK also highlighted the large number of deaths (20.7%) attributed to suicide where mephedrone was found in post mortem biological samples. (*40, 41*) It is not known whether these individuals experienced suicidal thoughts prior to intoxication and therefore a direct correlation between mephedrone use and suicide cannot yet be made.

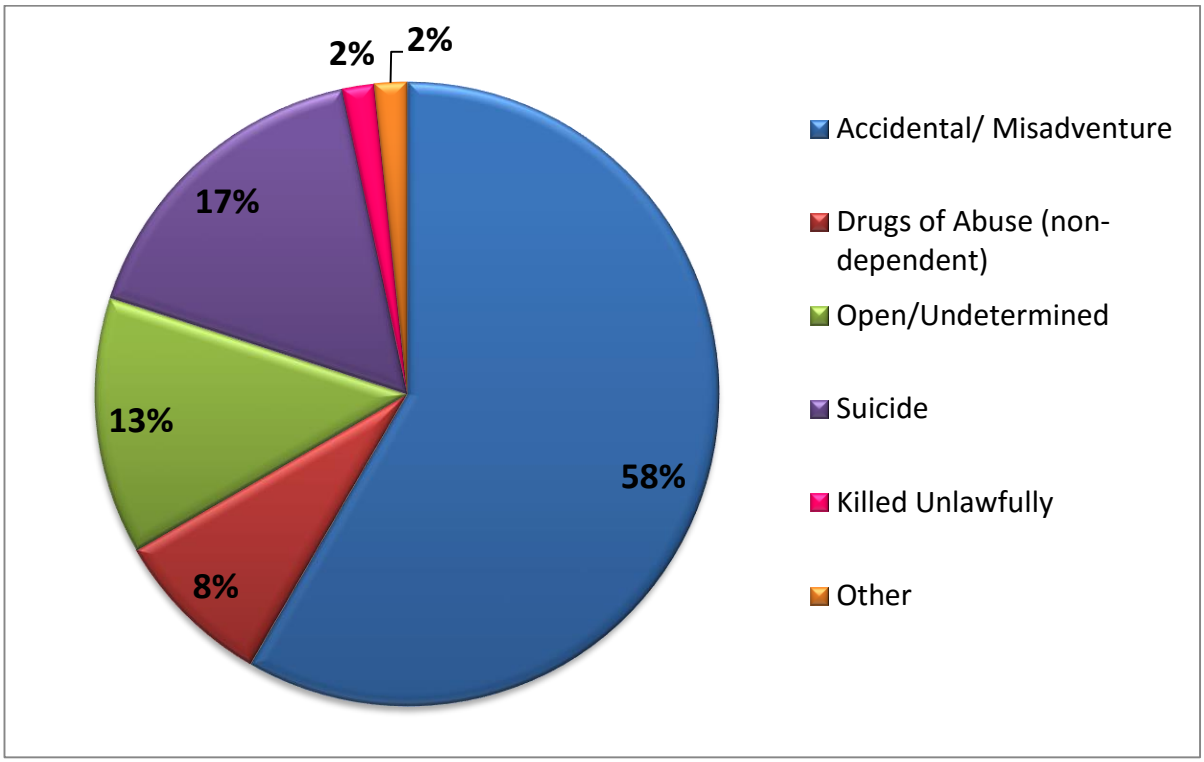


Figure 1-6: Cause of death verdict in 60 cases identified in the UK.

MDPV was detected in 13 fatalities in Finland in 2010. All of the deceased were male known drug users. Urine concentrations ranged from 0.02-4.8 mg/L; however MDPV was not listed as the sole cause of death in any of these cases.(*42*) In Michigan, (USA) a number of MDPV intoxications were reported in 2010 along with 1 fatality where MDPV was noted as the main factor contributing to death.(*24*) Although toxicology was done on this case the concentrations detected were not provided in the paper.

Fatal serotonin syndrome was reported in a 24-year old female who ingested 2 "ecstasy" pills. On analysis, these pills were found to contain methylone and butylone only. On arrival at A&E the patient had a temperature of 41.8^{o}C alongside other previously discussed side effects such as tachycardia and minimal reactivity. Despite extensive effort by the A&E team the patient suffered multi-organ failure and died.(*43*)

Methylone was also deemed the cause of death in three patients admitted to hospital in Florida. Methylone blood concentrations were found at 0.84, 3.3 and 0.56 mg/L. Using this data it was suggested that methylone blood concentrations above 0.5 mg/L may result in death.(*44*)

1.2.6 Pharmacology

Little information is available on the pharmacokinetics of many synthetic cathinones. Seven metabolites of mephedrone have been identified along with a suggested metabolic pathway for its breakdown.(*45*) Hydroxytolyl mephedrone and nor-hydroxytolyl mephedrone are thought to be partially excreted as glucuronides and sulphates.(*45, 46*) There is no information currently available on the stability of mephedrone metabolites or how long they can be detected in biological fluids.(*20*)

Urine samples were collected from individuals identified as recreational drug users through a previous telephone drug survey. Analysis of these samples showed limitations associated with mephedrone metabolite detection by GC-MS. This work stated that the peak areas of mephedrone and its metabolites did not appear to be dose related. This was not a controlled study however, and therefore the time of ingestion would have a much bigger impact upon the

parent drug to metabolite ratio than that of dose. At the time however there was no information regarding metabolic kinetics of mephedrone and thus it was not possible to confirm that metabolite peaks were dependent on the time mephedrone was taken.(*47*)

1.2.7 Prevalence

Despite the 2010 classification of mephedrone as a class B substance in the UK, its use in the UK is still widely reported both online and in recent publications.(*48*) A survey interviewing attendees of London "gay friendly" nightclubs showed that mephedrone was the most commonly used drug with 41% reporting they had taken the drug or were planning to take it over the course of the evening. During this survey individuals were also asked to name their favourite drug; again mephedrone came out on top at 20.4%, ahead of GHB/ GBL (24%) and cocaine (17%).(*49*)

A focus group study on mephedrone use amongst 145 Irish school children (14-15 years old) found that all pupils had heard of mephedrone. Of these teenagers, 40% admitted trying the drug, and 70% reported mephedrone use amongst friends. It should be noted that these pupils were selected from schools in areas known to have a high prevalence of drug use and where paramilitary violence was high and so are not necessarily a true indicator of mephedrone use in school children across the whole of Ireland.(*50*)

Analysis of naphyrone based products prior to its classification detected the already illegal substance mephedrone alongside other controlled substances. (*51*) Mephedrone was added to the British Crime Survey in 2010/11 with 1.4% of surveyed people reporting to have taken the drug within the last year.(*52*) LGC also published data on seizures received between 1st January and 10th June 2011, during which they recorded 176 seizures weighing 15.2 kg containing only mephedrone. They also reported 13 seizures of mephedrone and ketamine mixtures weighing 33.8 kg.(*53*)

One study in Finland analysed blood samples from 3000 drivers suspected of driving under the influence of drugs over the course of a year. Of these samples 8.6% tested positive for MDPV. The individuals who provided these samples were

noted at time of sample collection as being impaired (86%) or severely impaired (7%). The majority of individuals testing positive were male (87%) from 24-44 years of age (76%). As well as MDPV, 80% tested positive for amphetamines and 67% for benzodiazepines. Only 4 samples contained MDPV alone.(*54*)

Research into the prevalence of mephedrone, methylone and benzylpiperazine (BZP) amongst attendees of a drug treatment centre in the Republic of Ireland (ROI) found that 13.3% and 3.3% of 209 samples tested positive for mephedrone and methylone respectively. BZP was much less prevalent amongst subjects with only 0.5% of samples testing positive for this substance.(*55*)

Synthetic cathinones have also been problematic in the USA with phone calls to the American Association of Poison Control Centres (AAPCC) regarding "Bath Salts" sharply increasing. A total of 2371 calls had been made from Jan – May in 2011 in comparison to 303 calls in 2010, a 682% rise. (*11*) It is possible that the dramatic increase in calls was a result of the media attention these new substances were given. MDPV has been the most commonly encountered synthetic cathinone in the U.S.(*56*)

The UNODC Global SMART report (2013) found that all continents reported the emergence of synthetic cathinones with the exception of Africa, highlighting the world wide spread of these substances. The data in this report was collect via questionnaire from 80 different countries of which 10 were African. It is possible that although the African laboratories had not detected synthetic cathinones, their laboratories may not have been testing for them, or were sufficiently equipped to detect them. (*57*)

1.3 Ketamine, Phencyclidine-type substances and Methoxetamine

1.3.1 Background

Ketamine was developed in 1962 by Calvin Stevens and patented the following year in Belgium. It was originally marketed as a fast acting anaesthetic with fewer side effects than its precursor phencyclidine (PCP).(*58*) Despite this claim ketamine was shown to share some of the psychedelic properties although these

were not as extreme. Ketamine has now completely replaced PCP in the U.K. and is routinely used for both medical and veterinary purposes, most famously as a horse tranquillizer.(*59, 60*)

The first official record of ketamine misuse was in 1971. Ketamine misuse in the UK became more common during the 1990's as it was routinely sold in clubs. This correlates with data from a 1997 study where 32% of interviewed club goers had taken ketamine. Increased ketamine use correlated with the rise of the "club" and "rave" culture within the UK. Ketamine was routinely sold as Ecstasy as it has a similar appearance and was cheaper to synthesise.(*61*)

It has only recently become apparent that chronic and long-term ketamine abuse is associated with chronic bladder problems.(*62-66*) This recent bad press has resulted in an influx of new substances such as Methoxetamine (MXE), 3-methoxyphencyclidine (3-MeO-PCP) and 3-Methoxyeticyclidine (3-MeO-PCE), all marketed "bladder safe". This marketing was aimed at existing ketamine users wishing to change substance to avoid these negative side effects. Unfortunately current research shows that repeated administration of MXE is likely to produce similar renal and bladder harms to that of ketamine.(*67*)

1.3.2 Chemical Structures

MXE is structurally related to ketamine and PCP. The chemical structures and street names of ketamine, MXE and other phencyclidine-type substances are shown in Table 1-3.

Table 1-3: Chemical structures and street names of ketamine, phencyclidine-type substances and methoxetamine.

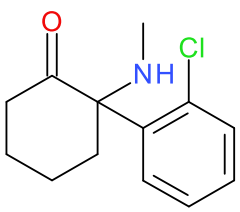
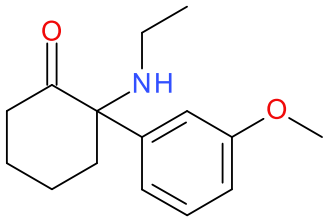
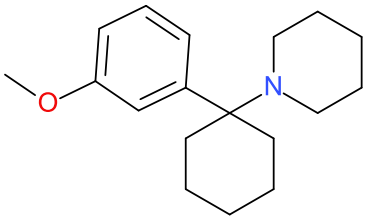
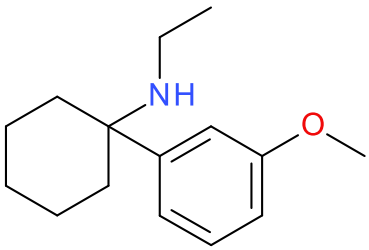
Name	Street Names	Chemical Structure
KETAMINE	K Special K Kit Kat Vitamin K Super Acid	

Table 1-3: Chemical structures and street names of ketamine, phencyclidine-type substances and methoxetamine (cont.)

Name	Street Names	Chemical Structure
METHOXETAMINE (MXE)	MXE ROFELCOPTER MexxyR	
3-MeO-PCP	3-MeO-PCP	
3-MeO-PCE	3-MeO-PCE	

1.3.3 Administration

The majority of "street" ketamine is diverted from legitimate veterinary supplies and is sold in either its original liquid form, or as a hydrochloric salt.(*68*) The issue of ketamine veterinary thefts was raised in 2009 by the House of Commons. The true prevalence of ketamine thefts remains unknown as these are currently listed under "other" theft and cannot be distinguished from other miscellaneous theft. As a result of this, impurities are rarely seen in seized ketamine.(*69, 70*)

Ketamine doses given via IV injection normally range between 1 to 4.5 mg/kg of body weight. Intramuscular doses normally range from 6.5 to 25 mg/kg body weight.(*58, 71*) Oral ketamine doses range from 40mg to over 500mg depending on tolerance, common doses range from 75-300 mg.(*72*)

MXE is routinely sold as a white or off-white crystalline powder which can be insufflated or dissolved in solution for oral and IV administration.(*73*) Oral and

insufflation doses typically range between 10-15 mg. There are also reports of rectal and IV administration.(*74*) Dependency is mentioned in the literature with chronic users expressing the need to re-dose on a frequent basis.(*75*)

3-MeO-PCE and 3-MeO-PCP are administered via the same routes as ketamine and MXE, although are reported to be slightly more potent than PCE, thus require lower doses. Doses of 3-4 mg are typical with strong dissociative effects felt after 10-20 mg.(*76*)

1.3.4 Effects

Ketamine is a known dissociative anaesthetic, meaning patients administered the drug although not unconscious, are unaware of their surroundings. It also works as an analgesic reducing pain and results in mild amnesia.(*68, 77-79*) Ketamine stimulates the cardiovascular system, increasing heart rate, cardiac output and blood pressure. There is a marked increase in muscle tone which in worst case scenarios produces seizure like symptoms by reducing the fluxes of sodium and potassium.(*58, 60, 80, 81*) It does not, however, have an effect on the pharyngeal and laryngeal reflexes; meaning that airways are easily maintained and there is no suppression of the gag reflex as seen in other anaesthetic drugs.(*82*) It is therefore advised that Ketamine should not be administered to any individual with existing cardiovascular problems such as high blood pressure. Ketamine also has the ability to cross the placenta, although to date there have been no reports of birth defects or abnormalities caused by ketamine administration during pregnancy.(*82*)

As ketamine is a central nervous system (CNS) depressant, mixing the drug with other CNS depressants is not advised. Combinations with alcohol and diazepam can result in respiratory problems at high doses. It can also cause nausea and vomiting in individuals who do mix these drugs, especially alcohol.(*82*) The psychedelic aspects of the drug are enhanced by the use of other similar drugs such as LSD.

Those who use ketamine recreationally, seek to gain “out of body experiences” where the dissociative effects of the drug make the user feel they are looking down upon themselves.(*60*) This is commonly referred to as the K-hole. Whilst in

this state users may lose the ability to move and struggle to communicate with those around them. There are also reports of ketamine being used as a drug to facilitate sexual assault.(*83*) The amnesia properties of the drug and the dissociative effects have made it an increasingly popular drug for carrying out these crimes along with its fast acting nature.(*84*)

Adverse side effects of ketamine use include cystitis, severe gastric cramping resulting in intense abdominal pain and memory impairment.(*59, 60, 62, 63, 65, 69, 85*) In 2007 Professor D Nutt published a rational scale to assess the harm of drugs of potential misuse in The Lancet. This paper named ketamine as the 8th most harmful drug tested in the research above ecstasy (16th) and LSD (17th).(*86*)

The main effects of MXE are similar to that of ketamine with users seeking dissociation, euphoria, and hallucinations. The onset and duration of effects are shown in Table 1-4, with after effects reported as lasting 2-48 hours after administration. Adverse effects have been reported in the literature, such as tachycardia, rotary nystagmus, nausea, paranoia, diarrhoea and anxiety.(*87*) There have also been reports of MXE alleviating phantom limb pain, again similar to ketamine.(*88*)

Table 1-4: Onset and duration of methoxetamine effects in relation to routes of administration.(*89*)

	ORAL/ SUBLINGUAL	INSUFFLATION
TOTAL DURATION	3-5 HOURS	2.5-4 HOURS
ONSET	10-20 MINS	10-20 MINS
COMING UP	15-30 MINS	15-30 MINS
COMING DOWN	60-120 MINS	60-120 MINS

Although similar to ketamine, the effects of MXE, 3-MeO-PCE and 3MeO-PCP are regarded by drug forum users as being inferior, however, they are a sufficient substitute should money and/or availability prevent the user from accessing ketamine.

There have been suggestions of ketamine dependence and many of the individuals questioned by Muetzefeldt et al (2008) hinted at this.(*90*) The book “The Scientist” also mentions John Lilly’s personal experience with ketamine

dependence.(*91*) Tolerance to ketamine can be quickly acquired and this may lead to psychological based dependence rather than physical. However, there have been no proven studies to back up these suggestions and the ACMD reported ketamine dependence to be a very rare phenomenon.(*69*) To date there have been no studies conducted on the dependency of MXE, 3-MeO-PCE or 3-MeO-PCP.

1.3.5 Toxicity

Ketamine has a wide therapeutic window and as a result overdose of the drug on its own is extremely unlikely. A review into 114 ketamine related deaths found blood concentrations ranging 2.4-3.6 mg/L.(*92*) Severe overdoses can be higher, with one case resulting in a blood concentration of 27.4 mg/L.(*93*) The EMCDDA reported only 12 deaths where ketamine contributed or was the cause of death between 1987 and 2000. Only 3 of these were from ketamine abuse alone.(*82*)

MXE use was attributed to the death of a male in Milwaukee, USA, although no toxicological data was provided.(*94*) Despite this instance, MXE fatalities have been rare to date with the majority of case reports focusing on acute toxicity. Acute toxicity was seen in 3 patients in London with serum levels ranging from 0.09 to 0.2 mg/L. One of the patients admitted also had 6-APB/5-APB present in their system; highlighting poly drug use amongst this population.(*95*)

Hofer published the case of a 19-year old male with a history of drug abuse and attention deficit hyperactivity disorder (ADHD) who injected an unknown quantity of MXE. MXE was analytically confirmed by liquid chromatography tandem mass spectrometry (LC-MS/MS), however, concentrations were not reported.(*96*) A review of 6 patients identified MXE urine concentrations ranging from 2µg/mL to 165.3µg/mL.(*97*)

Another post-mortem case identified MXE concentrations of 8.6 µg/g in femoral blood.(*98*) This was significantly higher than non-fatal concentrations identified in 4 cases which ranged from 0.21-0.49 µg/g.(*98*) As of yet there is no ED_{50}, TD_{50} or LD_{50} information for MXE, 3-MeO-PCE and 3-MeO-PCP.

1.3.6 Pharmacology

Ketamine exists as 2 separate isomers, R and S, with the S isomer believed to be 3-4 times more potent than the R isomer. Ketamine is lipid soluble and is quickly distributed throughout the body following injection or infusion with a half-life ranging from 2 – 3 hours.(*92*) The elimination of ketamine occurs through two phases, alpha and beta with half-lives of 10-15 minutes and 2.5 hours respectively. It is during the alpha phase that ketamine affects the CNS.(*82*) Once in the body ketamine is metabolized via N-demethylation to norketamine which is then dehydrogenated to form dehydronorketamine. Metabolism takes place in the liver by CYP3A4, CYP2B6 and CYP2C9 enzymes.(*99*)

As MXE is structurally similar to ketamine it is predicted to have a similar mechanism of action, acting as a NMDA receptor antagonist, thus inhibiting dopamine reuptake.(*28, 100, 101*) Unlike ketamine, MXE also has affinity to the serotonin receptor. MXE does not possess the same analgesic and anaesthetic properties as ketamine due to the alteration from 2-chloro to a 3-methoxy group.(*28*) 3-MeO-PCE and 3-MeO-PCP have also been shown to be NMDA receptor agonists with 3-MeO-PCP being particularly active.(*102*)

The metabolism of MXE was identified in rat and human urine by Meyer. This research identified nor-MXE as the most important human metabolites for aiding identification.(*101*) CYP2B6 and CYP3A4 are thought to be involved in the initial metabolism of this compound.(*101*) No pharmacokinetic data is currently available for 3-MeO-PCE or 3-MeO-PCP. The absorption, distribution or excretion of these compounds has yet to be investigated.

1.3.7 Prevalence

Due to the length of time ketamine has been used, prevalence for this drug has been well documented. Its use continues to increase in the UK, in particular with the “underground dance scene”. Despite its popularity, negative-effects from long-term use are now common knowledge with underground DJ Seth Troxler claiming “Ketamine is the heroin of our time, it’s horrible”.(*103*)

MXE was first detected and reported to the EMCDDA in 2010. Initially reports originated from the UK with other EU countries reporting this substance shortly after. UK research analysing pooled urine from portable urinals found that 50% of the urinals contained ketamine, with 1 urinal tested positive for the presence of MXE.(*104*) Urinals were placed in 12 sites throughout an area of London and used for 1 night during March. This data is limited because the urinals could only be used by men and it is unknown whether MXE was purposely ingested or used as an adulterant in a ketamine sample. Despite its limitations this research did provide a strong indication of drugs currently being abused within the night time economy.

A total of 120 non-fatal intoxications of MXE were reported to the EMCDDA by 5 member states, highlighting the spread of this drug throughout Europe.(*73*)

1.4 Aminoindanes & Methiopropamine

1.4.1 Background

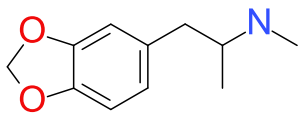
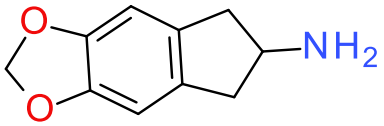
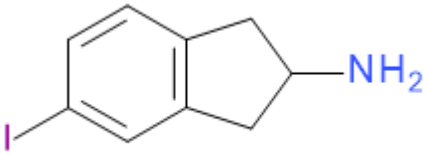
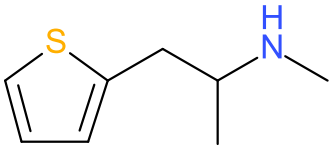
Aminoindanes, are a sub-group of phenethylamine and include substances such as 5,6-methylenedioxy-2-aminoindane (MDAI) and 5-Iodo-2-aminoindane (5-IAI), which have both been detected in Australia, Belgium, Finland, Latvia and the UK.(*57*) Although not an aminoindane, methiopropamine (MPA) is commonly encountered alongside these compound types, and thus is discussed here.

5-IAI was developed in the 1990's by David Nichols at Purdue University as was MDAI during research into the pharmokinetics of MDMA. Methiopropamine (MPA) was synthesized much earlier in the 1940's and again is another failed pharmaceutical product.(*105*) Recreational abuse of these substances was not recognised until 2010. This is likely to have been a response to the banning of mephedrone and the aggressive marketing taking place offering these products as its alternative.

1.4.2 Chemical Structures

The chemical structures and street names of aminoindanes (discussed in this thesis) and MPA are shown in Table 1-5. These are chemically similar to MDMA which is also shown for comparison.

Table 1-5: Chemical structures and street names of aminoindanes.

Name	Street Names	Chemical Structure
MDMA	Ecstasy E	
MDAI	Woof Woof Stardust Sheet Mystic	
5-IAI	Bounce Charge	
METHIOPROPAMINE	MPA Blow	

1.4.3 Administration

MPA is an off-white powder with a bitter taste and slight aniseed odour. It can be administered orally, insufflated, or inhaled as a vapour with doses varying depending on the route taken.(*106*) Smoking MPA has also been mentioned in literature with this providing the quickest onset of effects.(*105*)

The first MDAI samples to be detected in the UK were brown powders with further seizures of capsules (n=17) in 2010. MDAI is typically administered orally, although it can also be snorted, and administered rectally. Typical doses range from 150-200 mg, and re-dosing appears to be common.(*107*)

1.4.4 Effects

Positive effects for MPA users include mild euphoria, increased alertness and sexual arousal. Negative effects include anxiety, difficulty urinating, laboured breathing, heart palpitations and nausea. Effects are thought to be similar to methamphetamine, effects are shown in Table 1-6.

Table 1-6: Onset and duration of MPA effects following insufflation. (*89*)

	INSUFFLATED
TOTAL DURATION	2-4 hrs
ONSET	5-10 mins
COMING UP	5-10 mins
COMING DOWN	30-120 mins

The effects of MDAI are similar to that of MPA, with the effects and their duration being dependent on the route of administration. Effects are felt normally within the first 10 minutes, although this can change depending on the users own tolerance of this substance. The effects of 5-IAI are also similar to MDMA, without the long-term neurotoxicity. (*108*)

1.4.5 Toxicity

Analytically confirmed toxic cases for these substances remain rare. The cause of death for a 27-year old female was linked to MPA use with this substance detected at a concentration of 400 ng/mL in urine. Although this was not the only drug detected it was thought to have played a significant role in her death.(*109*)

A&E presentations due to MDAI are rare, as are fatalities. A MDAI concentration of 26.3 mg/L was identified by HPLC-DAD in one female fatality; ethanol was also identified in this case at a concentration of 14 mg/dL. A second fatality involving a male identified a MDAI blood concentration of 3.3 mg/L. A urine screen identified the presence of other substances which were later confirmed using the blood sample; APB (0.34 mg/L), BZP (0.19 mg/L, MDMA (0.1 mg/L) and caffeine (19.2 mg/L). (*107*)

1.4.6 Pharmacology

MPA for the most part remains un-metabolised with the parent compound excreted in both rat and human studies.(*110*) MPA works on the CNS, acting as a norepinephrine and dopamine reuptake inhibitor as well as a serotonin reuptake inhibitor.(*111*) As of yet there have been no studies on the dependence or tolerance of this substance, however it is likely that this will be similar to other stimulant compounds.(*111*)

To date there is very little information on the pharmacology of MDAI or 5-IAI, although they are thought to be similar to that of MDMA.

1.4.7 Prevalence

The prevalence of these drugs is especially difficult to determine due to the large number of products mis-sold online. 5-IAI was one of the most sold NPS on websites between 2011 and 2012, however it may be that although many retailers were offering this product, they were in fact selling something different. Analysis of aminoindane products purchased online found that 6/7 contained large amounts of caffeine, with only 1 containing the intended aminoindane (MDAI).(*112*) Case reports involving these substances have also been comparatively low suggesting that use of these substances is not as common, or that few laboratories are screening for these substances.

The use of MPA has been identified in the night-time economy being the 12th most commonly detected drug in a London study of urine collected from public urinals.(*104*)

1.5 Benzofurans

1.5.1 Background

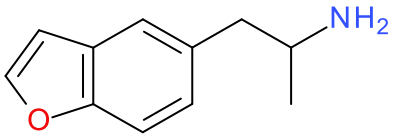
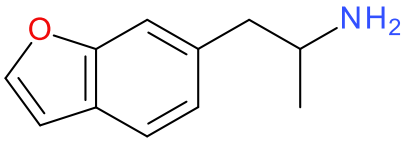
Identified in 2010 in the UK 5-(2-aminopropyl)benzofuran (5-APB), and its positional isomer 6-(2-aminopropyl)benzofuran (6-APB) (identified in 2011 in Hungary) are phenylethylamine analogues, specifically MDMA. Benzofurans were initially investigated in the early 1990's as potential anti-depressants. This work

is ongoing with some compounds of this class now having patents for such use.(*113-115*)

1.5.2 Chemical Structure

The chemical structures and street names of 5-APB and 6-APB are shown in Table 1-7. These compounds differ only in the position of the oxygen atom in the furan ring, relative to the amine. These compounds are similar to methylenedioxyamphetamine, with the oxygen in the methylenedioxy ring replaced by a carbon, and an additional double bond.

Table 1-7: Chemical structures and street names of benzofurans.

Name	Street Names	Chemical Structure
5-APB	Benzo Fury White Pearl	
6-APB	Benzo Fury White Pearl	

1.5.3 Administration

Benzofurans are sold in powder, pill, pellet or gel capsule form, with powders being white or slightly tan in colour depending on purity. Benzofurans can be ingested orally, insufflated or taken rectally. Typical oral doses range from 50-100 mg however stronger doses over 170 mg have been mentioned in drug forums.(*116*)

1.5.4 Effects

Both 5 and 6-APB have amphetamine like effects on the body. Positive effects result in increased visual and tactile stimulation, mild euphoria, hallucinations both auditory and visual. Common side effects include bruxism, nystagmus, headaches and nausea. The onset and duration of these effects are shown in Table 1-8.

Table 1-8: Duration of APB effects.

	ORAL/ SUBLINGUAL
TOTAL DURATION	Up to 14 hours
ONSET	15-90 mins
COMING UP	2-3 hours

1.5.5 Toxicity

Acute psychosis was reported in 1 patient with a 6-APB urine concentration of 2 mg/L. Psychological treatment and diazepam administration was used to treat symptoms before the patient was discharged. Synthetic cannabinoid metabolites were detected at much lower concentrations, and deemed not to have played a significant part in the patient's symptoms.

Post mortem toxicology was carried out on a 21-year-old male who collapsed, and eventually died despite intensive medical intervention. Analysis was done on peripheral blood, central blood and urine with 5-APB detected at 2.5 mg/L, 2.9 mg/L and 23 mg/L respectively. Other matrices were also tested including liver, vitreous and gastric contents and found to contain 16 mg/kg, 1.3 mg/L and 6 mg/L respectively.

1.5.6 Pharmacology

5-APB has been shown to inhibit the dopamine transporter system and act as an agonist to both the 5-HT_{2A} and 5-HT_{2B} receptors. It was also noted in this study that 5-APB caused vasoconstriction of the aorta, and suggested that its activity at the 5-HT_{2B} receptor may result in cardiotoxicity.(*117*)

1.5.7 Prevalence

There is little prevalence data regarding these compounds due to their apparent limited use. The National Poisons Information Service TOXBASE received 65 phone enquiries and 741 online requests for information relating to 5 and 6-APB between 2009-2013.

1.6 NBOMe's

1.6.1 Background

Another group derived from phenethylamines are the NBOMe's, a derivative of the 2C-X series (developed by Alexander Shulgin in the 70's and 80's as part of his research on phenethlamines), the first NBOMe was mentioned in Ralf Heim's PhD thesis in 2004.(*118*) NBOMe's were further developed by David Nichol with the addition of a 2-methoxybenzyl (MeOB) on the nitrogen (N) of the phenethylamine, hence the term NBOMe. Recreational abuse is thought to have begun in 2010.(*119*)

1.6.2 Chemical Structures

The generic chemical structure of the NBOMe series is shown in Table 1-9 along with their street names.

Table 1-9: The generic chemical structure for the NBOMe series, alongside street names.

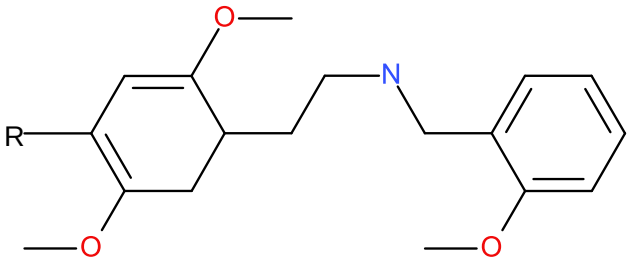
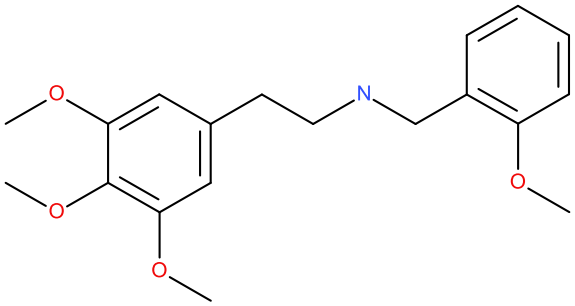
Name	R=	Street Names	Chemical Structure
25I-NBOMe	I	Smiles Bomes	
25B-NBOMe	B		
25N-NBOMe	N		
25P-NBOMe	P		
25D-NBOMe	D		
25E-NBOMe	E		
25H—NBOMe	H		
25T2-NBOMe	Methylthio Group		
25T4-NBOMe	Isopropylthio Group		
25T7-NBOMe	Propylthio Group		

Table1-9: The generic chemical structure for the NBOMe series, alongside street names (cont.)

Name	R=	Street Names	Chemical Structure
25C-NBOMe	C		
Mescaline-NBOMe	Mescaline group		

1.6.3 Administration

Phenethylamines are routinely associated with the "club drug" scene and as a result are administered commonly using routes compatible with these settings, i.e. oral or insufflation.

NBOMes are administered either in liquid form or on blotters due to their high potency and similarities to LSD. Oral and sublingual administrations were the reported methods of administration by 81.2% of users taking part in the Global Drug Survey.(*120*) Insufflation has been noted in the literature although this route of administration is associated with toxicity and even death.

Oral doses of all NBOMes (except mescaline-NBOMe) reportedly range from 50-1200 µg with hallucinogenic effects felt at doses of 50-200 µg. Sublingual doses for hallucinogenic effects range from 100-250 µg with strong effects being felt above 800 µg. Low insufflation doses are in the range of 50-200 µg with doses over 700 µg considered as extremely strong. Alternative administration routes have been mentioned in drug forums, such as injection (830 µg), smoking (50-200 µg), rectal (400 µg), and vaginal (500 µg).(*121*)

Mescaline-NBOMe appears to be an anomaly due to the fact that drug users in forums report requiring a much higher dose in order to achieve psychoactive effects, with one forum user reporting to have insufflated 75 mg over the course of a 20 minute period.(*122*)

1.6.4 Effects

The NBOMe and 2C-X series differs from other phenethylamines as these produce hallucinogenic effects which are not normally observed for other phenethylamines such as MDMA unless very strong doses are administered. Sublingual users of NBOMes report an unpleasant metallic taste, alongside numbness. According to user forums it is this metallic taste and sublingual numbness that allows users to differentiate this substance from LSD. Psychedelic effects differ from user to user, however many report only visual hallucinations with less of the "spiritual" high experienced when taking LSD.(*123*)

Table 1-10: Onset and duration of 25C-NBOMe effects in relation to routes of administration.

	ORAL/ SUBLINGUAL	INSUFFLATION
TOTAL DURATION	6-10 hrs	4-8 hrs
ONSET	0-15 mins	0-5 mins
COMING UP	30-90 mins	15-30 mins
COMING DOWN	1-4 hrs	1-3 hrs

1.6.5 Toxicity

Due to the high potency of NBOMe's adverse effects, fatalities have been reported. The case of a 19-year-old man was reported by Poklis *et al* who was found to have overdosed on 25I-NBOMe. A range of matrices were tested including blood and urine, identifying concentrations of 0.441 ng/mL and 2.860 ng/mL respectively.(*124*) Kueppers and Cooke reported a fatality attributed to 25I-NBOMe. In this case 3 NBOMes were identified, 25I-NBOMe (2.800 ng/mL), 25H-NBOMe (1 ng/mL) and 25C-NBOMe (0.7 ng/mL).(*125*) This is the second publication where individuals have ingested multiple NBOMes with Stellpflug *et al*. identifying 25I-NBOMe (7.5 ng/mL) and 25H-NBOMe (0.9 ng/mL).(*126*)

1.6.6 Pharmacology

Rat and mice studies have shown NBOMes to be potent 5-HT_{2A} receptor agonists and it is this activity at the 5-HT_{2A} receptor which leads to the hallucinogenic effects of these drugs.(*127, 128*) The metabolism of the NBOMe series is reported to be similar to that of the 2C-X series. Screening of urine case samples revealed the presence of *O*-demethylated metabolites, although the parent drug

was still the most abundant. Other metabolites include N-dealkylation, phenolic hydroxylation and conjugation.(*126, 129*)

1.6.7 Prevalence

Due to the recent launch of NBOMe's onto the market, their prevalence remains unknown. They are not included as yet in major surveys such as the Crime Survey for England, but were added to the MixMag drug survey in 2013.This survey was completed by 22,289 individuals of which 2.6% had reported ever using NBOMe drugs.(*120, 130*)

Information provided to the ACMD by the Serious Organized Crime Agency suggested that large quantities of the drug had already arrived into the UK from China.(*123, 131, 132*) Prevalence studies are further complicated by the false advertising of this substance as LSD, and as a result many users may unwillingly be taking this substance.

Reports of mescaline-NBOMe use are rare and there are no scientific papers published on this compound. It has been suggested that its relatively short life-span as a recreational drug was due to the high cost in synthesis. This coupled with low potency has resulted in manufacturers ceasing production in favour of more lucrative compounds. (*133*)

1.7 Pipradols

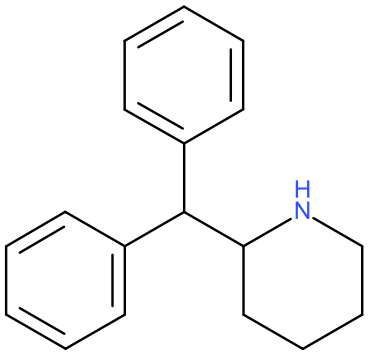
1.7.1 Background

2-desoxypipradol (2-DPMP) was first synthesized for the treatment of narcolepsy and ADHD in 1954, although it was never used for these purposes due to its long elimination time (16-20 hours).(*2*) Recreational abuse of this substance seems to have begun in 2007; however drug seizures were not reported until 2009 in Finland.(*134, 135*) Due to the relatively low prevalence of this substance very little is known about it and the number of individuals reporting to A&E, or drug treatment programmes appears to be few. (*136*)

1.7.2 Chemical Structure

The chemical structure of 2-DPMP and its associated street names is shown in Table 1-11. This compound is structurally similar to both methylphenidate and pipradol.

Table 1-11: Chemical structures and street names of 2-DPMP.

Name	Street Names	Chemical Structure
2-DESOXYPIPRADOL 2-(DPMP)	Ivory Wave Purple Wave Vanilla Sky Whack	

1.7.3 Pharmacology

There is little pharmacological data reported for 2-DPMP, however it appears to be metabolised via hydroxylation, dehydrogenation and oxidation, with these processes resulting in 6 phase I metabolites.(*137*) Although these metabolites have been identified in urine case samples, the levels detected varied between cases and thus the main metabolite could not be identified. This may in part be due to drug-drug interactions taking place as all case studies contained other substances.

1.7.4 Administration

2-DPMP is a white crystalline powder typically snorted or administered orally. Doses range from 0.5 mg-10 mg depending on the tolerance of the user and the method of administration.(*138*) Again due to the limited use of this substance information seems to be mainly from drug forums and thus may not be accurate.

1.7.5 Effects

Effects are typically felt within the first 60 minutes after ingestion, and can be felt up to 48 hours later. Desired effects are similar to MDMA with users seeking

euphoria, increased energy, alertness and sociability.(*134*) Table 1-12 shows the duration of these effects.

Table 1-12: Onset and duration of 2- DPMP effects when insufflated.

	INSUFFLATION
TOTAL DURATION	Up to 48 hrs
ONSET	0-20 mins
COMING UP	1-2 hrs
COMING DOWN	12-24 hrs

Adverse side effects following recreational use of 2-DPMP have been reported and these include hallucinations, paranoia, agitation, chest pain, palpitations and tachycardia. It has also been noted that adverse effects may still be felt 5-7 days after initial ingestion.(*139*)

1.7.6 Toxicity

Several deaths and hospitalisations have been reported as a result of 2-DPMP abuse. The UK reported 3 deaths due to 2-DPMP in 2010 involving 2 males and 1 female. 2-DPMP concentrations were 1.16 mg/L, 0.79 mg/L and 0.025 mg/L respectively. In the third case, the individual had lost consciousness, gone into a coma, and developed severe swelling of the brain. She was kept in intensive care for 12 days on life-support which was removed 2 days later resulting in heart failure. Due to the extended time period between death and ingestion, initial concentrations would have been significantly higher than was determined post-mortem.(*134*)

Five post mortem cases from Finland were reported positive for 2-DPMP between Oct 2010 and May 2012. Of these 5 cases, 2-DPMP was listed as contributing to death in 2, with both of these cases having blood concentrations of 1.4 mg/L.(*140*)

1.7.7 Pharmacology

2-DPMP is metabolised by the liver with a bioavailability of over 90%. Six metabolites have been identified using human liver microsome (HLM) and

analysing human urine samples. These metabolites are formed through hydroxylation, dehydrogenation and oxidation.(*137*) Animal studies have shown that 2-DPMP increases the release of dopamine and decreases dopamine re-uptake similar to cocaine.(*141*)

1.7.8 Prevalence

From the literature, it would appear that 2-DPMP use is most prevalent in the UK and Ireland, however it does not currently appear on any national surveys and thus assessment of prevalence is difficult. 2-DPMP was found to be contained within the "legal high" product Ivory Wave which had originally contained MDPV, further complicating prevalence assessment as many individuals may not be aware that this was the substance they were ingesting.

Searching google trends with the key words "2-DPMP" and "ivory wave" shows main interest in this substance peaked in 2011, with ivory wave having a second peak in June 2012 as shown in Figure 1-7. The blue line represents google searches involving the term "ivory wave" whereas the red relates to the 2-DPMP.

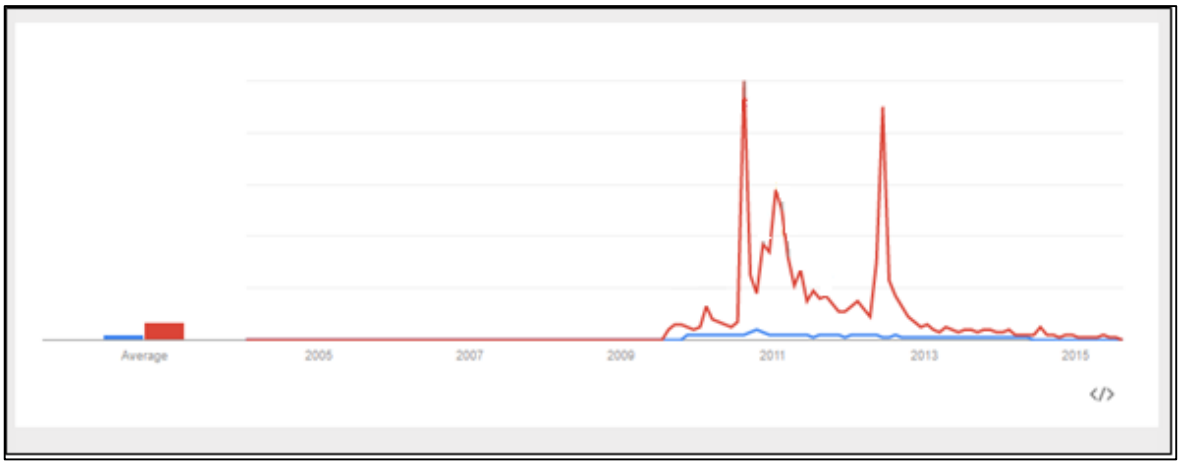


Figure 1-7: Screenshot of Google Trends search data for Ivory Wave and 2-DPMP (last accessed 06/08/2015)

A study carried out in Finland examined the prevalence of 2-DPMP in drugged drivers and found 1.7% of cases examined between October 2010 and May 2012 to be positive for this drug.(*140*) All of the 106 cases involving 2-DPMP also tested positive for other substances (mainly amphetamines and

benzodiazapines), again highlighting polydrug use amongst this drug-taking population.

1.8 Marketing

The rise of legal highs has been increased by the ease at which products can be bought and sold. The internet has played a key part in this with many drugs being marketed online as “bath salts”, “plant food” or “research chemicals” labelled “not fit for human consumption” to avoid penalties under the Medicines Act 1968.(*142*)

NPSs are normally sold as white powders or pressed into pills, similar to MDMA. Products are sold in packaging which is already familiar to the user as seen below in Figure 1-8. It is thought that this may increase the appeal of these products and falsely enhance the idea of safety.(*143*)

Figure 1-8: Legal high packaging. This product was found to contain MDAI (*143*).

The internet has also allowed users to discuss these drugs in forums, providing information on side effects and suitable doses.(*144*) These on-line community forums have also given rise to the cyber-pyschonaut- individuals who specifically seek out new substances and document their experiences in detail. These individuals are committed to harm-reduction and therefore view it as their duty to provide as much information as possible to these online forums, viewing their drug use as research. (*145*)

Internet retailing allows companies to monitor purchases and build personalized customer profiles. These marketing strategies have also been applied to NPSs, with customers being notified of flash sales, given free samples to try and being alerted to new products which the retailer feels may be of interest to the customer.(*146*) This aggressive marketing could in theory lead to individuals trying products which they otherwise would not have considered and which may pose higher health risks than what they had originally ordered.

A 2010 investigation into the source of legal highs online found that 52.2% of shops identified were based in the UK, further emphasizing the popularity of these drugs in the UK.(*142*) In 2013 the EMCDDA identified 651 websites openly selling 'legal highs' to Europeans illustrating the rapid growth of this market. This figure does not include sites found only in the deep web such as Silk Road, Agora and Evolution. These websites can only be accessed using onion browsers such as Tor which provide added anonymity for the user by clouding IP addresses. Illicit substances are then sold using bitcoin, an electronic currency which the user purchases and which cannot be easily traced back to the individual.(*147*)

Studies into the use of these dark net sites have shown that the majority of purchases are made in bulk for re-sale. Although transactions of this nature are currently small in comparison to traditional routes it is thought that these will increase as new regulation is passed and drug use is pushed further underground.

In 2015, the Global Drug Survey focused on purchases made through the "dark net" and found that 30% of individuals purchased a wider range of drugs than they would previously have done. Despite this, the most commonly purchased "dark-net" products were MDMA, LSD and cannabis, with 37%, 30% and 26% of individuals opting to purchase these. In contrast only 7% and 4% of people surveyed purchased 25I and 25C-NBOMe, indicating that the majority of legal high purchases are made when traditional alternatives are not available.

Manufacturing of these drugs seems to be restricted to Asia, in particular China and India. There is no indication of manufacturing taking place in Europe although packaging does appear to take place, with three tableting machines

being seized in Holland, one in 2009 and two in 2010.(*148*) It is likely that after tableting, these pills would have entered the market to be sold as Ecstasy.

1.9 Legality

Due to the speed at which NPSs appear on the recreational drug market governing bodies have struggled to control these. As a result, the number of uncontrolled substances now detected outnumbers those that are controlled by the UN. Many countries have tried to solve this problem with none having found a solution. A range of measures have been used throughout Europe to control and limit the supply of NPSs such as consumer safety legislation, the application of existing legislation or the creation of new legislation.

UK law requires that substances need only pose a threat to public health, or society before scheduling. This has meant that the UK government is in a position to quickly respond when a new substance is detected without having to identify its pharmacology etc. This may result in compounds being unnecessarily controlled, impeding the pharmaceutical industry, however, this is viewed as a worth-while risk.

The UK government, in response to NPS use, created a temporary drug class order, which places a compound temporarily under control while further scientific work is carried out to assess its potential threat. Possession of substances controlled under this legislation will not be an offense, however importing these substances or supplying them will remain an offence. This again has resulted in faster scheduling of compounds; however, it is unlikely that a drug placed in this category would ever be removed from it without further classification as this would be viewed as the government effectively “okay-ing” a psychoactive compound. Methoxetamine and 2-DPMP were the first compounds to be subject to this level of control, both are now class B substances. The current classifications for drugs mentioned in this thesis are shown in Figure 1-9.

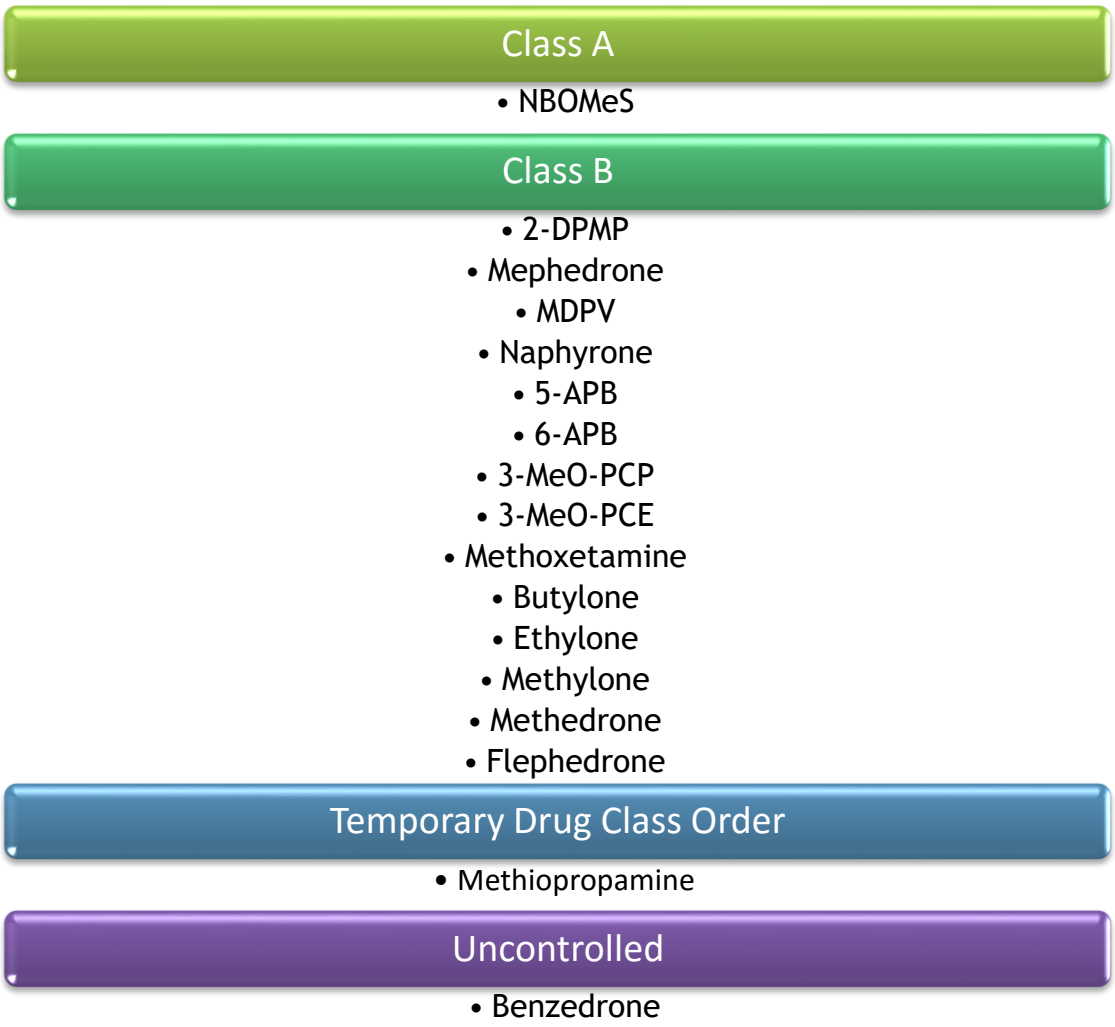


Figure 1-9: MoDA classification for the NPSs discussed in this thesis.

The Home Office announced in the Queens Speech that there is to be a blanket ban on NPSs through the implementation of the Psychoactive Substances Bill.(*149*) This Bill currently proposes to outlaw the importation, exportation and supply of psychoactive substances, or substances suspected of having psychoactive effects. Anyone convicted of such offences will face up to 7 years imprisonment depending on the severity of the case. Personal possession carries no penalties and is not controlled in this Bill. Due to the wide range of this Bill there are exclusions such as substances which are already under control, medicinal products, alcohol, nicotine, tobacco, caffeine and food.(*150*) This Bill is similar to that implemented in the ROI to curve the number of NPS retailers.(*151*) Although based on the ROI legislation, the Bill as it currently stands has been met with strong opposition and criticism and changes are likely to occur before it is finalized and implemented by early April 2016.(*152-154*)

1.10 Literature Review & Gap Analysis

To date there have been several methods published for the analysis of NPS compounds however these only focus on single chemical groups such as synthetic cathinones or NBOMes as shown in Table 1-13. This means that should analysts encounter more than 1 classification of substance in a sample, not all may be detected. This limitation also results in existing methods becoming outdated rapidly, or several methods having to be run, thus increasing laboratory turn-around times. This is particularly true when looking at GC-MS methods, as a lot of the new screening methods utilise quadrupole time of flight (QToF) instruments and LC-MS/MS which not all laboratories can afford.

Although the different isomers of APB have been identified for drug analysis purposes, there are very limited publications quantifying doses in biological matrices. Publications which do quantify the levels present, report only the presence of APB unable to determine which isomer is present, i.e. 5-APB or 6-APB.(*155, 156*)

Publications for NBOMes are scarce with methods only available for traditional matrices such as blood and urine as shown in Table 1-13. The majority of publications focus on a single NBOMe rather than the simultaneous detection of several different NBOMes. (*125, 126, 128, 131, 157, 158*) To date, all NBOMe analysis methods have used LC-MS/MS or QToF instruments with GC-MS only being used when analysing powders for drug analysis. As prevalence of these compounds remains unknown and limiting detection to these 2 matrices may result in users going undetected.

The lack of publications including toxicological data not only causes problems for medical practitioners, but also for forensic laboratories, as without this information forensic toxicologists are left to guestimate appropriate calibration concentrations. This is particularly true for alternative matrices such as hair.

Table 1-13: Summary of screening methods published for the detection of multiple NBOMe's

	(159)	(160)	(161)	(162)	(163)	(164)	(165)	(166)	(160)	(167)	(168)
INSTRUMENT	LC-MS/MS	UPLC-QToF	LC-MS/MS	LC-MS/MS	UHPLC-MS/MS	GC-MS & LC-MS/MS	LC-MS/MS	GC-MS	UPLC-QToF	HPLC-MS/MS	LC-MS/MS
MATRIX	BLOOD	BLOOD	BLOOD & URINE	URINE	BLOOD	BLOOD & URINE	BLOOD	DRUG POWDER	BLOOD	URINE	HAIR
25B-NBOME	X	X	X	X						X	
25C-NBOME	X	X	X	X						X	
25D-NBOME			X	X						X	
25E-NBOME			X								
25H-NBOME	X	X	X	X						X	
25I-NBOME	X	X	X	X						X	
25N-NBOME			X								
25P-NBOME			X								
25T2-NBOMe			X							X	
25T4-NBOME			X								
25T7-NBOME			X								
MECALINE-NBOME			X								
2-DPMP											
3-MeO-PCE											
3-MeO-PCP											
5-APB											
6-APB					X						

Table 1-13: Summary of screening methods published for the detection of multiple NBOMe's (cont.)

	(159)	(160)	(161)	(162)	(163)	(164)	(165)	(166)	(160)	(167)	(168)
BENZEDRONE								X			
BUTYLONE					X	X	X	X	X		X
ETHYLONE					X						X
FLEPHEDRONE							X	X	X		X
MDPV					X	X		X	X		X
MEPHEDRONE					X	X	X	X	X		
METHEDRONE					X	X	X	X	X		
METHIOPROPAMINE											
METHYLONE					X	X	X	X			X
MXE					X						
NAPHYRONE					X			X	X		

1.11 Aims & Objectives

The literature indicates that although substantial work has been carried out and published in the field of NPSs clearly more is required. Although there are several published methods for the detection of single NPS groups, there is a lack of methods covering a wide range of NPS groups. The speed at which additional NPSs appear on the recreational drug market means that laboratories who are able to screen for a wide variety of NPS groups using singular methods are much more likely to detect substances not commonly encountered. The aims and objectives of this study and how they will be achieved are listed below:

1. To develop a GC-MS method for the detection and quantification of a wide variety of NPSs compounds in biological matrices.

    - This involves the optimisation of GC conditions such as column type, injection port temperatures and oven temperatures, as well as altering MS parameters.

    - To determine the optimum extraction technique for the analysis of a wide range of NPSs. This will be achieved by comparing different SPE cartridges to identify the optimum cartridge for extraction, and comparing this against SLE and LLE methods for blood, urine, plasma and serum.

    - To identify the optimum derivatization method for the analysis of NPS compounds by GC-MS by altering the derivatization reagent, temperature and incubation period.

    - The validation of this method in accordance with ISO/IEC 17025 and SWGTOX validation guidelines.

    - Testing this method against “real-life” case samples.

2. To investigate the stability of mephedrone in blood under different temperature and preservative conditions.

- Analysing blank blood spiked with mephedrone with or without the addition of different preservatives and stored at room, fridge and freezer temperature over time.

3. To investigate the use of ELISA and other Point of Care Testing kits for the detection of NPSs.

    - Assessing the cross reactivity of ELISA with a wide selection of NPS compounds

    - To determine if any cross reactivity for ketamine POCT's is observed with any of the "newer" ketamine alternatives such as MXE.

4. To assess the effects of NBOMes and to test for their presence in rat hair and urine.

    - Administer 25B-, 25C, and 25I-NBOMe at different concentrations to Long Evans rats over a period of 8 days and assess if any analgesia is observed through tail flick tests.

    - To identify the presence of parent NBOMe compounds and their metabolites in rat urine after administration.

    - To identify the presence of NBOMes in rat hair, and determine whether hair colour has any impact on the incorporation of these drugs into the hair.

    - To validate the hair and urine methods in accordance with ISO/IEC 17025 and SWGTOX.

Chapter 2: GC-MS NPS Method Optimisation

2.1 Gas Chromatography- Mass Spectrometry

Gas chromatography (GC) was first mentioned in 1941 by Martin and Synge who suggested using gas-liquid partition chromatography for analytical purposes.(*169*) A liquid sample is injected into the system where it is volatilised into the gaseous phase. This gas sample is then transported through the column by the carrier gas (mobile phase). Analytes are separated out whilst travelling through the column based on the degree of their interaction with silica particles lining the inside of the column (stationary phase). The stronger the chemical interactions between analytes and stationary phase, the longer the analytes will be retained in the column. Analyte size and shape can also affect retention time in the column, with smaller analytes retained less than larger analytes.

In mass spectrometry the molecules contained within the sample are ionised either by electron ionisation (EI) or chemical ionisation (CI) (*170*). EI is the most commonly used ionisation technique and is the technique used in this research. In EI, molecules travel through the interface to the ionisation chamber, where they are bombarded by a stream of electrons. These electrons are emitted from a heated filament and propelled across the ionisation chamber within the source. By colliding with the electrons the molecules lose an electron, therefore becoming positively charged as shown in Equation 2-1. *(171)*

Equation 2-1: The electron ionisation of an atom or molecule.

$$M + e^- \rightarrow M^+ + 2e^-$$

The ionised molecules are then pushed through the ionisation chamber by a positively charged repeller, before travelling through a series of focusing lenses eventually reaching the mass analyser. In this case a quadrupole mass spectrometer (MS) as shown in Figure 2-1.(*172*)

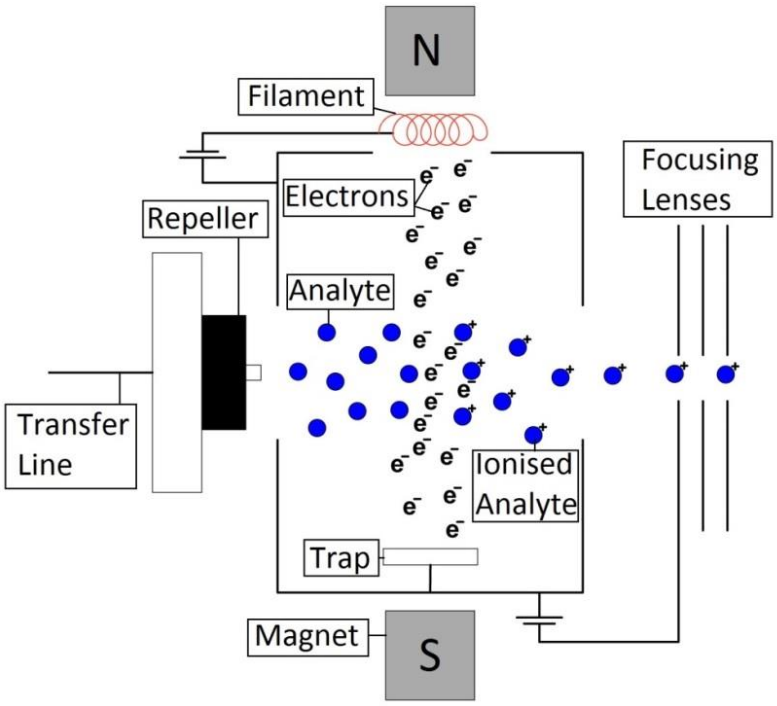


Figure 2-1: Schematic of an EI chamber.

The majority of mass analysers separate out positively charged molecules by their mass to charge ration (m/z).(*173*) A quadrupole MS consists of 4 parallel rods, to which a direct current (DC) and an oscillating radio frequency (RF) signal are applied. To one set of oppositely paired rods a DC current is applied and to the other 2 oppositely paired rods a RF signal is applied. This creates an electrostatic field inside the rods, to which only ions with the correct m/z ratio can travel through. Ions which do not have the correct m/z ratio collide with the rods, become discharged and go to waste.(*172, 174*)

The mass spectrometer can either be used in total ion chromatogram mode (TIC) where all ions over a set range are scanned or in selective ion monitoring (SIM) mode where pre-selected ions only are monitored. TIC is advantageous when it is not known what compounds may be present, whereas SIM analysis is more useful for targeted analysis providing greater sensitivity as only selected ions are being monitored.

The fragmented ions are then detected by an electron multiplier diode which converts the ionized mass fragment to an electronic signal. A schematic of a GC coupled to a mass spectrometer is shown in Figure 2-2.

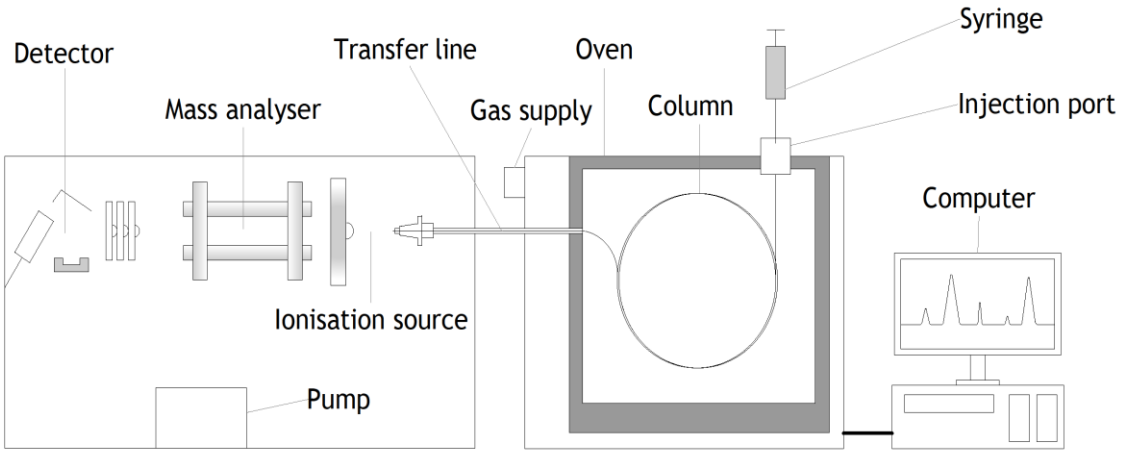


Figure 2-2: Schematic of a GC-MS instrument.

2.2 Sample Preparation

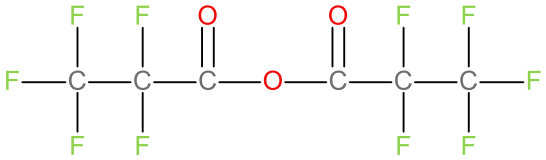
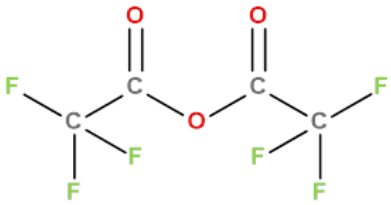
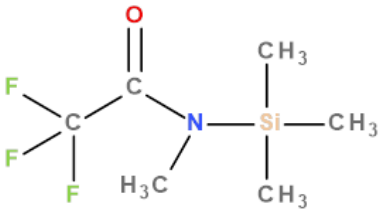
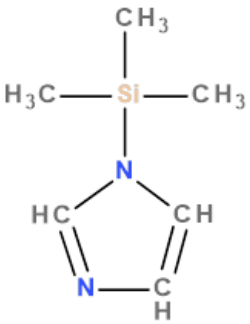
Prior to analysis biological samples must undergo an extraction "clean-up" process, removing proteins and other contaminants. This allows for better analyte detection, increases the life span of the analytical instrument and improves the productivity of the laboratory by reducing maintenance time.(*175*) As part of the sample clean-up process it is necessary to remove any particulate matter, such as proteins from blood and urine, by centrifugation or filtration. Chemical adjustments may be required in order to free any bound analytes, or to enhance the analyte retention if the sample is to be passed through a sorbent during extraction. There are various sample clean-up methods available, varying in their effectiveness, cost and the time they take to carry out. Recovery of analytes from the sample matrix will differ depending on the method chosen and as a result it is important to test as many possible options to ensure that optimum analyte recovery is achieved. As sample preparation is the timeliest part of sample analysis, typically accounting for 80% of sample analysis time, it is important to ensure that the best method chosen is also suitable for the specific needs of the laboratory and that unnecessary steps are avoided.(*176*)

2.2.1 Derivatisation

In order to improve analyte detection it is sometimes necessary to derivatize analytes prior to injection to improve the volatility, thermal stability of the analytes and to make them amenable to GC-MS analysis. Silylation, alkylation

and acetylation are the most commonly used derivatisation reactions and the appropriate method is chosen based upon the chemical structure of the analyte. The chemical structures of the 4 derivatisation agents, pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TMSI), used in this study are shown in Table 2-1. TMSI is used alongside pyridine for the derivatisation of wet sugar samples, hindered hydroxyl groups and alongside fluorinated acylation reagents.

Table 2-1: Chemical structures of derivatisation agents used.

Derivatisation Agent	Reagent Type	Structure
Pentafluoropropionic anhydride (PFPA)	Acylation	
trifluoroacetic anhydride (TFAA)	Acylation	
N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)	Silylation	
N-trimethylsilylimidazole (TMSI)	Silylation	

The limited analytical application notes for the detection of synthetic cathinones via GC-MS also make use of derivatisation to improve peak shape and resolution. Although acetylation appears to be the dominant derivatisation reaction for the detection of NPSs, reagents, temperatures and incubation times vary.(*45, 131, 177-180*)

2.2.2 LLE

Liquid liquid extraction (LLE) is the crudest method of extraction tested here. LLE involves the addition of an immiscible solvent to the sample resulting in the partitioning of analytes and contaminants between the 2 phases.(*181*) The main advantage of LLE is that it is extremely simple to carry out and allows for rapid method development. LLE is however difficult to automate and as a result labour costs can be quite high. This is particularly important for high-throughput laboratories.(*182*) Although the glassware and chemicals used themselves are relatively inexpensive, LLE generally uses higher solvent volumes than the other clean up methods. The disposal costs of these chemicals is an additional factor which must be considered when deciding on an extraction method. Another downfall of LLE is the possibility of emulsion formation, where the organic and inorganic layers do not fully separate, these can usually be broken, but again this leads to additional labour costs.(*183*)

2.2.3 SPE

Solid phase extraction (SPE) is probably the most commonly used sample preparation technique, especially in forensic toxicology. A sample is passed through a column containing a chromatographic sorbent which retains the desired analytes and other sample components.(*184*) The column is then washed to remove additional contaminants and the clean extract is then eluted. Alternatively, the contaminants may be retained by the sorbent, allowing the analyte to pass through.(*185*) There is now a wide variety of SPE columns available commercially, using different sorbent materials, different bed sizes and varying amount of steps. SPE although more complex does allow for automation and the varying sorbent types available also allow for cleaner extracts than using LLE.(*186*)

2.2.4 SLE

Supported liquid extraction (SLE), sometimes referred to as support assisted liquid liquid extraction is a technique available as an alternative to LLE. SLE comprises of cartridges packed with diatomaceous earth which the sample is passed through. Although not a new technique its application to forensic toxicology is relatively recent. This is in part due to the development of

chemically reproducible diatomaceous earth which previously hindered the reproducibility of this method. SLE offers cleaner extracts than LLE as either contaminants or analytes are retained in the diatomaceous earth, depending on solvents used. Based on similar principles to LLE, SLE is also extremely simple to perform, however it uses less solvent and can be automated.(*187*) Another main advantage of SLE is that due to the gravitational flow of sample through the cartridge, the possibility of emulsion formation is significantly reduced.(*188*)

2.3 Aims

This research aimed to develop a method for the detection of a wide range of NPSs by GC-MS, and determine the optimum sample preparation conditions to enhance sensitivity. This was to be achieved by determining the optimum derivatisation conditions, i.e. reagent, incubation time and incubation temperature for their simultaneous analysis. In order to determine the optimum sample extraction procedure, the recoveries of various NPSs using LLE, SPE and SLE methods were to be assessed and compared.

2.4 Materials & Methods

2.4.1 Materials

2.4.1.1 Chemicals & Reagents

Mescaline, 25P, 25T4, and 25T7-NBOMe reference standards were purchased from LIPOMED (MA, USA), as were ethylone, MDPV, naphyrone and butylone. 25B, 25C, 25D, 25E, 25H, 25I, 25N and 25T2-NBOMes were purchased from Cayman Chemical (MI, USA). Flephedrone, mephedrone, methylone, 2-DPMP and MXE were purchased from Cerilliant (TX, USA). 3-MeO-PCE, 3-MeO-PCP, 5-APB, 6-APB, benzedrone, methedrone and methiopropamine were purchased from Logical (MA, USA).

PFPA and TFAA were purchased from Sigma Aldrich (MO, USA). MSTFA was purchased from Pierce (Ill, USA), and TMSI:pyridine (1:4) was purchased from Supelco (PA, USA). All other chemicals were of analytical grade and purchased from Honeywell (MI, USA).

Blank human whole blood, plasma and serum were purchased from Golden West Biologicals Inc® (CA, USA). Blank human urine was collected in house from a willing donor.

All SPE cartridges were supplied by United Chemical Technologies (PA, USA), except Oasis® cartridges which were supplied by Waters (MA, USA). SLE ISOLUTE® cartridges were supplied by Biotage (MN, USA).

2.4.1.2 Saline Solution (0.95%)

Saline solution was prepared by weighing 9.5 g of sodium chloride in a beaker to which approximately 500 mL of dH_2O was added. The solution was then transferred to a 1 L volumetric flask and made up to the 1 L mark using dH_2O.

2.4.1.3 Blank Blood Preparation

Blank (drug-free) human whole blood (500 mL) was measured in a volumetric cylinder. To this an equal amount of 1% saline solution was added. The blood saline solution was then mixed thoroughly, transferred to an amber bottle, and stored in the fridge (4 ^oC) for a maximum of 3 months.

2.4.1.4 Phosphate Buffer Preparation

Phosphate buffer (0.1 M, pH 6) was prepared by dissolving 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 in 500 mL of dH_2O in a beaker. This solution was then transferred to a 1 L volumetric flask and made up to the mark using dH_2O used to rinse the original beaker. The volumetric flask was then stoppered and inverted several times to ensure thorough mixing. The pH was adjusted using monobasic sodium phosphate (to lower pH) or dibasic sodium phosphate (to increase pH). The buffer was then stored at 4^oC for a maximum of 3 months.

2.4.1.5 0.1M Acetic Acid

2.9 mL of glacial acetic acid was transferred to a 500 mL volumetric flask in a fume hood. This was then made up to the mark with dH_2O and mixed. This solution was then stored for up to 6 months at room temperature.

2.4.1.6 2% Acetic Acid

2 mL of glacial acetic acid was transferred to a 100 mL volumetric flask in a fume hood. To this 98 mL of dH_2O was added. The solution was then mixed thoroughly and stored for up to 6 months at room temperature.

2.4.1.7 2% Acetic Acid in Methanol

2 mL of glacial acetic acid was transferred to a 100 mL volumetric flask in a fume hood. This was then made up to the mark with the addition of 98 mL of MeOH. The solution was then mixed thoroughly and stored for up to 6 months at room temperature.

2.4.1.8 MeOH/NH_4OH

To a 100 mL measuring cylinder 95 mL of MeOH was added. To this a further 5 mL of 28% NH_4OH was added. This was then transferred to a glass bottle and stored for 6 months at room temperature.

2.4.1.9 CH_2Cl_2/IPA/NH_4OH

Concentrated 28% NH_4OH solution (2 mL) was added to 20 mL of isopropanol and mixed. To this solution 78 mL of methylene chloride was then added before mixing again. This solution was stored at room temperature and made fresh each day of use. Solution preparation was carried out in a fume hood.

2.4.1.10 PFPA/EtOAc (2:1)

PFPA (2 mL) was transferred to a 7 mL glass vial in a fume hood. To this 1 mL of EtOAc was added. The vial was then capped and inverted several times to ensure thorough mixing. This solution was prepared fresh upon use as required.

2.4.1.11 Tris solution (0.02M)

Tris(hydroxymethyl)aminomethane (Tris) (2.43 g) was weighed and transferred to a 500 mL beaker. To this approximately 300 mL of dH_2O was added. This solution was then transferred to a 1 L volumetric flask which was then made up to the mark using additional dH_2O. This solution was then inverted several times to ensure through mixing.

2.4.1.12 Preparation of Stock Solutions

Stock solutions were produced for each drug individually (25B, 25C, 25D, 25E, 25H, 25I, Mescaline, 25N, 25P, 25T2, 25T4, 25T7-NBOMe, 2-DPMP, 3-MeO-PCE, 3-MeO-PCP, 5-APB, 6-APB, benzedrone, butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methiopropamine methylone, MXE and naphyrone) giving a concentration of 100 µg/mL. This was achieved by transferring 1 mL of each reference drug (1 mg/mL) to a 10 mL volumetric flask and making the solution up to the meniscus using MeOH. The flasks were then inverted several times before each solution was transferred to individual amber glass bottles and stored for 1 year at -20^{o}C.

I.S. stock solutions of mephedrone-D_3, methylone-D_3, ethylone-D_5 and MDPV-D_8 were made by transferring 1 mL of each 1 mg/mL solution into separate 10 mL flasks. These were then made up to the mark using MeOH, inverted several times and stored in amber bottles for 1 year at -20^{o}C. This was not necessary for 25I-NBOMe-D_3 as this was purchased as a 100 µg/mL reference solution.

2.4.1.13 Working Solutions

Using the stock solutions as outlined in 2.4.1.12 four working solution mixtures (10 µg/mL) were made. These mixtures were prepared by transferring 1 mL of each working solution to a 10 mL volumetric flask and making it up to the mark using MeOH. The resulting mixtures were then stored at -20^{o}C for up to 3 months and replaced as and when necessary. **Table** 2-2 shows the drugs contained in each drug mixture. Several working solutions ran out throughout the course of these experiments which could not be replaced due to time restrictions, and thus the contents of each mixture slightly varied over time. Analytes which were not consistently present for all experimental procedures are highlighted with an * and additional information provided below Table 2-2.

Table 2-2: Analytes contained in each working mix solution.

MIX 1	MIX 2	MIX 3	MIX 4
Benzedrone	**2-DPMP	25B-NBOMe	Mescaline-NBOMe
Butylone	3-MeO-PCE	25C-NBOMe	25N-NBOMe
Ethylone	***3-MeO-PCP	25D-NBOMe	25P-NBOMe
Flephedrone	5-APB	25E-NBOMe	**25T2-NBOMe
Mephedrone	6-APB	25H-NBOMe	**25T4-NBOMe
*Methedrone	MDPV	25I-NBOME	**25T7-NBOMe
***Methylone	Methiopropamine		
Naphyrone	MXE		

*Only used for LLE work.
**Unavailable at time of LLE extraction work.
*** Unavailable at time of SPE work.

2.4.1.14 I.S. Working Mix

An internal standard (I.S) working solution mixture was prepared by transferring 1 mL of each 100 µg/mL I.S. working solutions described in section 2.4.1.12 to a 10 mL volumetric flask. To this flask 1 mL of a 100µg/mL of 25I-NBOMe-D_3 reference solution was also added. This flask was then made up to the meniscus using MeOH before being stoppered and inverted several times to ensure thorough mixing to produce a working solution of 10 µg/mL. This mixture contained all 5 I.Ss (mephedrone-D_3, methylone-D_3, ethylone-D_5, MDPV-D_8 and 25-NBOMe-D_3).This was then transferred to a labelled amber glass bottle and stored in the freezer (-20^{o}C) for a maximum of 6 months.

2.4.1.15 Instrumentation

2.4.1.15.1 GC-MS Method Optimisation

Initial method development was carried out on a Thermo Trace GC 2000, equipped with an AS2000 autosampler and Trace Plus mass spectrometer. The GC was fitted with a J&W Agilent DB-5ms low bleed column (30 m x 0.32 mm i.d.; film thickness 0.25 µm). The injection port was operated in splitless mode initially at 250^{o}C. Helium was used as a carrier gas at a flow rate of 1.5 mL/min. The GC transfer line was operated at 250^{o}C and the source at 200^{o}C. The mass spectrometer was operated in EI mode (scan range 40-500 m/z). Data was processed using Xcaliber 1.4 software. Initial method optimisation was carried

out at the University of Glasgow before the method was transferred to a new instrument at the Fredric Rieders Family Renaissance Foundation (FRFRF).

2.4.1.15.2 Sample Preparation

Sample preparation optimisation analysis was carried out using an Agilent GC-MSD 5975C series instrument fitted with a J&W DB-5ms low bleed column (30 m x 0.32 mm i.d.; film thickness 0.25 µm). The GC-MS was operated in full scan mode with an EI source and a splitless injection. The injection port temperature was 225°C; the transfer line temperature was 250°C with a MS source temperature of 200°C. The initial oven temperature was 80°C, which was held for 2 mins, before ramping to 170°C at a rate of 25°C /min and being held for 1 min. The temperature was then further increased to 200°C at 5°C /min and held for 1 min before being increased to 250°C at a rate of 15°C /min. Finally, the oven temperature was increased to 300°C at a rate of 5°C and held for 3 minutes. The total run time was 30 minutes. Data was analysed using Agilent's ChemStation software.

Samples were either evaporated by nitrogen using a Pierce 18830 Reacti-Therm III heating module with Pierce Reacti-Vap or air-dried using a TurboVap® LV sample concentrator (American Instrument Exchange, MA). Samples were spun using a Combo V24 centrifuge (LW Scientific, GA). All pipetting was carried out using Capp Bravo accurate pipettes (Pipette.com, CA). Positive displacement pipettes used were Gilson Microman® (Pipette.com, CA).

2.4.2 Methods

2.4.2.1 GC-MS method optimisation

All drugs were run individually as standards in order to obtain their mass spectra and retention time. This was achieved by evaporating 10 µl of each stock solution (100 µg/mL) at room temperature, derivatising using 50 µl PFPA:EtOAc (2:1) for 40 minutes at 70°C before evaporating to dryness again. These reference standards were then reconstituted in 100 µl of EtOAc, and transferred to labelled GC-MS vials.

In order to separate compounds as fully as possible different oven temperature programs and injector port temperatures were tested. An adapted version of the in-house FMS amphetamine method (method 1) was used as a starting point, having previously resulted in successful identification and quantification of mephedrone.(*189*) Varying GC-MS oven parameters were tested (Table 2-3) during initial phases of method development. The GC oven parameters were altered several times as new compounds were added to the method to ensure chromatographic separation where possible. The injection port temperature was altered during method development, using 250°C, 225°C and 200°C.

Table 2-3: Optimisation of GC oven conditions

	Method Number				
	1	2	3	4	5
Initial Temperature	55°C	80°C	80°C	80°C	80°C
Hold Time	2 min	2 min	2 min	2 min	2 min
Ramp 1	20°C/min to 200°C	20°C/min to 200°C	25°C/min to 170°C	25°C/min to 170°C	25°C/min to 170°C
Hold	-	-	3 min	3 min	1 min
Ramp 2	10°C/min to 250°C	10°C/min to 250°C	5°C/min to 200°C	5°C/min to 250°C	5°C/min to 200°C
Hold	-	-	2.5 min	2.5 min	1 min
Ramp 3	-	-	-	-	15°C/min to 300°C
Final Ramp	25°C/min to 300°C	25°C/min to 300°C	25°C/min to 300°C	50°C/min to 300°C	5°C/min to 300°C
Final Hold	3 min	3 min	2 min	2 min	3 min
Total Run Time	18.5 min	17 min	23 min	30 min	30 min

2.4.2.2 Derivatisation

To 120 test tubes 100 µl of each drug mix (10 µg/mL) was added. Samples were then evaporated to dryness using the TurboVap® LV sample concentrator. To 30 test tubes, 50 µl of PFPA:EtOAc (2:1) was added. To another 30 samples 100 µl of MSTFA:Toluene (1:3) mixture was added. A further 30 samples were derivatized using 100 µl TMSI:pyridine (1:4) and the final 30 samples were derivatized using 100 µl of TFAA:EtOAc (2:1). Each set of derivatized samples was then incubated at room temperature (24°C), 37°C, 50°C or 70°C for 20 or 40

minutes as illustrated by Figure 2-3. Each temperature and incubation time was analysed in triplicate. Samples were then evaporated to dryness using the TurboVap® LC before being reconstituted in 100 µl of EtOAc and analysed by GC-MS. The GC-MS was operated in full scan mode as each derivatisation agent would result in its own specific ions.

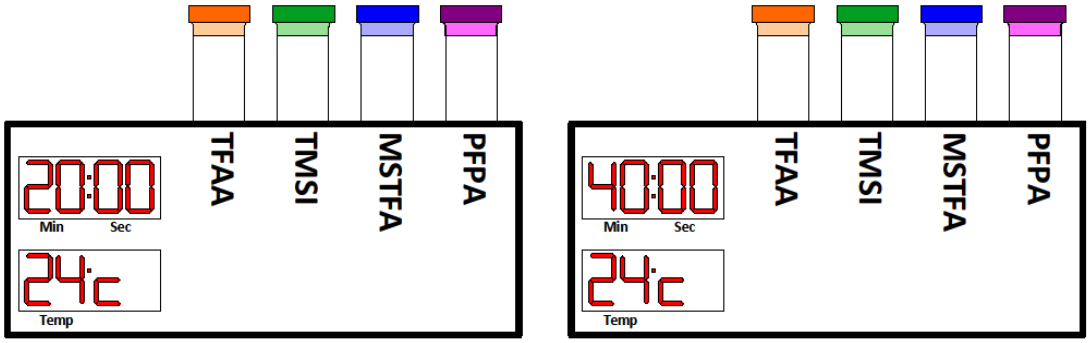


Figure 2-3: Diagram of derivatization at 24°C for 20 minutes and 40 minutes.

2.4.2.3 Unextracted standards

In order to assess the recovery for each extraction method, unextracted standards were prepared. These were made separately for each of the experiments so that any inter day instrumentation differences would not affect the comparison results. Unextracted standards were made by pipetting 100 µl of each 10 µg/mL solution mixture and I.S. mixture into a glass culture tube. This was completed in triplicate, before evaporating each tube to dryness using a steady stream of nitrogen, with no added heat. The unextracted standards were then derivatised using 50 µl of PFPA:EtOAc for 40 minutes at 70°C before being evaporated to dryness once again. The unextracted standards were run on GC-MS alongside their extracted counterparts.

2.4.2.4 LLE Methods

LLE was investigated as an extraction method for urine only. This is due to the relative simplicity of this matrix in comparison to blood which requires much more clean up prior to analysis by GC-MS. As LLE is a relatively crude extraction method it is typically used when analysing by LC-MS rather than GC-MS.

Two different LLE methods were analysed and compared for the extraction of analytes from urine. Method 1 had previously been published for the extraction of NBOMes from blood and urine; however this method only examined the use of this method for this specific group. The second method was based upon that published for the detection of 2,500 toxic compounds.(*190*) As this method had been utilised for the detection of such a wide and varied number of compounds it was surmised that it may be applicable to NPSs. Although this method used CH_2Cl_2 it was exchanged for t-MTBE which has been shown to be effective on the extraction of amines.(*191*)

Blank urine (1 mL) was added to 6 different culture tubes. These were then each spiked with 100 µl of all 4 working solutions (10 µg/mL) detailed in section 2.4.1.1. Three of these were then extracted using method 1, 0.1 M NaOH solution and 3 mL of extraction solvent (hexane:EtOAc (50:50)). The remaining 3 were extracted using method 2, by adding 3 mL of t-MTBE and 200 µl of tris solution. These methods are shown in Figure 2-4.

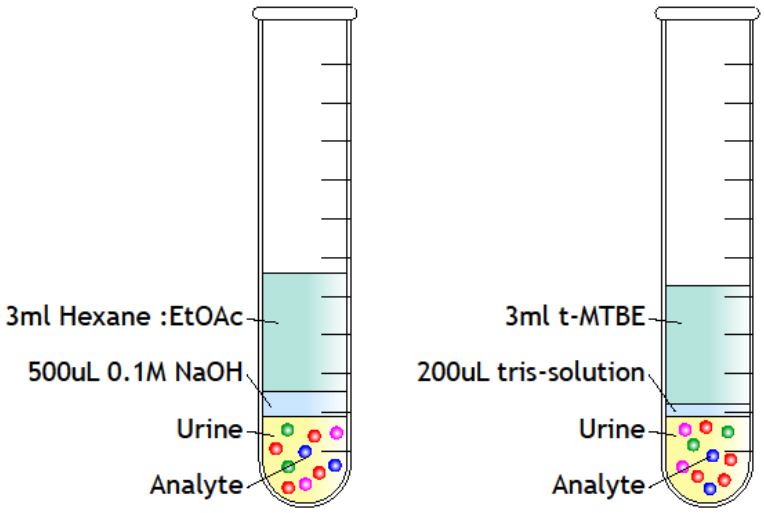


Figure 2-4: Schematic of the two LLE methods.

The culture tubes were then capped and placed on the sample rotator for 10 minutes after which they were centrifuged at 4000 rpm for 10 minutes. After centrifugation the top layer was transferred to a new culture tube, spiked with 100 µl of I.S. mixture and vortexed for 30 seconds. The contents of each culture

tube were then evaporated to dryness under a steady stream of nitrogen at room temperature (23ºC). PFPA:EtOAc derivatising agent (50 µl) was then added and each culture tube was then re-capped and left to incubate for 20 minutes at 37ºC. The derivatising agent was then evaporated off and samples were reconstituted in 100 µl of EtOAc and transferred to GC autosampler vials for analysis. Unextracted standards were analysed alongside these.

2.4.2.5 SPE Method

Blank MeOH, urine, blood, plasma and serum samples (1 mL) were all spiked with 100 µL of each 10 µg/mL working solution mixture. To each sample, 1 mL of 0.1 M phosphate buffer (pH 6) was added before centrifugation for 10 minutes at 4000 rpm. Samples were then extracted in triplicate using various solid phase extraction cartridges; UCT's XCEL® I, ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z as well as Waters Oasis® MCX.

ZSDAU020 cartridges are routinely used at the University of Glasgow where as CSDAU133 are the cartridge of choice at the Frederic Rieders Family Renaissance Foundation, hence the comparison of these cartridges. DAU cartridges are UCT's main cartridge type for forensic analysis and are the most commonly encountered in publication applicable to acid, basic and neutral drugs. XCEL® I cartridges require no cartridge conditioning and so are popular in clinical settings due to the reduced extraction time. As it has been shown that many NPS users present to A&E with adverse effects, it is important to assess how clinical SPE cartridges may perform for the extraction of these substances. XTRACKT cartridges use similar co-polymeric bonded phases to that of the Clean Screen varieties; however the sorbent bed weight is much larger as these cartridges are designed for larger particles. Oasis® MCX cartridges claim to be able to "achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups". The individual specifications of each cartridge are shown below in Table 2-4.

Table 2-4: Cartridge specifications

Cartridge	Sorbent Type	Sorbet Bed Weight (mg)	Load Volume (mL)	Phase
Clean Screen®	ZSDAU020	200	10	Co-polymeric bonded phase
Clean Screen®	CSDAU133	130	3	Reverse phase, ion exchange co-polymeric bonded phase
Clean Screen®	XCEL® I	130	3	Sample prep phase designed to reduce extraction steps
XTRACKT®	XRDAH206	200	6	Co-polymeric bonded phase, gravity flow-large particle
XTRACKT®	XRDAH502	500	3	Copolymeric bonded phase, gravity flow-large particle
XTRACKT®	XRPCH50z	500	10	Propylsulfonic acid cation exchanger
Oasis®	MCX 6cc	500	6	Mixed-mode polymeric sorbent

ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z cartridges were conditioned using 2 mL MeOH, 2 mL dH_2O and 2 mL phosphate buffer before loading samples. Cartridges were washed using 2 mL dH_2O, 1 mL 0.1 M acetic acid and 2 mL MeOH. The cartridges were then left to dry at full vacuum for 5 minutes. Samples were eluted using 3 mL CH_2Cl_2/IPA/NH_4OH (78:20:2) solution.

Samples were loaded directly to the XCEL® I cartridges and washed with 1 mL 2% acetic acid/98% MeOH before elution with 3 mL CH_2Cl_2/IPA/NH_4OH (78:20:2).

Oasis® cartridges were conditioned with 1 mL MeOH and 1 mL dH_2O prior to loading samples. These were then washed using 2 mL 2% acetic acid and 1 mL MeOH prior to elution with 2 mL MeOH/NH_4OH (95:5) solution. Due to a limited number of available cartridges only blood and urine were extracted using these. The different SPE procedures are illustrated in Figure 2-5.

I.S. mix (50 µl) was added to the collection tubes prior to elution. Post extraction, samples were evaporated using a stream of nitrogen, derivatised

using 50 µL of PFPA:EtOAc (2:1) at 70°C for 40 minutes, before being evaporated again and reconstituted in 100 µL of EtOAc. Samples were analysed in triplicate by GC-MS. Fresh unextracted standards were analysed alongside these samples.

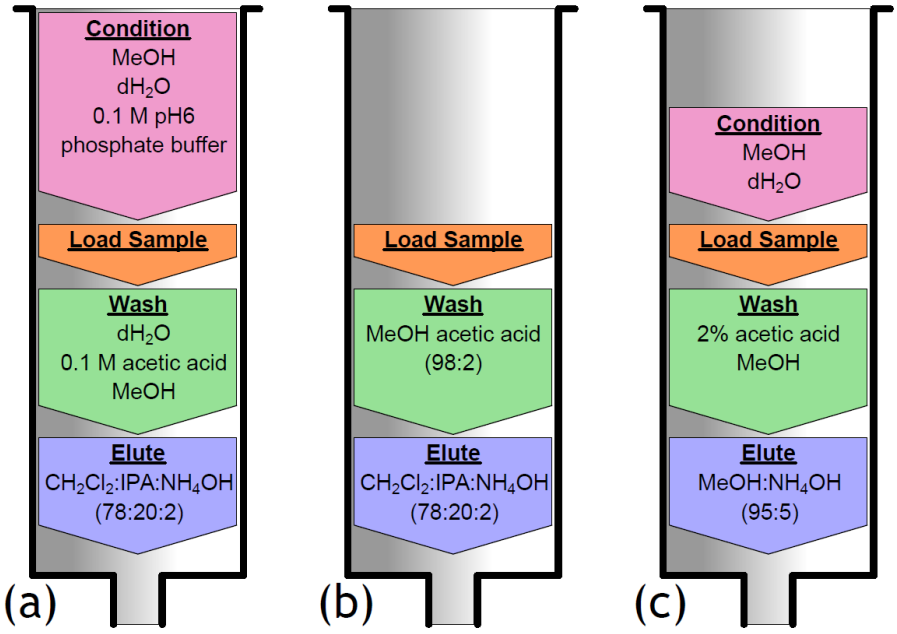


Figure 2-5: Diagram showing different extraction procedures for each type of SPE cartridge. ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z were extracted using method (a). XCEL® I were extracted using method (b) and Oasis® were extracted using method (c).

The recovery for each extraction method was calculated using Equation 2-2. The recovery values were then averaged across all 3 replicates.

Equation 2-2: Calculation used to determine analyte recovery

$$Recovery = \frac{PAR\ extracted}{PAR\ Unextracted} \times 100\%$$

2.4.2.6 SLE Method

Blank MeOH, urine, blood, plasma and serum samples (1 mL) were all spiked with 100 µL of each 10 µg/mL work solution mixtures (section 2.4.1.1). Samples were pH adjusted using 1% NH_4OH, centrifuged for 10 minutes at 4500 rpm and loaded directly to Biotage's® ISOLUTE® SLE+ columns. These columns have a sample load volume of 2 mL (including any pre-treatment solution required) and allow for the extraction of a wide range of analytes from aqueous samples.(*192*) Previous application notes have been published for the extraction of NBOMes

from oral fluid using these cartridges and thus they were investigated for their use of these compounds plus additional NPSs from additional matrices. (*193*)

The samples were held on the columns for 5 minutes before being eluted twice with 4 mL of EtOAc as depicted in Figure 2-6. I.S. mix (50 µl) was added to the collection tubes prior to elution. Post extraction, samples were evaporated using a stream of nitrogen, derivatised using 50 µL of PFPA:EtOAc at 70°C for 40 minutes, before being evaporated again and reconstituted in 100 µL of EtOAc. Samples were analysed in triplicate by GC-MS.

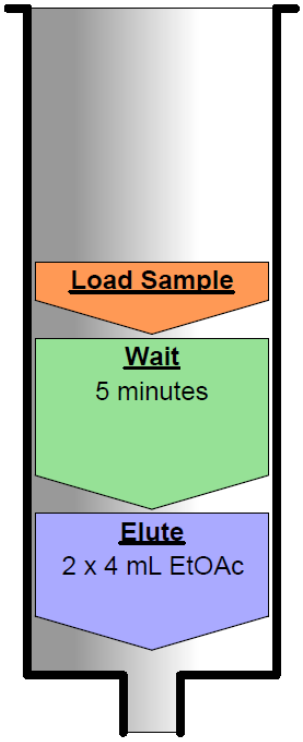


Figure 2-6: Diagram of SLE method

The extraction efficiency of the SLE cartridges was compared to that of SPE. SPE extraction was carried out in triplicate using CSDAU020 cartridges using the method described in section 2.4.2.5. SPE and SLE results were compared against methanolic unextracted standards to determine % recovery using Equation 2-2. The difference between the SLE and SPE results was calculated using Equation 2-3.

Equation 2-3: Calculation used to determine the difference between SPE and SLE recovery

$$Difference\% = SLE\ Recovery\ (\%) - SPE\ Recovery\ (\%)$$

2.5 Results & Discussion

2.5.1 GC-MS Method Optimisation Results

The GC-MS temperature alterations listed in Table 2-3 occurred as more drugs were added to the method. The stage at which each drug was added to the method is shown in Figure 2-7. The corresponding deuterated I.S. was added at the same time as their un-deuterated counterparts. Drugs were added in stages as reference standards became available, and as standards were received by FMS as part of the EWS.

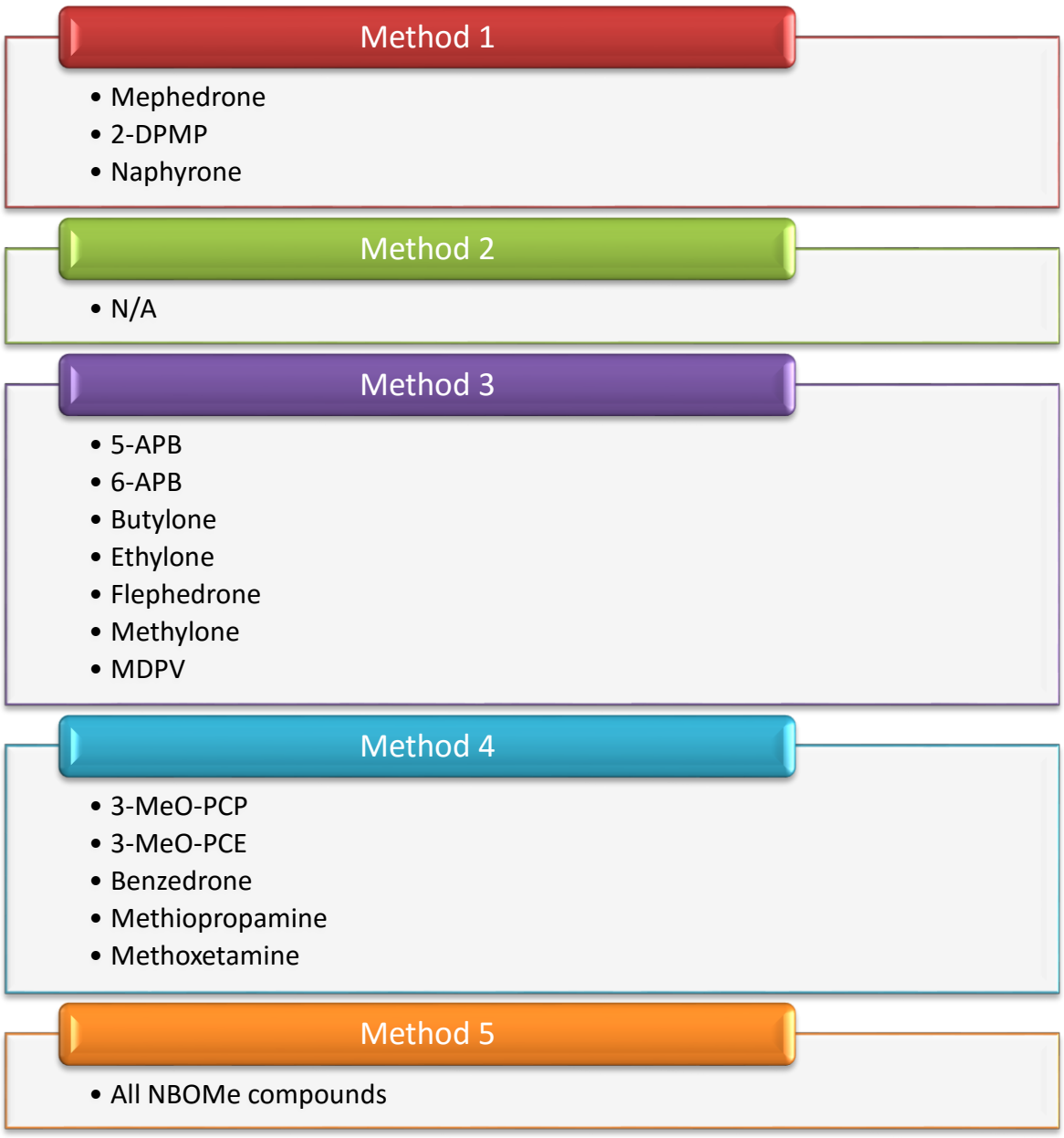


Figure 2-7: Point at which each drug was added during method development.

Method 1 was used when only 2-DPMP, mephedrone, and naphyrone were included. Initially mephedrone eluted at 13 minutes, and so the starting temperature was increased from 50°C to 80°C as shown in method 2.

After the development of method 2, 5-APB, 6-APB, butylone, ethylone, flephedrone, methylone and MDPV were added to the method. The initial temperature ramp was increased to 25°C until 170°C was reached and a 3-minute hold was added to enhance separation of 5-APB and 6-APB. In order to separate butylone and ethylone, the 2nd temperature ramp rate was reduced to 5°C/min and a 2.5-minute hold was added. The initial ramp was increased to compensate for the additional hold times to keep the method as short as possible.

The addition of 3-MeO-PCE, 3-MeO-PCP, benzedrone, methiopropamine and methoxetamine to the method meant that the 2nd ramp of 5°C/min was extended until the oven temperature reached 250°C. This was to try and chromatographically separate out 3-MeO-PCP and 2-DPMP.

Finally, the addition of NBOMe compounds to the method resulted in oven method 5 in Table 2-3. Chromatographic separation of these compounds was extremely difficult as they eluted during the final hold at 300°C. As higher temperatures are required to elute NBOMes it was necessary to slow the final oven ramp to 5°C/min and extend the final hold time to ensure maximum chromatographic separation. It was also necessary to add in a third ramp of 15°C/min prior to this to help separate earlier eluting NBOMe compounds. Temperature ramps 1 and 2 were then reduced where possible in order to keep the total run time at 30 minutes. Using a lower injection port temperature (225°C) was deemed more favourable in order to avoid any analyte degradation in the injection port.

Alterations to the GC-MS oven methods were limited by a maximum column temperature of 325°C and as a result total chromatographic separation of all NBOMe compounds was not possible. It was also not possible to fully chromatographically separate 2-DPMP and 3-MeO-PCE either. The separation of these analytes however was possible using their mass spectra which was significantly different after derivatization using PFP:EtOAc as highlighted in

Figure 2-8. The mass spectra of each PFPA derivatised analyte in order of elution time is shown in Appendix 1.

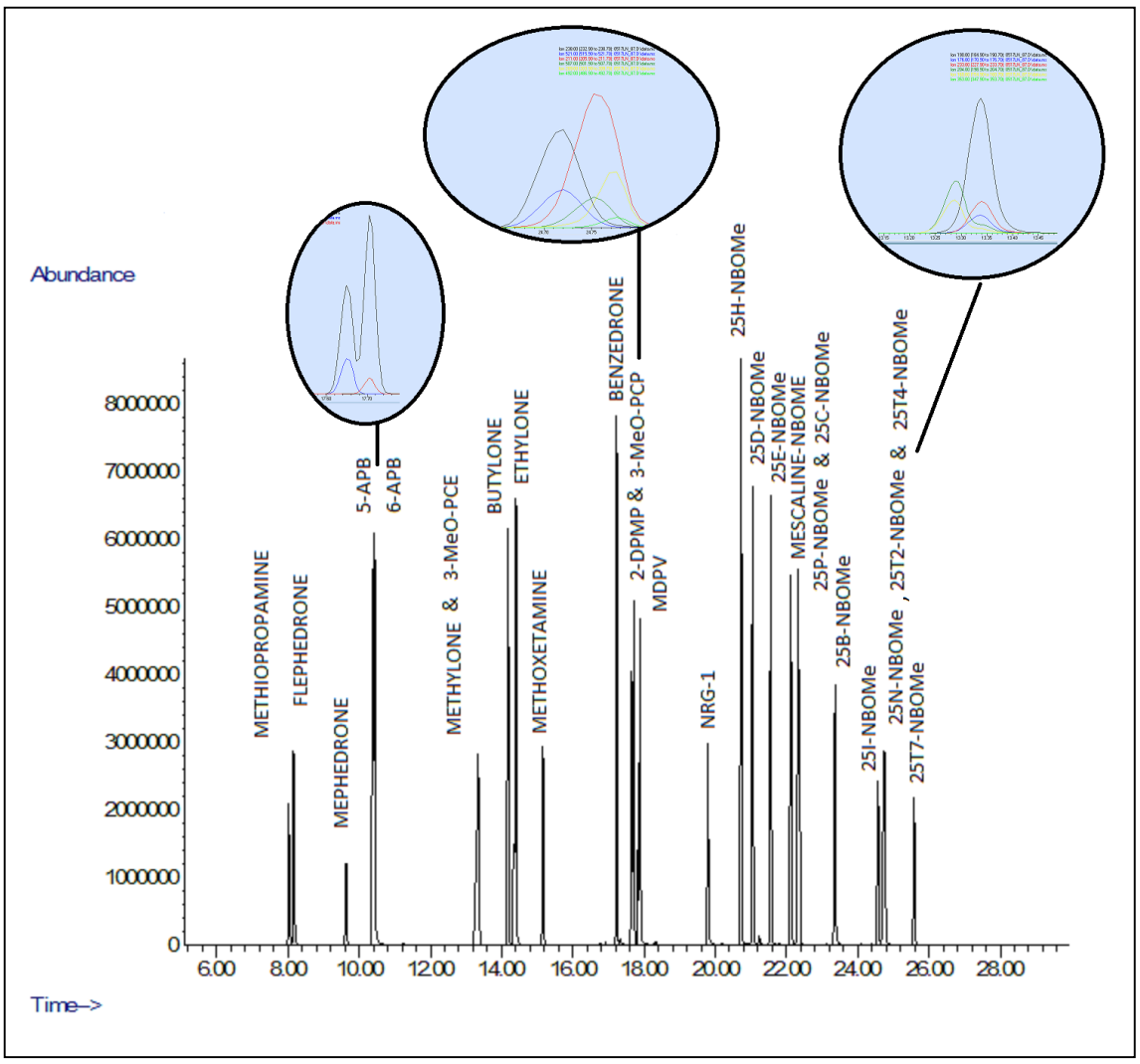


Figure 2-8: Chromatogram showing the elution of all compounds. Closely eluting compounds are highlighted, and their separation is shown using ions.

Due to the high potency of NBOMes and the low concentrations detected in reported fatalities the GC-MS method was altered from total ion chromatogram (TIC) to selective ion mode (SIM), thus allowing for lower limits of detection (LOD). The reconstitution amount was also lowered to 50 µl rather than the initial 100 µl to further enhance sensitivity. A chromatogram showing all drugs and their retention times when possible is shown in Figure 2-8.

The retention times, target ions and qualifier ions used for the detection and identification of each analyte are shown in Table 2-5.

Table 2-5: Retention times and ions used for the identification of each analyte.

Drug	RT (min)	TGT Ion	Q1	Q2
METHIOPROPAMINE	7.99	124	204	160
FLEPHEDRONE	8.11	123	204	160
MEPHEDRONE-D_3	9.56	207	163	326
MEPHEDRONE	9.61	204	160	323
5-APB	10.33	131	158	190
6-APB	10.39	131	158	190
METHYLONE-D_3	13.21	207	163	356
METHYLONE	13.26	204	160	353
3-MeO-PCE	13.30	190	233	176
BUTYLONE	14.14	149	218	121
ETHYLONE-D_5	14.29	223	191	372
ETHYLONE	14.36	218	190	367
MXE	15.14	190	219	134
BENZEDRONE	17.19	91	119	148
3-MeO-PCP*	17.63	272	230	188
2-DPMP*	17.68	165	230	176
MDPV-D_8	17.80	134	133	135
MDPV*	17.86	126	149	110
NAPHYRONE	19.76	126	155	127
25H-NBOMe	20.74	164	121	447
25D-NBOMe	21.01	178	121	461
25E-NBOMe	21.52	192	475	121
Mescaline-NBOMe	22.06	194	181	477
25P-NBOMe	22.28	206	193	489
25C-NBOMe	22.32	198	481	185
25B-NBOMe	23.30	121	244	525
25I-NBOMe-D_3	24.43	124	576	188
25I-NBOMe	24.52	121	573	185
25T4-NBOMe	24.68	238	521	183
25T2-NBOMe	24.71	211	507	224
25T7-NBOMe	25.54	225	238	521

2.5.2 Derivatization

The optimum derivatization temperature and time for each compound using each reagent is shown in Table 2-6, alongside the peak area response. From this table it is evident that PFPA is the optimum derivatising agent which should be used when derivatising NPSs, as it provided the most abundant peak areas for the majority of compounds, correlating with previously published work.(*194*)

Cathinones generally derivatised better at lower temperatures (24-37^{o}C) with the exception of PFPA:EtOAc where they were able to withstand much higher temperatures, with 5 out of 8 compounds having their largest peak area at 70^{o}C. It is unsurprising that lower temperatures were favoured with some of the more amphetamine type compounds, as it is known that these drugs are extremely volatile. Longer incubation times also appeared more favourable for MSTFA and TFAA derivatizations.

NBOMes also preferred lower incubation temperatures, which contradicts previously published methods using higher temperatures. Previously published literature incubated these samples at 70^{o}C for 40 minutes, which this study found not to be necessary and could in fact be detrimental to the concentrations detected. The previous research focused on the detection of NBOMes in blotter papers, thus concentrations are much higher and as a result it may be that any compound loss would only have an effect on GC-MS analysis when trying to identify these compounds for toxicological purposes.

Out of the remaining analytes (2-DPMP, 3-MeO-PCE, 3-MeO-PCP, 5-APB, 6-APB, methiopropamine and methoxetamine) all except 2-DPMP were best derivatised using PFPA:EtOAc. Again lower temperatures (24/37^{o}C) were favoured by all compounds with the exception of methiopropamine (70^{o}C) using PFPA:EtOAc.

Table 2-6: Optimum conditions for each derivatization agent.

Drug	PFPA			MSTFA			TFAA			TMSI		
	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)	Time (mins)	Peak Area
25B-NBOMe	37	20	4244681	50	20	1099776	50	40	943432	37	20	1570304
25C-NBOMe	37	20	5022450	50	20	1198676	50	40	1277952	37	20	1144834
25D-NBOMe	37	20	7346805	50	20	2302976	50	40	1189888	37	20	1333760
25E-NBOMe	37	20	364609	50	20	2108928	50	40	754488	37	20	1756672
25H-NBOMe	24	40	7005366	50	20	2195968	50	40	1187328	37	20	1421312
25I-NBOMe	37	20	5491689	50	20	1747968	50	40	746481	37	20	1193472
25N-NBOMe	70	20	1612858	50	20	420480	50	40	292736	37	20	1637376
25P-NBOMe	37	40	5828767	50	20	2041856	50	40	1161216	37	20	535616
25T2-NBOMe	37	20	1866410	50	20	420480	50	40	292736	37	20	1637376
25T4-NBOMe	37	20	1868777	50	20	1181184	50	40	207744	37	20	790656
25T7-NBOMe	37	20	1188352	50	20	423040	50	40	190720	37	20	612736
2-DPMP	24	40	247792	24	40	1246720	37	40	1907712	37	20	1475072
3-MeO-PCE	24	40	5064590	24	40	971392	37	40	621056	37	20	794624
3-MeO-PCP	37	20	2806468	24	40	387648	50	40	141440	37	40	1052160
5-APB	24	40	7344962	24	40	1523712	24	40	3688627	UNDETECTED		
6-APB	24	40	7630914	24	40	845681	24	40	3761448	UNDETECTED		
BENZEDRONE	70	20	7931314	24	40	455424	37	40	2001920	UNDETECTED		

Table 2-6: Optimum conditions for each derivatization agent (cont.)

Drug	PFPA			MSTFA			TFAA			TMSI		
	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)	Time (mins)	Peak Area	Temp (^{O}C)		
BUTYLONE	24	40	917665	24	40	139712	37	40	1661440	UNDETECTED		
ETHYLONE	24	40	2831935	24	40	676800	37	40	1603072	UNDETECTED		
FLEPHEDRONE	70	20	2635889	UNDETECTED			37	40	520128	UNDETECTED		
MDPV	24	40	8579133	24	40	1329664	37	40	2387968	37	20	2707456
MEPHEDRONE	70	20	1148247	24	40	561088	37	40	886208	UNDETECTED		
MESCALINE-NBOMe	37	40	5826486	50	20	1088512	50	40	1156608	37	20	4419072
METHIOPROPAMINE	70	20	2668526	UNDETECTED			37	40	307264	UNDETECTED		
METHOXETAMINE	24	40	3509882	24	40	351168	37	40	330816	37	20	648260
METHYLONE	70	20	1892738	24	40	905728	37	40	1285623	UNDETECTED		
NAPHYRONE	70	20	7107450	24	40	2045440	37	40	914496	37	20	2280448

Figure 2-9 shows the percentage of analytes which had an optimum incubation temperature of 24°C, 37°C, 50°C and 70°C with PFPA derivatisation. This shows that 37°C was the overall optimum incubation temperature for analytes derivatised with PFPA:EtOAc. At higher temperatures the caps on the culture tubes would "pop" off due to the build-up of gases inside, and as a result we see much higher %CVs for 70°C, as shown in

Appendix 1 through Appendix 4 . This could be remedied by using screw top culture tubes although these are costlier. The optimum incubation time was 20 minutes which accounted for 80% of optimum PFPA:EtOAc derivatisations as shown in Figure 2-10.

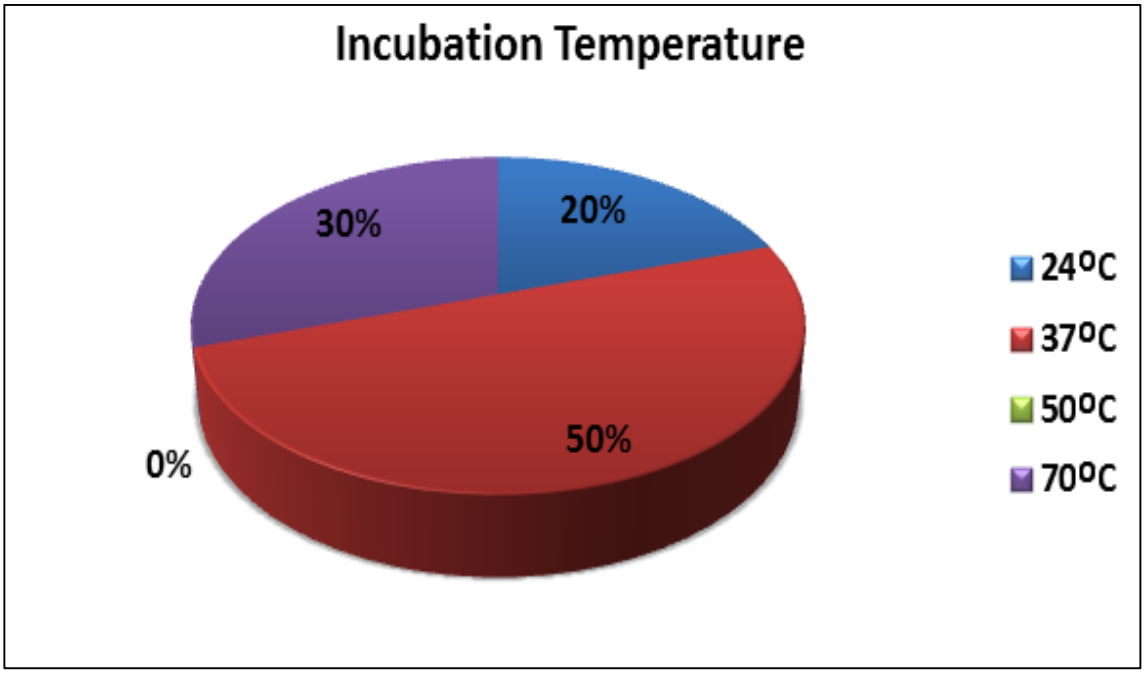


Figure 2-9: Chart showing % of NPS analytes (n=20) with optimum incubation temperatures of 24°C, 37°C,50°C, or 70°C

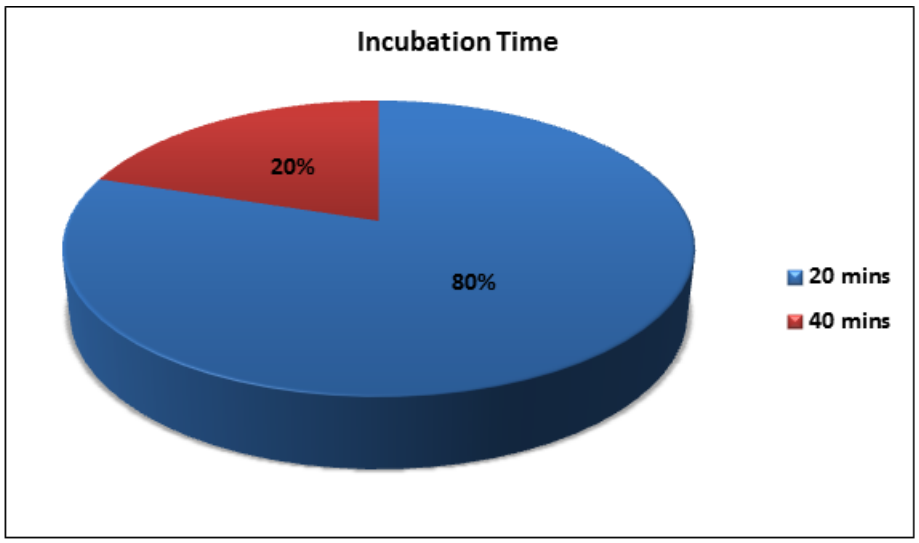


Figure 2-10: Chart showing % of NPS analytes (n=20) with optimum incubation times of 20 or 40 minutes.

Overall PFPA:EtOAc (Figure 2-11) worked much better as a derivatising agent than MSTFA, TFAA or TMSI. TFAA did however allow for the detection of all NPSs contained within the GC-MS method which was not the case for MSTFA or TMSI.

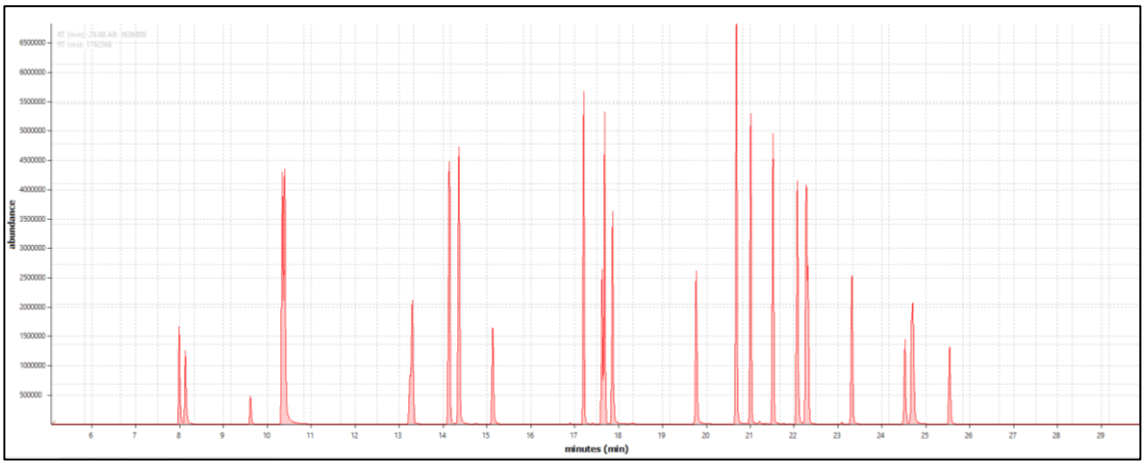


Figure 2-11: Chromatogram of various NPSs derivatised using PFPA:EtOAc (1 mg/L).

A chromatogram of all analytes after TFAA derivatisation is shown in Figure 2-12. TFAA produced similar mass spectra to that of PFPA:EtOAc which is unsurprising as both are acylation agents. The peak areas however, were much lower than those of PFPA:EtOAc showing that PFPA:EtOAc is the preferred acylation agent out of the two. This could be in part due to the use of EtOAc with PFPA which acts as an acid receptor and promotes reactivity. Had a suitable base been used alongside TFAA an improvement in the resulting peak areas may have been seen.

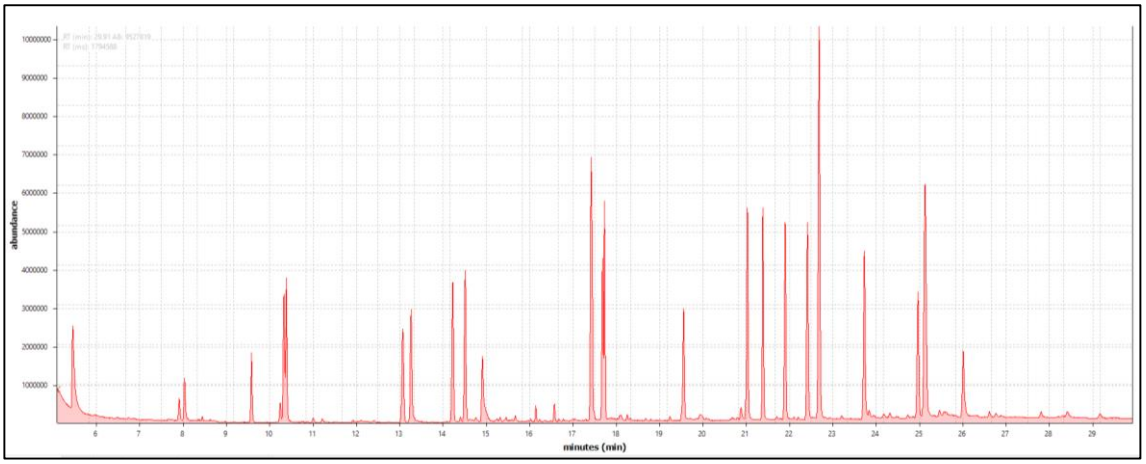


Figure 2-12: Chromatogram of various NPSs derivatised using TFAA (1 mg/L)

A chromatogram of all analytes after derivatisation with MSTFA is shown in Figure 2-13. This derivatisation agent led to the identification of all analytes with the exception of methiopropamine and flephedrone. This may be due to the increased interferences found at the beginning of this chromatogram which could have masked these analytes or that these analytes eluted before the 5minute solvent window. The use of MSTFA lead to the NBOMe compounds all producing similar mass spectra, and thus do not allow for the identification of all NBOMes when in a mixture. This is due in part to the derivatising agents having no effect on the structure of these analytes failing to actually derivatise. This highlights the need to derivatise analytes to enhance their uniqueness.

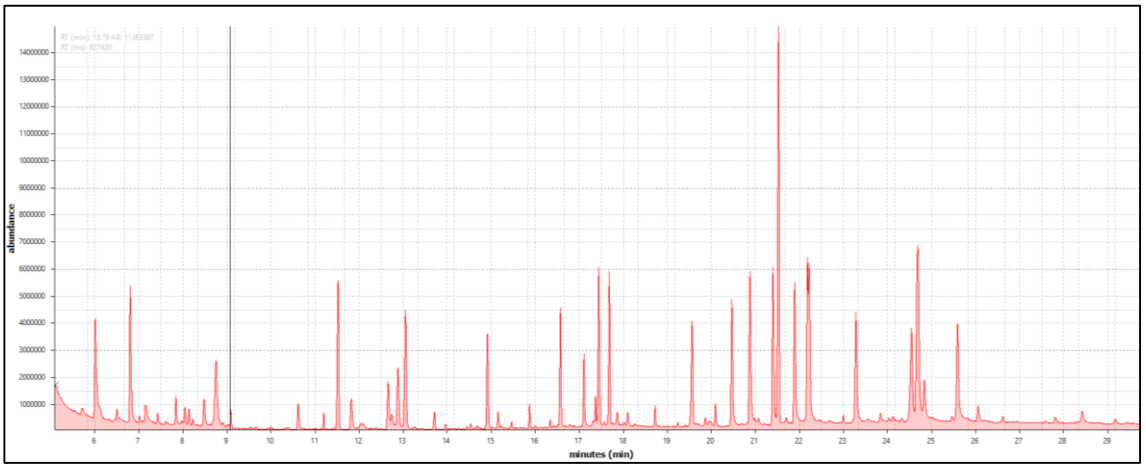


Figure 2-13: Chromatogram of various NPSs derivatised using MSTFA (1 mg/L).

The use of TMSI is not advised as 7 of the 25 analytes tested were not detected after derivatisation with this reagent. This is unsurprising as TMSI directly does

not react with amines and amides and hence would have no impact on these substances. The use of TMSI does however highlight the importance of derivatising these compounds in order to detect them. In order for TMSI to derivatise amines and amides the reagent must be used as part of a multi-derivatising protocol, which is both time consuming and more costly.(*195*) The use of TMSI also resulted in a large solvent peak lasting 8 minutes as shown in Figure 2-14, which could lead to the masking of early eluting compounds such as methiopropamine and flephedrone.

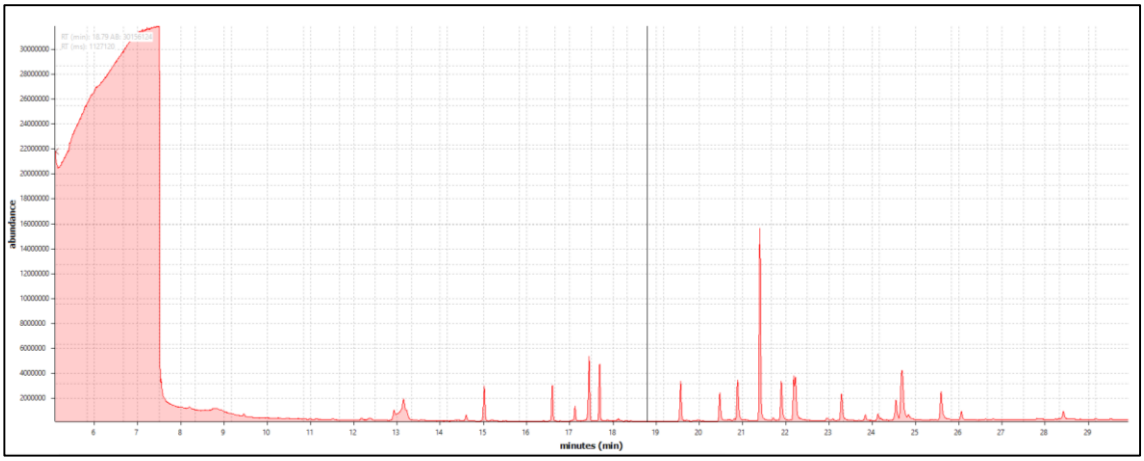


Figure 2-14: Chromatogram of 28 NPSs derivatised with TMSI (1 mg/L).

The NBOMe spectra produced using TMSI also failed to produce unique spectra, with the main ions repeating throughout the series (91, 121, 150 and 194) as shown in Appendix 4. This is particularly problematic for compounds such as 25P-NBOMe and 25C-NBOMe which could not be chromatographically resolved, and therefore depending on unique ions being extracted to confirm identify of the NBOMe in question.

One limitation of this study is the lack of suitable I.S. available resulting in the comparison of peak areas rather than PARs. This is particularly important as these results were obtained over a 3-day period. In order to overcome this, system suitability mixtures ran before each batch were compared and assessed to ensure that the instrument was working to the same standard each day. When overlaid (Figure 2-15) it is clear that the instrument was performing to the same standard and thus the differences observed during this study are not due to instrumental deterioration but due to the effects of the derivatising agents

themselves. The system suitability mixtures contained various amphetamines and benzodiazepines.

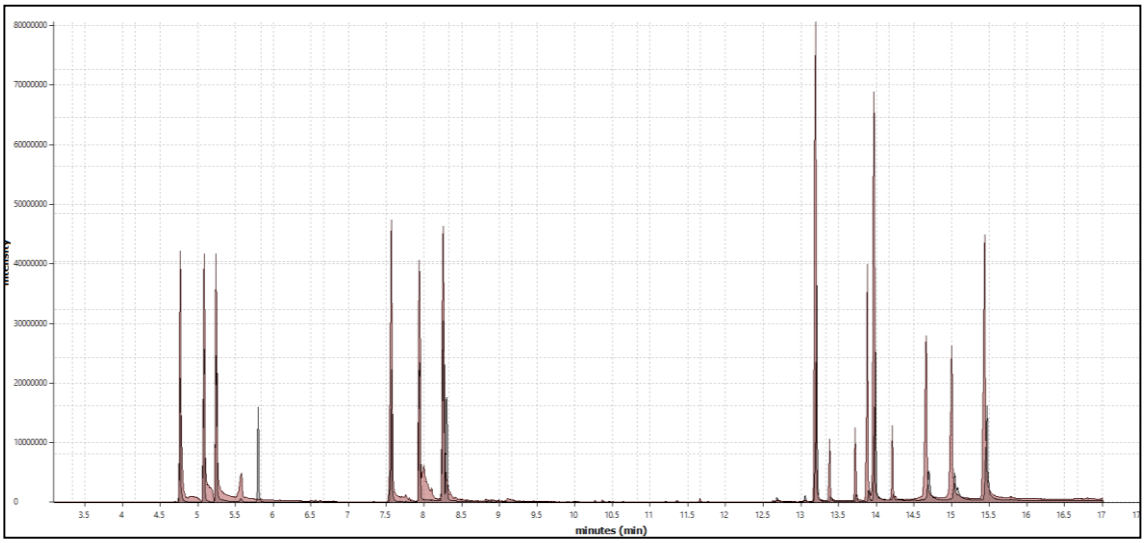


Figure 2-15: Overlaid chromatograms of system suitability mixtures ran for each of the 3 days (1 mg/mL)

2.5.3 LLE

Table 2-7 shows a comparison of the two LLE methods used for the 23 NPSs studied in urine. These results show that for 15 of the 23 analytes tested method 2 was the better LLE method; neither method allowed for the extraction of all analytes from urine. Method 1 was unable to extract flephedrone or methiopropamine and method 2 was unable to extract methoxetamine from urine.

Method 1 resulted in an average recovery of 55.4% (ranging 0-88.4%), whereas method 2 resulted in an average recovery of 68.9% (ranging 0- 129.5%), a 13.5% improvement. 25I-NBOME saw the highest recovery of 88.4% using method 1, whereas flephedrone was the highest recovered analyte of method 2 (129.5%).

Synthetic cathinones failed particularly using LLE method 1, with average recovery rates of 40.3%. NBOMes compared to other NPSs fared much better with recovery rates of 81.2% and 75.8% respectively for each method. This is less surprising as this method has been previously used in the literature for the analysis of NBOMes using LC-MS/MS.(*161*) Both methods produced clean extracts with no visible matrix contamination.

Table 2-7: LLE extraction recovery (%) results

Analyte	Method 1		Method 2		Difference between method 1& 2 (%)
	Recovery (%)	%CV	Recovery (%)	%CV	
25C-NBOMe	84.5	8.6	100.5	4.5	(-16.0)
25D-NBOMe	86.3	5.4	88.0	7.6	(-1.7)
25ENBOMe	79.9	9.6	93.9	9.2	(-14.0)
25H-NBOMe	87.3	10.5	79.6	5.6	7.8
25I-NBOMe	88.4	13.7	118.9	3.1	(-30.5)
25N-NBOMe	79.9	6.5	102.7	10.5	(-22.8)
25P-NBOMe	68.7	8.6	112.6	6.5	(-43.9)
3-MeO-PCE	67.0	7.3	12.8	7.9	54.2
3-MeO-PCP	85.2	14.2	13.2	8.9	72.0
5-APB	11.2	13.9	16.8	3.4	(-5.6)
6-APB	11.1	12.8	18.6	6.7	(-7.5)
BENZEDRONE	87.5	6.3	74.2	9.2	13.3
BUTYLONE	65.6	9.7	66.3	6.2	(-0.8)
ETHYLONE	41.6	9.6	29.0	13.6	12.7
FLEPHEDRONE	N.D.	12.1	129.5	12.6	(-129.5)
MDPV	87.4	9.2	63.0	10.5	24.4
MEPHEDRONE	2.6	9.2	82.0	5.5	(-79.4)
MESCALINE-NBOMe	87.7	5.6	60.1	9.5	27.6
METHEDRONE	29.1	7.9	38.4	11.5	(-9.4)
METHIOPROPAMINE	N.D.	9.5	46.4	5.6	(-46.4)
METHOXETAMINE	15.5	10.2	N.D.	6.8	15.5
METHYLONE	13.7	13.1	69.5	4.5	(-55.9)
NAPHYRONE	93.8	7.5	100.0	9.6	(-6.2)

***N.D. - none detected**

2.5.4 SPE Cartridges

All cartridges were able to extract each NPS from each matrix, with the exception of the XRPCH50z cartridge which failed to extract any of the 25 NPSs tested as shown in Table 2-8.

Table 2-8: Optimum cartridge for the extraction of various NPSs from blood, urine, plasma and serum (% recovery).

DRUG	BLOOD		URINE		PLASMA		SERUM	
	Cartridge Type	% Recovery	Cartridge Type	% Recovery	Cartridge Type	% Recovery	Cartridge Type	% Recovery
25B-NBOMe	CSDAU	86	CSDAU	85	ZSDAU	117	XCEL 1	71
25C-NBOMe	CSDAU	83	CSDAU	85	ZSDAU	76	ZSDAU	85
25D-NBOMe	CSDAU	102	XRDAH 206	109	ZSDAU	117	ZSDAU	111
25ENBOMe	CSDAU	110	CSDAU	110	ZSDAU	111	ZSDAU	108
25H-NBOMe	CSDAU	99	CSDAU	139	XCEL 1	51	ZSDAU	81
25I-NBOMe	CSDAU	90	ZSDAU	90	XCEL 1	47	ZSDAU	118
25N-NBOMe	ZSDAU	81	ZSDAU	98	ZSDAU	105	ZSDAU	118
25P-NBOMe	XRDAH 206	69	ZSDAU	76	ZSDAU	63	ZSDAU	62
25T2-NBOMe	XRDAH 206	111	XRDAH 206	108	ZSDAU	66	XCEL 1	81
25T4-NBOMe	CSDAU	105	CSDAU	105	ZSDAU	64	XCEL 1	70
25T7-NBOMe	XCEL 1	106	XCEL 1	106	ZSDAU	115	ZSDAU	107
2-DPMP	CSDAU	88	CSDAU	115	CSDAU	89	CSDAU	97
3-MeO-PCE	XRDAH 206	45	XCEL 1	96	XRDAH 206	60	ZSDAU	50

Table2-8: Optimum cartridge for the extraction of various NPSs from blood, urine, plasma and serum (% recovery).

Drug	BLOOD		URINE		PLASMA		SERUM	
	Cartridge Type	% Recovery	Cartridge Type	% Recovery	Cartridge Type	% Recovery	Cartridge Type	% Recovery
5-APB	CSDAU	49	XCEL 1	95	CSDAU	63	CSDAU	71
6-APB	ZSDAU	27	XCEL 1	94	ZSDAU	45	CSDAU	51
BENZEDRONE	XRDAH 206	47	OASIS®	124	XRDAH 206	48	XRDAH 502	47
BUTYLONE	XCEL 1	80	OASIS®	119	XCEL 1	101	XCEL 1	87
ETHYLONE	ZSDAU	115	OASIS®	87	XCEL 1	79	XCEL 1	79
FLEPHEDRONE	CSDAU	65	CSDAU	81	CSDAU	67	CSDAU	63
MDPV	CSDAU	106	XCEL 1	105	XCEL 1	109	XCEL 1	100
MEPHEDRONE	CSDAU	112	CSDAU	101	CSDAU	88	CSDAU	126
Mescaline-NBOMe	XCEL 1	49	XCEL 1	50	ZSDAU	41	XRDAH 502	90
METHIOPROPAMINE	OASIS®	52	XCEL 1	80	XCEL 1	27	ZSDAU	43
METHOXETAMINE	XRDAH 206	56	XCEL 1	70	XRDAH 206	66	ZSDAU	58
NAPHYRONE	XRDAH 502	40	XCEL 1	113	XRDAH 502	42	XRDAH 502	47

Figure 2-16 shows the cartridge which provided the highest % recovery for each analyte from blood. From this data it is shown that the CSDAU033 cartridge was the most efficient overall, accounting for 48%. This was followed by the ZSDAU020 cartridge which accounted for 12%. Both these cartridges contain a reverse phase and an ion exchange co-polymeric bonded phase, and only differ by their bed weight, 130mg and 200mg respectively. Both have already been used for the extraction of NPSs from biological matrices in literature and it is therefore unsurprising that these provided the best results. (*189, 196, 197*)

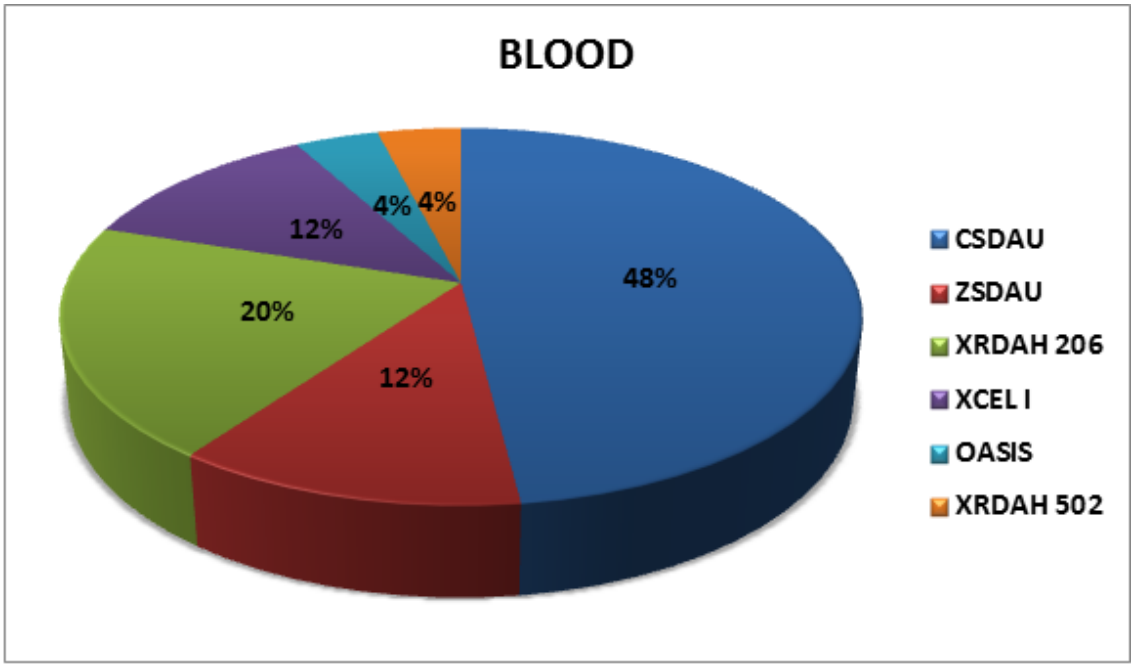


Figure 2-16: The % of analytes (n=25) with the highest recovery using each cartridge from blood.

Figure 2-17 shows the cartridge which provided the highest % recovery for each analyte from urine. From this data it is apparent that XCEL I cartridges favoured this matrix (36%), followed by CSDAU cartridges (32%). This cartridge is commonly used in clinical toxicology as it requires no column conditioning, importantly reducing sample analysis time. As a result however it typically performs better for simple matrices.

Waters Oasis® cartridges although able to extract drugs from blood (4%), were unable to do so without significant sample breakthrough, leading to discoloured samples. This in turn would increase instrument maintenance down-time which is an additional cost to consider, especially for high-throughput laboratories.

These cartridges did however favour slightly better when extracting analytes from urine (12%), providing the highest recoveries for benzedrone (124%), butylone (119%) and ethylone (87%). Vazquez-Roig was able to analyse 14 different illicit drugs from surface water samples using the Waters Oasis® MCX cartridge. This research combined with previous research suggests that these cartridges could be more useful when analysing less complex matrices. This cartridge had a bed size of 500 mg and this may have played a role in sample breakthrough.

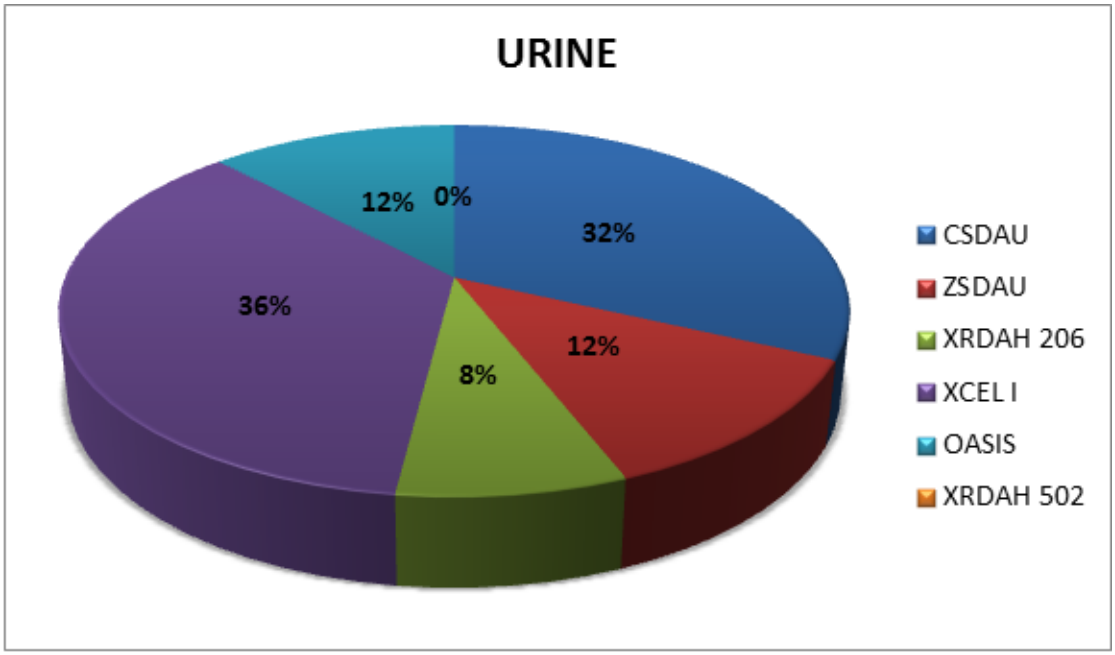


Figure 2-17: The % of analytes (n=25) with the highest recovery using each cartridge from urine.

Figure 2-18 shows the cartridge which provided the highest % recovery for each analyte from plasma. ZSDAU performed significantly stronger than any other cartridge type here, providing the highest recovery for 44% of analytes. XCEL I performed better extracting the 25 NPSs from plasma (24%) in comparison to blood (12%).

No difference was observed when using XRDAH 502 cartridges to extract these substances from blood or plasma with only 4% of analytes having the highest recovery using these cartridges. Previous research showed the successful use of the XRDAH506 cartridges for the recovery of MDPV, mephedrone and other stimulant compounds from waste water, however this was not the case here, as shown in Table 2-8.(*198*)

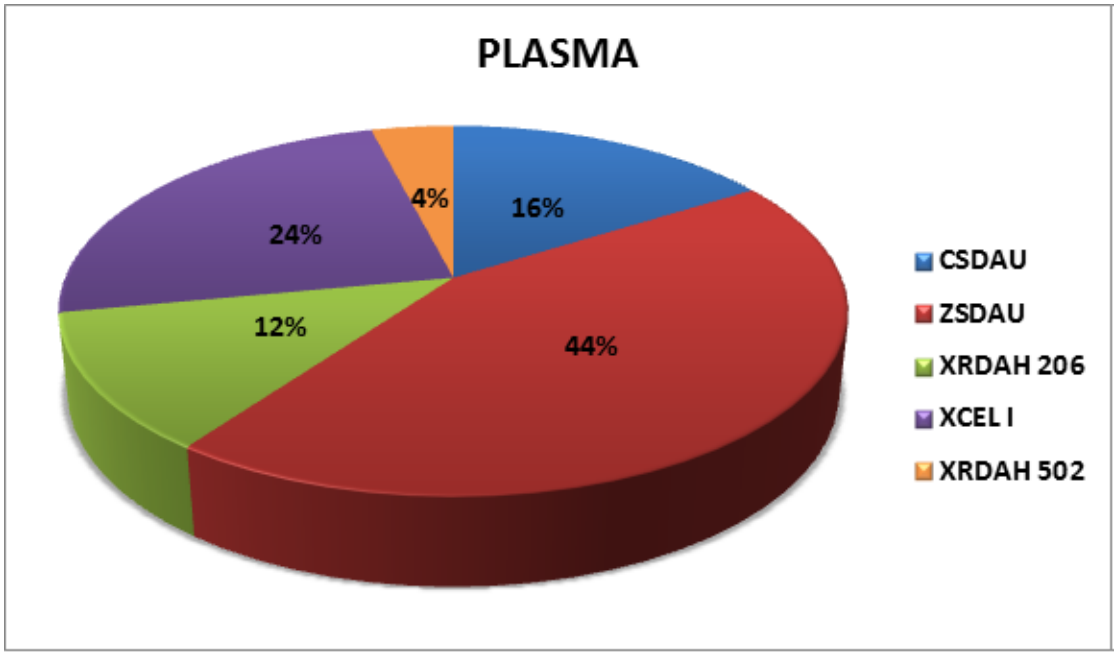


Figure 2-18: The % of analytes (n=25) with the highest recovery using each cartridge from plasma.

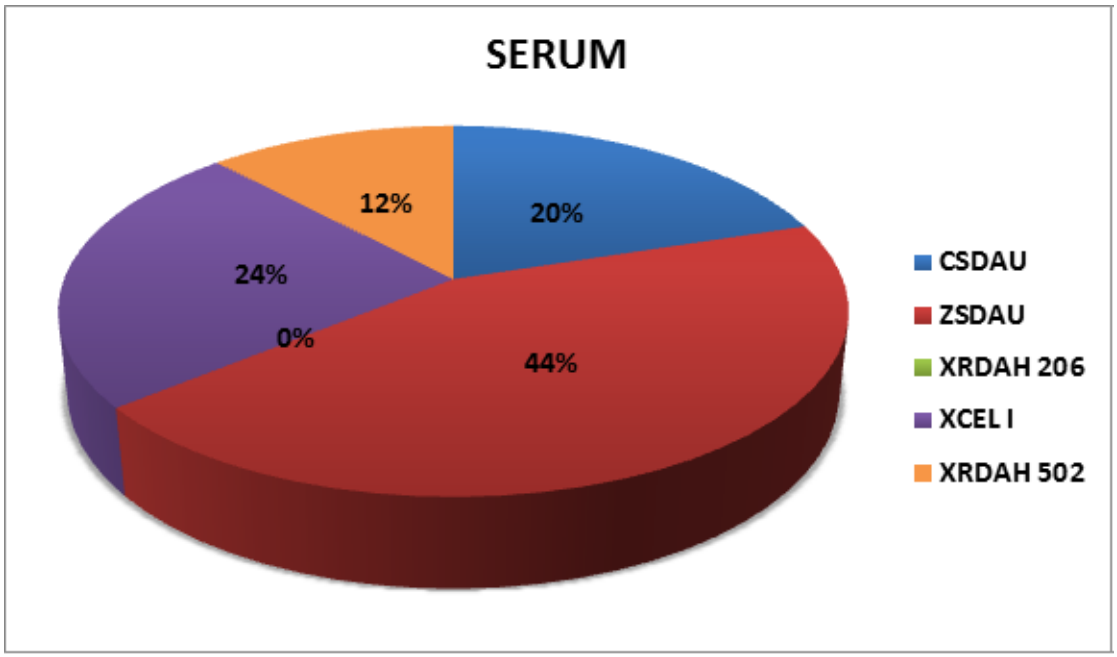


Figure 2-19: The % of analytes (n=25) with the highest recovery using each cartridge from serum.

Figure 2-19 shows the cartridge which provided the highest % recovery for each analyte from serum. XRDAH 502 cartridges performed slightly better than previous matrices (12%). ZSDAU cartridges again performed strongly providing the strongest recovery for 44% of analytes tested. CSDAU cartridges performed slightly better extracting the various NPSs from serum (20%) in comparison to

plasma (16%). XRDAH 206 cartridges did not provide the highest recovery for any analytes extracted from serum.

2.5.5 SLE / SPE Cartridge Comparison

Similar to SPE, SLE extracted all analytes from all matrices. Results are shown in Figure 2-20 through to Figure 2-23. SLE provided higher recoveries than SPE when extracting drugs from blood as shown in Figure 2-20, with 17 out of the 26 analytes (65%) having higher recoveries when SLE was used. Naphyrone saw the biggest increase (47%) when compared to SPE. The poorest recovered analyte using SLE in comparison to SPE was methiopropamine which decreased 47% against SPE.

SPE proved to be the better technique when extracting from urine, producing higher recoveries for 17 (65%) of the 26 analytes tested, as shown in Figure 2-21. SPE was significantly better at extracting ethylone from urine showing a 51% increase versus SLE. Conversely to blood, methiopropamine was the most successfully recovered analyte versus SPE, with the SLE recovery showing a 47% increase.

Plasma samples showed a strong preference for SPE with all except 3 analytes, 25N-NBOMe, flephedrone and methiopropamine, having higher recoveries using this method versus SLE (+88%) as shown in Figure 2-22. 25N-NBOMe was the best analyte recovered from SLE (143%), and ethylone was the poorest (15%). 2-DPMP showed a recovery of 70% using both methods. The recovery of 6-APB from plasma using SLE and SPE saw the biggest difference in the comparative % recoveries. When extracted using SPE, 98% of this analyte was recovered in comparison to using SLE where only 34% of analyte was able to be recovered, a decrease of (-64%). Similar results were seen with MXE (-63%), ethylone (-58%) and 5-APB (-52%), showing that SPE really is the favoured extraction method for these analytes from plasma. Oppositely methiopropamine saw the largest comparative difference between the 2 methods, with SLE improving recovery by 62% in comparison to SPE.

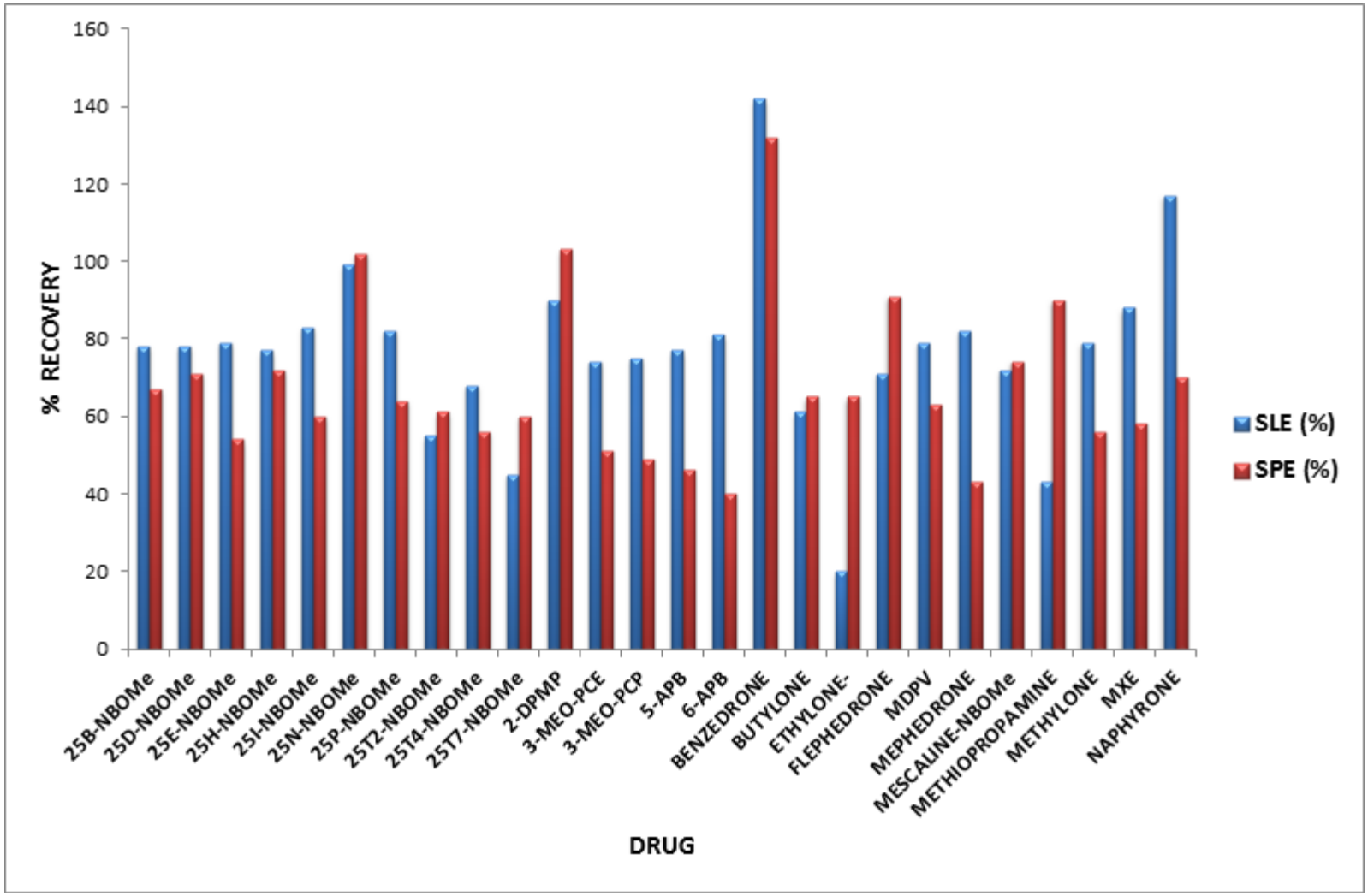


Figure 2-20: Comparison of SPE and SLE for the extraction of NPSs from blood.

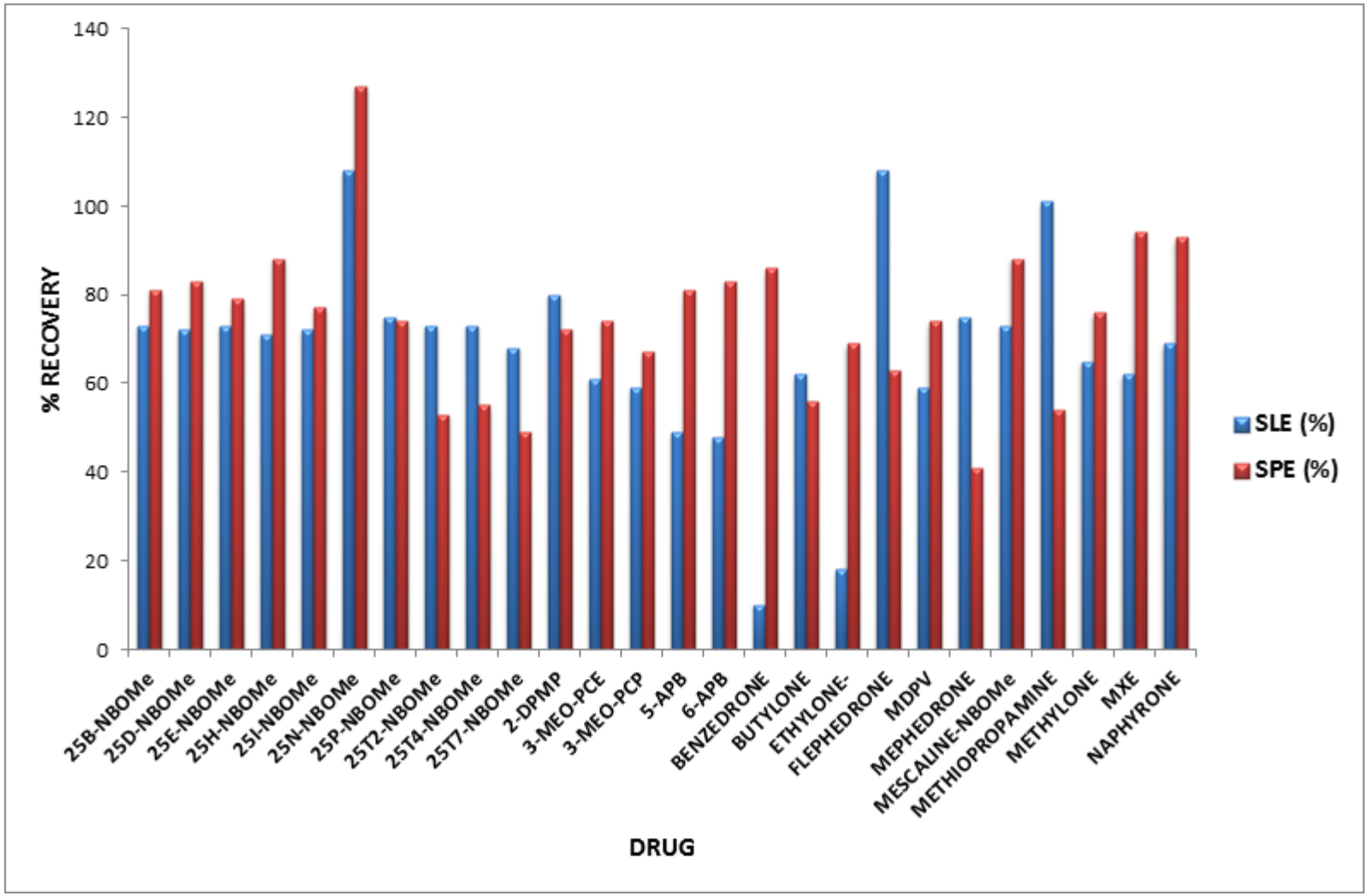


Figure 2-21: Comparison of SPE and SLE for the extraction of NPSs from Urine.

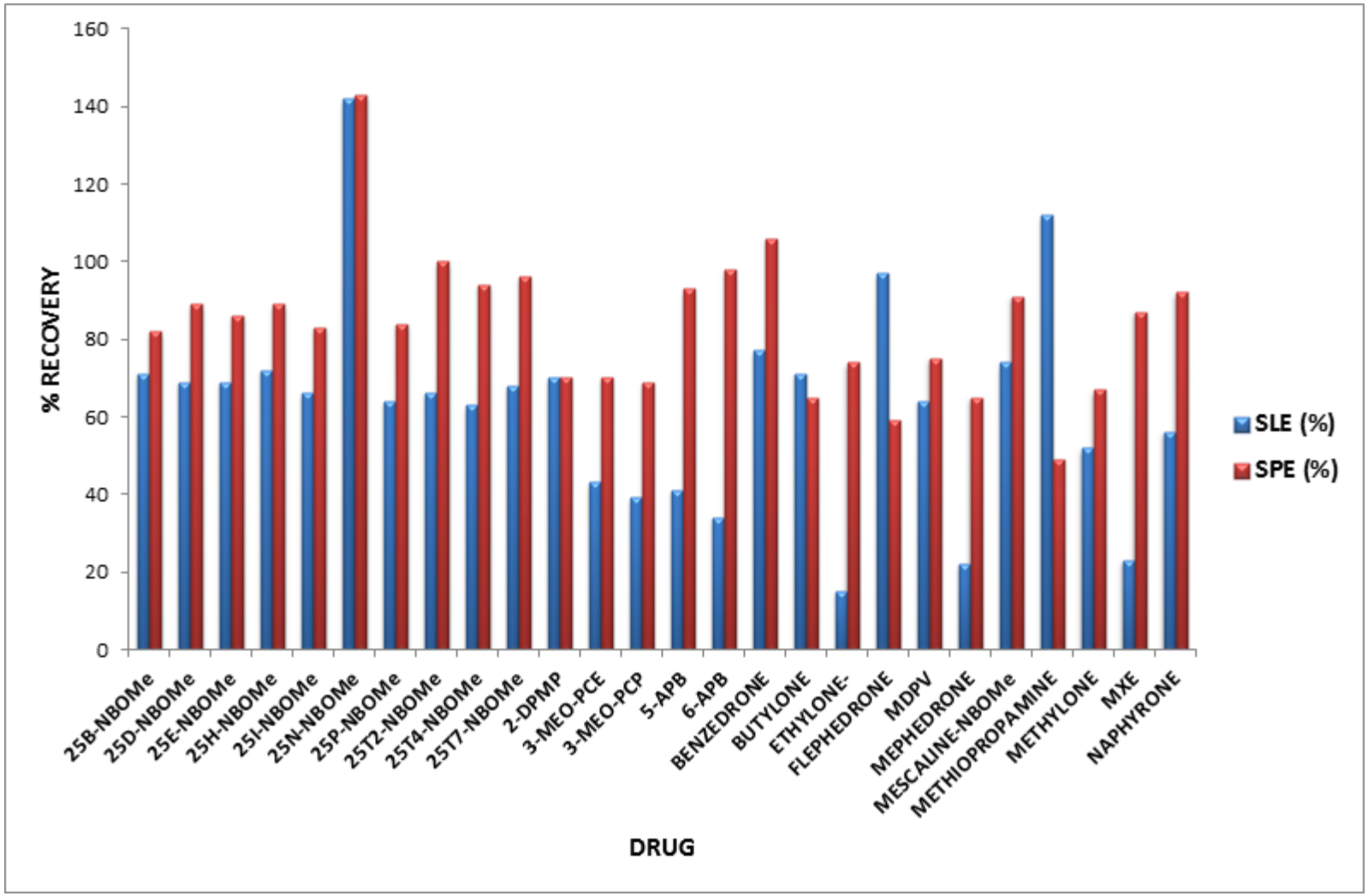


Figure 2-22: Comparison of SPE and SLE for the extraction of NPSs from plasma.

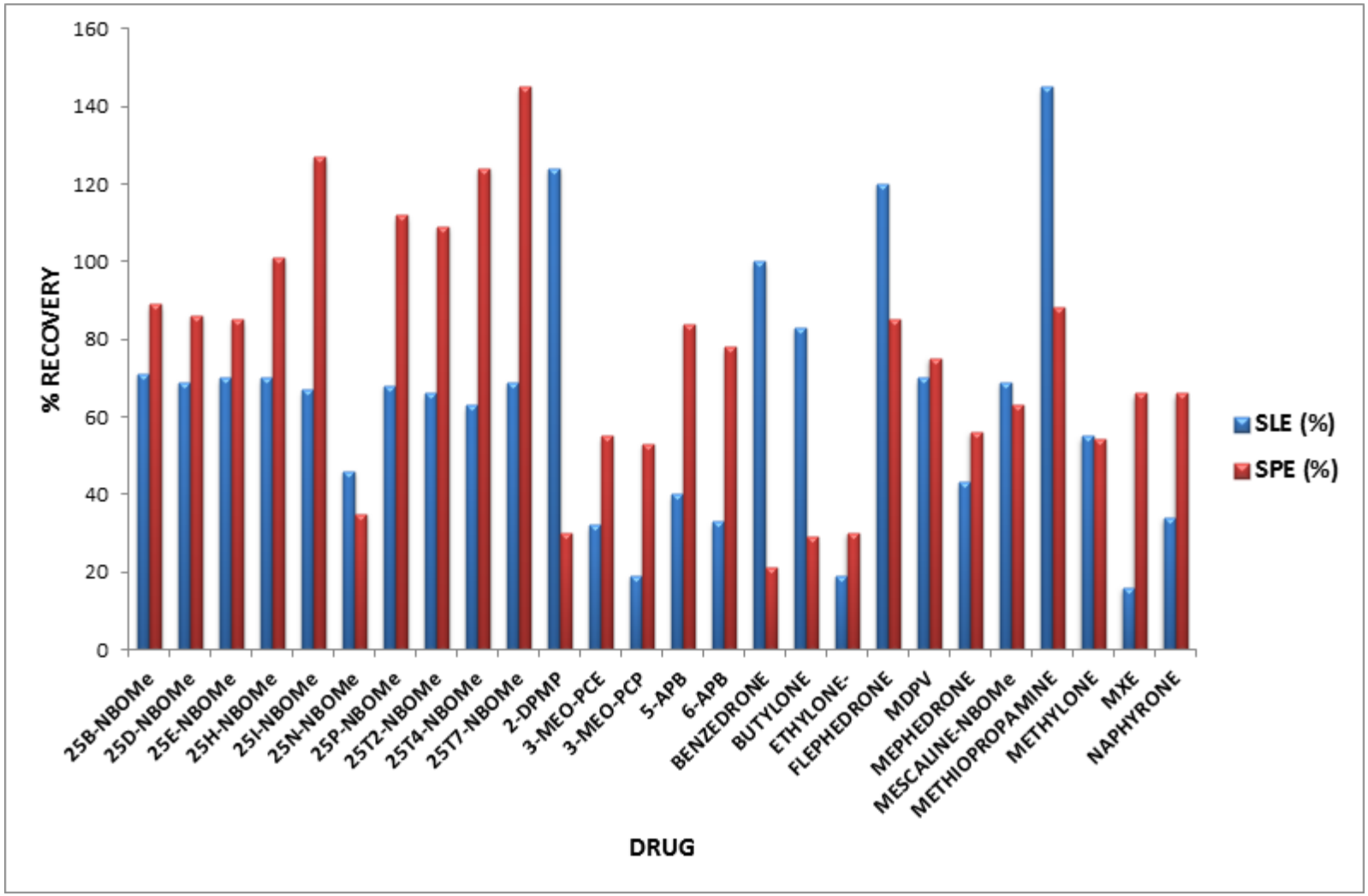


Figure 2-23: Comparison of SPE and SLE for the extraction of NPSs from serum.

Again all analytes were extracted from serum samples as shown in Figure 2-23. 2-DPMP gave a good recovery with SLE, showing a 94% improvement when compared against SPE. 25T7-NBOMe on the other hand showed a strong affinity for SPE, with recovery improving 76% when this technique was used. Overall samples were better extracted using SPE with 17 analytes (68%) showing better recoveries using SPE than SLE.

The differences observed between blood, plasma and serum may be due to the different components contained within each matrix. Blood contains plasma, serum, white blood cells and red blood cells. Plasma and serum both consist of water, albumin, globulins, amino acids, hormones, enzymes, nitrogenous waste nutrients and gases. In addition to this, plasma also contains fibrinogen which is responsible for the clotting of blood. It is therefore normal that serum, in comparison to plasma and blood, produces the higher extraction recovery results, as this has had the majority of the matrix already removed. This was not the case for the SLE experiments carried out here. No information is available regarding the size of diatomaceous earth particles biotage use. These particle sizes can vary and thus this could be favouring the retention of larger molecules such as whole blood particles than that of plasma and serum, leading to more sample break through. Alternatively, it could be that the method was not optimised to these alternative matrices and that slight variances in pH may also be impacting upon the recovery values. Although plasma, blood and serum all have pH values typically ranging from 7.35-7.45, the blood, plasma and serum purchased were from different donors and therefore small differences may have been present.

The chromatograms of the resulting serum and plasma samples had more contamination at the front of the chromatogram, and dirtier base lines than those of the whole blood and urine. This suggests that matrix breakthrough may be a contributing factor for the poor recoveries seen for the NPS's tested as shown in Figure 2-24 through Figure 2-27.

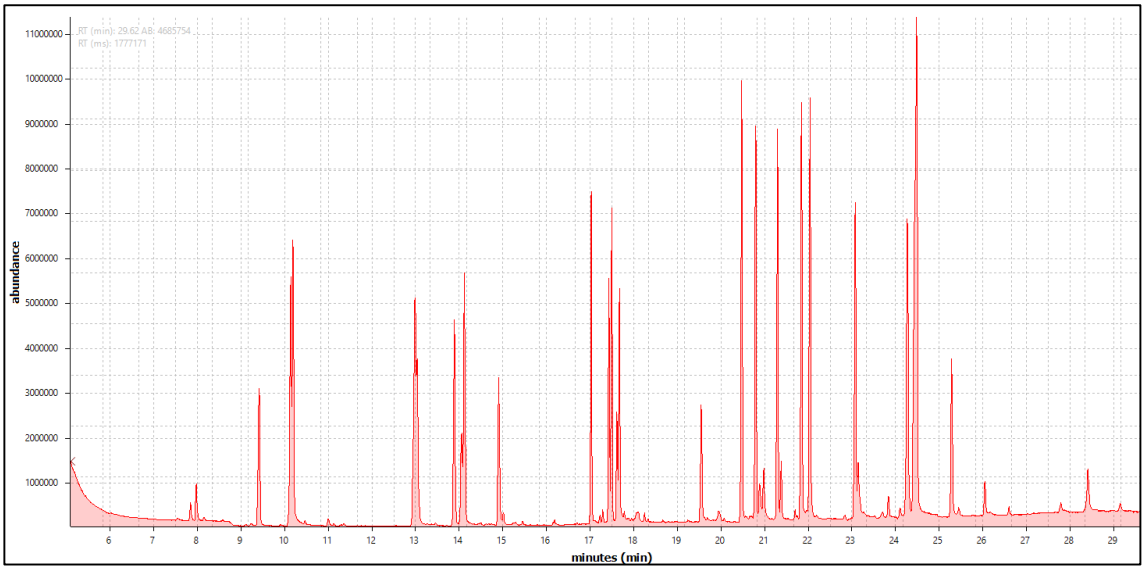


Figure 2-24: Chromatogram of all 26 NPSs extracted from blood by SLE (1 mg/L).

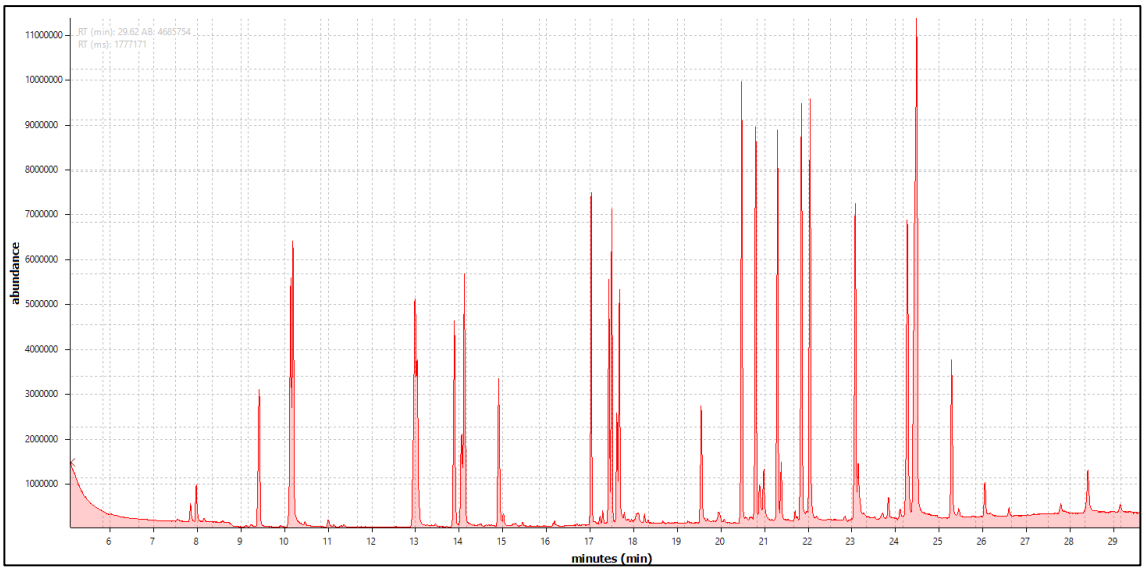


Figure 2-25: Chromatogram of all 26 NPSs extracted from urine by SLE (1 mg/L).

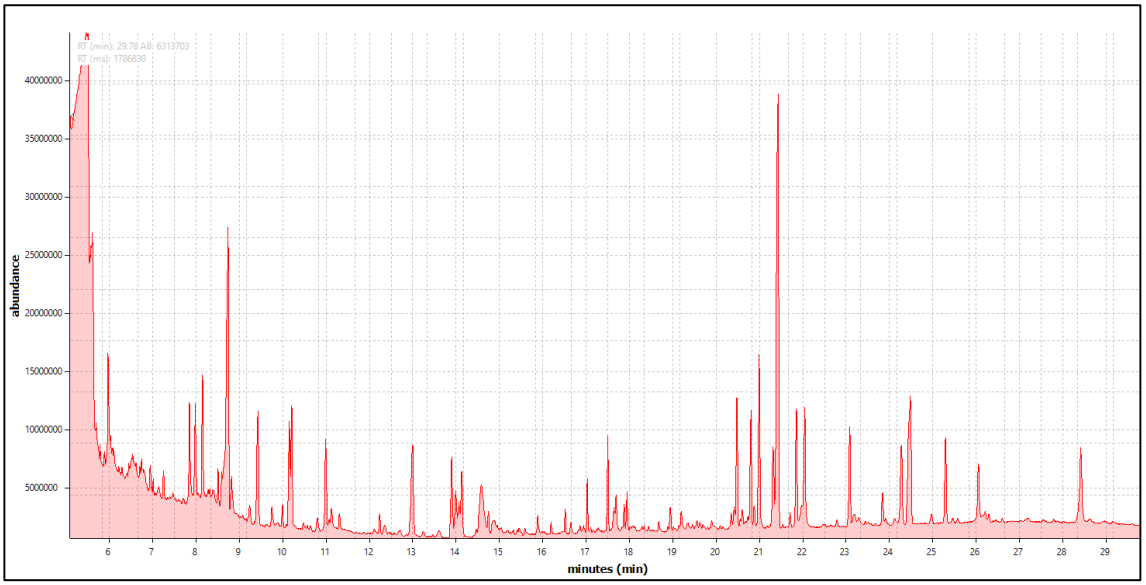


Figure 2-26: Chromatogram of all 26 NPSs extracted from plasma by SLE (1 mg/L).

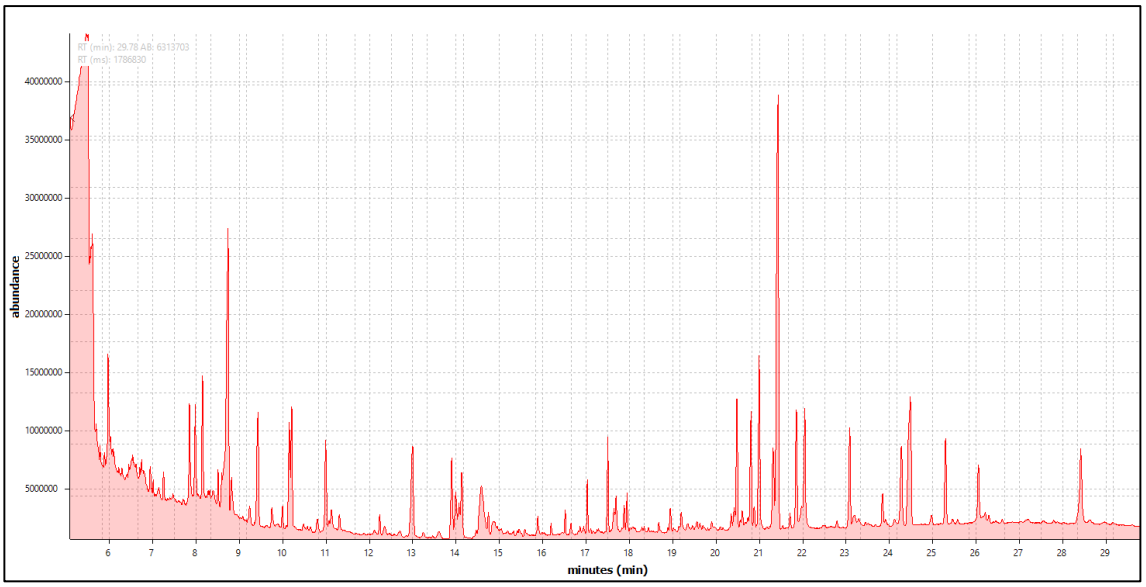


Figure 2-27: Chromatogram of all 26 NPSs extracted from serum by SLE (1 mg/L).

Another anomaly of this research was the difference in recoveries between ethylone and butylone. Although both were recovered to relatively the same degree using SPE there were significant differences between these two analytes when extracted using SLE. Butylone recovery ranged 61-83% in comparison to ethylone which ranged 15-20% across all 4 matrices. There is no real explanation for these observed differences, as the chemistry between these two compounds is very similar, with the only variation being the position of a methyl group. In order to assess this better these drugs should be repeated, thus determining if this was a “one off” anomaly.

When choosing extraction methods analyte recovery and cleanliness of extract must be weighed against sample preparation time and cost. Although SPE involves many more steps than SLE overall both techniques took equal time from start to finish. This is because 2x 4 mL of EtOAc was used as the elution solvent for the SLE cartridges, and this took significantly longer to evaporate than the 3 mL of CH_2Cl_2:IPA:NH_4OH (78:20:2) elution solvent used in SPE. This is due to the different volatilities of each solvent with EtOAc being less volatile than the CH_2Cl_2:IPA:NH_4OH mixture. Solvent waste was heavily reduced however when using SLE in comparison to LLE as although a high volume of solvent is required for elution in SLE this is evaporated off, and thus solvent disposal is not required. This differs to LLE where the bottom aqueous layer is left behind which will need to be disposed of and depending on the number of samples being processed each day, this could result in a significant volume of waste.

2.6 Conclusion

By adapting the in-house FMS amphetamine method the detection of a much wider range of compounds was possible, in particular that of NBOMes. Altering the injection port temperature parameters prevented degradation of compounds in the injection port. Derivatisation using PFPA:EtOAc (2:1) resulted in individual spectra for all compounds with the exception of ethylone and butylone, 5-APB and 6-APB and 25T4 and 25T7-NBOMe. These were however separated chromatographically. The altering of the oven temperature programme provided significant chromatographic separation for the identification of each of these analytes. This meant that all compounds were easily identifiable, using both chromatographic RTs and mass spectra.

Across all of the drugs evaluated, PFPA:EtOAc (2:1) at 37°C yielded the most consistently high recoveries and therefore it is recommended that these conditions are used when multiple cathinones and NBOMEs are being analysed simultaneously by GC/MS. Due to the low concentrations typically detected during the analysis of NBOMes these compounds take priority. For specific drug analysis other conditions may improve detectability as shown in Table 2-4.

TMSI should not be used for the analysis of synthetic cathinones as the majority of these were undetected using this derivatising agent. It also failed to produce

unique spectra for each NBOMe. As the NBOMes all elute during the final hold time some such as 25P and 25C-NBOMe are unable to be separated chromatographically through retention times. This in turn makes the identification of a specific NBOMe more difficult or inconclusive depending on the NBOMe present.

From the data it has been shown that the LLE methods were not suitable for the extraction of NPSs in urine. This is particularly true if the NPS is unknown as neither method allowed for the extraction of all analytes tested. LLE is however a suitable method for the extraction of NBOMes from urine.

This SPE study showed that when analysing urine and blood samples, the smaller bed size of the CSDAU cartridges is preferable, in contrast to analysing plasma and serum samples where ZSDAU cartridges should be used. XRPCH50z cartridges, using the method tested, are not recommended for sample clean-up of NPS compounds.

From the SLE/SPE comparison data it is clear that SPE remains the favoured extraction technique when extracting a wide range of NPSs from urine, serum and plasma. SLE did however prove to be the better technique when extracting these analytes from blood. Although carrying out the SLE process was rapid, the evaporation of such large amounts of elution solvent took additional time, making the time scale of SLE versus SPE similar. Further work should be done to optimise the SLE protocol for these drugs as altering the elution solvent may have a positive impact on the urine, serum and plasma recoveries.

2.7 Future Work

Although this research indicates which SPE cartridge should be used for 25 different analytes in different matrices, there are some limitations to these results which require further work. The cartridges themselves were not optimised, therefore they each only had one method applied, which did or did not work. Thus it might be that one cartridge performed much better than another and that this might not be the case had different solvents been used. This may be particularly important when evaluating the XRPCH50z cartridge which failed to extract any analytes from all matrices tested.

This study did not examine the potential loss of analytes during evaporating steps. This is something which could be further investigated as this has been shown to occur for amphetamine, methamphetamine and MDMA.(*199*)

As with all research there is a need to keep up to date with commercial developments, and this research should be repeated as new cartridges are released.

Chapter 3: GC-MS NPS Method Validation

3.1 Introduction

In order to determine whether an analytical method is fit for purpose, it should be thoroughly tested and validated. This is extremely important in the context of forensic toxicology where the results will have significant impact on individuals coming into contact with the criminal justice system.

The need for validating scientific methods has grown in recent years with the U.S. Department of Justice and the U.K. Home Office highlighting the lack of standards within the field of forensic science. There are a number of governing bodies who produce standards for the work carried out in laboratories ensuring work is of the highest quality, such as the U.K. Accreditation Service (UKAS) and the Scientific Working Group for Forensic Toxicology (SGWTOX). The latter is now under the control of the Organisation of Scientific Area Committee (OSAC).(*200*)

3.2 Aims

The aim of this work was to develop a GC-MS method for the detection of multiple NPS's and validate it in accordance with SWGTOX guidelines, investigating the below parameters –

- Precision & Bias
- Linearity and linear range
- Carryover
- Specificity
- Limit of detection (LOD)
- Limit of quantitation (LOQ)
- Stability
    - Cool/ warm and freeze/ thaw cycles
    - Autosampler stability

This method will be validated for both blood and urine as these are the 2 most commonly encountered matrices. It will then be further tested using “real- life” case samples supplied by NMS laboratories.

3.3 Materials & Methods

3.3.1 Chemicals & Reagents

All reference standards, derivatising agents, blood and other analytical grade chemicals were purchased from the same suppliers as listed in section 2.4.1.12. Again urine was collected in house. Clean Screen ZSDAU020 SPE cartridges were purchased from United Chemical Technologies (PA, USA).

3.3.2 Preparation of Blank (drug-free) Blood and Urine

Blood and urine were screened for common drugs of abuse prior to use by GC-MS. Blank blood was prepared as outlined in section 2.4.1.3.

3.3.3 I.S. Stock solutions

I.S. stock solutions (100 µg/mL) were prepared by transferring 1 mL of each 1 mg/mL I.S. reference solution (mephedrone-D_3, methylone-D_3, ethylone-D_5, and MDPV-D_8) to a 10 mL volumetric flask. This flask was then made up to the mark using MeOH, inverted several times and transferred to labelled amber glass bottles. An I.S. stock solution (10 µg/mL) of 25I-NBOMe-D_3 was prepared by transferring 1mL of a 0.1 mg/mL reference solution to a 10 mL volumetric flask. This was then made up to the mark using MeOH, inverted several times and transferred to a glass amber bottle.

3.3.4 Preparation of Working Solutions

Where possible working solutions for calibrators and quality controls (QCs) were produced using drugs from different manufacturers or with different lot numbers. QC working and stock solutions were also produced by a different analyst. This was possible for all compounds with the exception of 25P-NBOME which was available only from one manufacture who at the time of this validation had only produced one lot. A second vial of this drug (same lot) was

purchased and both the QC stock solution and working solution were made by a different analyst.

Stock solutions were prepared as detailed in section 2.4.1.12. Using these stock solutions, working solution mixtures were produced as detailed in section 2.4.1.1. The drugs contained in each of the 4 mixes are shown in Table 3-1.

The NBOMe solutions (working solutions 3 & 4) were further diluted by taking 1 mL of the 10 µg/mL solution, transferring this up to a 10 mL volumetric flask and making up to the mark with MeOH, producing a 1 µg/mL solution. A 0.1 µg/mL solution was then subsequently produced by transferring 1 mL of the 1 µg/mL solution to a 10 mL volumetric flask and making it up to the meniscus using MeOH. It was these 1 and 0.1 µg/mL solutions which were used to produce calibrators and controls as the concentrations detected are much lower than those of the drugs contained in working solutions 1 & 2 due to their higher potency. Calibrator and QC solutions were stored for a maximum of 6 months, and stored at -20°C.

Table 3-1: Analytes contained in each of the 4 drug mixes.

Working Solution Number			
1	2	3	4
Benzedrone	2-DPMP	25B-NBOMe	Mescaline-NBOMe
Butylone	3-MeO-PCE	25C-NBOMe	25P-NBOMe
Ethylone	3-MeO-PCP	25D-NBOMe	25T4-NBOMe
Flephedrone	5-APB	25E-NBOMe	25T7-NBOMe
MDPV	6-APB	25H-NBOMe	
Mephedrone	Methiopropamine	25I-NBOMe	
Methedrone	Methoxetamine		
Methylone	Naphyrone		

3.3.5 I.S. Working Solution

A mixed I.S. working solution was then prepared by transferring 1 mL of each of the five I.S. stock solutions to a 10 mL volumetric flask. This flask was then made up to the mark using MeOH, thus giving a final concentration of 10 µg/mL for all I.S's with the exception of 25I-NBOMe-D_3 which had a final concentration of 1 µg/mL.

3.3.6 LOD & LLOQ Solutions

In order to assess the methods LOD and LLOQ further solutions were made. To a new 10 mL volumetric flask, 1 mL of working solution 1 and 1 mL of working solution 2 was added. This was then made up to the mark using MeOH and inverted several times to give a final concentration of 1 µg/mL. To another clean 10 mL volumetric flask, 1 mL of working solution 3 and 1 mL of working solution 4 was added. This was again made up to the mark using MeOH and inverted several times producing a final concentration of 0.01 µg/mL.

3.3.7 Calibration & QC Preparations

Urine and blood calibrators and QCs were prepared by spiking 1 mL of either blood or urine with the volumes of each working solution or QC solution as outlined in Table 3-2 and Table 3-3.

Table 3-2: Preparation of Calibrators: Volume of NPS and NBOMe solutions used when spiking 1 mL of blood or urine.

Level	Amount Added (µL)			Final Concentration	
	Working Solutions 1 & 2	Working Solutions 3 & 4	Working Solutions 3 & 4	Working Solution 1 & 2	Working Solution 3 & 4
	10 µg/mL	0.1 µg/mL	1 µg/mL	mg/L	µg/L
1	5	5	0	0.05	0.5
2	10	10	0	0.10	1
3	20	20	0	0.20	2
4	50	50	0	0.50	5
5	100	0	10	1.00	10
6	200	0	50	2.00	50

Table 3-3: Preparation of QCs: Volume of each NPS solution used when spiking 1 mL of blood or urine.

QC	Amount Added (µL)			Final Concentration	
	Working Solutions 1 & 2	Working Solutions 3 & 4	Working Solutions 3 & 4	Working Solution 1 & 2	Working Solution 3 & 4
	10 µg/mL	0.1 µg/mL	1 µg/mL	mg/L	µg/L
1	8	8	0	0.08	0.8
2	42	42	0	0.42	4.2
3	142	0	28	1.42	28.0

3.3.8 Sample preparation

I.S. mix (50 µl) and 1 mL of phosphate buffer (pH6) was added to all calibrators, QCs and samples before being vortexed for 30 seconds. Calibrators, QCs and samples were then centrifuged for 10 minutes at 4000 rpm. The supernatant of each calibrator, QC and sample was then transferred to ZSDAU020 Clean Screen SPE cartridges which had been conditioned using 2 mL MeOH, 2 mL dH_2O followed by 2 mL phosphate buffer (pH 6). Columns were washed using 2 mL dH_2O, 1 mL 0.1 M acetic acid and 2 mL MeOH. Analytes were eluted using 3 mL CH_2Cl_2/IPA/NH_4OH (78:20:2) solution. Excess elution solvent was then evaporated to dryness using a gentle steam of nitrogen with no heat added, before derivatisation using 50 µL of PFPA:EtOAc (2:1). These were left to incubate at 70°C for 40 minutes before being evaporated to dryness once again under a gentle stream of nitrogen (room temperature). All calibrators and QCs were then reconstituted using 50 µL of EtOAc, vortexed for 30 seconds and transferred to labelled glass GC-MS vials containing limited volume inserts.

3.3.9 Instrumentation

Method validation was carried out using an Agilent GC-MSD 5975C series instrument in accordance with SWGTOX guidelines. Data was analysed using Agilent ChemStation software (version 02.02.1431). The instrument was fitted with a J&W Agilent DB-5ms low bleed column (30 m x 0.32 mm i.d.; film thickness 0.25 µm). The GC-MS parameters used were as summarised in Table 3-4. The ions monitored were those listed in Table 2-5. Additional instrumentation was the same as that listed in section 2.4.1.12

Table 3-4: Summary of GC-MS Parameters.

GC Parameters	
Column	J&W Agilent DB-5ms low bleed column (30 m x 0.32 mm i.d.; film thickness 0.25 µm)
Injection Port Mode	Splitless
Injection Port Temperature	225°C
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Transfer Line Temperature	250°C
Initial Temperature	80°C
Hold Time	2 min
Ramp 1	25°C/min to 170°C
Hold	1 min
Ramp 2	5°C/min to 200°C
Hold	1 min
Ramp 3	15°C/min to 300°C
Final Ramp	5°C/min to 300°C
Final Hold	3 min
MS Parameters	
Source Temperature	200°C
Source Mode	EI
Scan Mode	SIM
Total Run Time	30 min

3.3.10 Limit of Detection (LOD), Lower Limit of Quantification (LOQ)

The limit of detection (LOD) was assessed by determining the lowest concentration at which the drug could be detected with a signal to noise (S/N) ratio greater than 3. S/N was calculated using Equation 3-1. Blood and urine (1 mL) was spiked with various volumes (10-100 µl) of the 1 µg/mL LOD & LLOQ mixtures (section 3.3.6). The analytes were then extracted as detailed in section 3.3.8 and analysed. All LODs were assessed after analyte extraction from the matrix as this could heavily impact upon the concentrations identified.

Equation 3-1: Signal to Noise Ratio Calculation.

$$Signal\ to\ Noise = \frac{height\ of\ analtye}{amplitude\ of\ noise}$$

In order to determine the lower level of quantitation (LLOQ) for each compound calibration standards and QCs were spiked with the LOD and LLOQ solutions described in section 3.3.6.(10-100 µl) and extracted. These were run alongside a set of calibrators and QC's. The LLOQ was determined as the lowest concentration with a S/N ratio greater than 10 and at which identification bias and precision criteria were met. In the case of the NBOMe analytes this was decided as being the lowest non-zero calibrator.

As the NBOMe concentrations previously identified in the literature show concentrations ranging from range from 0.441-7.5 ng/mL, it was important that the NBOMe method be sensitive enough to be applicable to forensic cases. Therefore, a cut-off level of 0.5 ng/mL was set when deciding which NBOMe compounds to validate the method for.

3.3.11 Linearity

Calibration standards were prepared by spiking blank blood and urine with NPSs as outlined in 3.3.7. The PAR of each analyte to its I.S. was calculated as per Equation 3-2.

Equation 3-2: Peak Area Ratio Equation

$$PAR = \frac{Anayte\ Peak\ Area}{I.S.\ Peak\ Area}$$

The I.S. used in this calculation for each analyte is shown in Figure 3-1.The PAR was then plotted against its concentration to give a calibration curve. The equation of the line and correlation coefficient (R^2) was measured.

In order for calibration curves to comply with SWGTOX guidelines, R^2 values must be greater than 0.99 and QCs when plotted should not give accuracy values more than ±20%.

Figure 3-1: Analytes with the corresponding I.S. used to calculate PARs.

The SIM ion ratios were monitored during the course of this validation to ensure that they remained constant throughout. This is important when developing a SIM method as the full mass spectrum is not available. Monitoring ion ratios aids with interpreting whether an analyte is positive or not. Ratios were calculated using the abundance of each ion in Table 2-5 and inputting this information into Equation 3-3,Equation 3-4 or Equation 3-5.

Equation 3-3: Target to qualifier 1 ion ratio

$$TGT\ to\ Q1\ Ratio = \frac{Average\ TGT\ Ion\ Abundance}{Average\ Qualifier\ 1\ Ion\ Abundance}$$

Equation 3-4: Target to qualifier 2 ion ratio

$$TGT\ to\ Q2\ Ratio = \frac{Average\ TGT\ Ion\ Abundance}{Average\ Qualifier\ 2\ Ion\ Abundance}$$

Equation 3-5: Qualifier 1 to qualifier 2 ratio

$$Q1\ to\ Q2\ Ratio = \frac{Average\ Qualifier\ 1\ Ion\ Abundance}{Average\ Qualifier\ 2\ Ion\ Abundance}$$

3.3.12 Bias, Precision and Accuracy

Bias and precision were calculated by running calibration standards alongside 3 replicates of each QC, low (QC1), medium (QC2) and high (QC3). The data from these QC's was then entered into Equation 3-6 and Equation 3-7. This was carried out for 6 batches to calculate both the within-run and between-run precision using Equation 3-8 and Equation 3-9. Acceptable criteria for each calculation was set at ≤20%.

The accuracy of the method was then determined using Equation 3-10 in order to determine how close to the true value the results from each QC were. In order to comply with SWGTOX guidelines an acceptable variance limit of ±20% was used. Again results were averaged across all 6 batches.

Equation 3-6: Bias Calculation.

$$Bias\ (\%) = \left[\frac{Mean\ of\ calculated\ conc._x - true\ conc._x}{True\ conc._x}\right] x\ 100$$

Equation 3-7: Precision Calculation.

$$\%\ Coefficient\ of\ Variation\ (CV) = \frac{Std\ Dev}{Mean\ Response}$$

Equation 3-8: Within-Run Precision Calculation

$$Within-run\ CV\ (\%) = \left[\frac{Std\ Dev\ of\ a\ single\ run\ of\ samples}{Mean\ Calculated\ value\ of\ a\ single\ run\ of\ samples}\right] \text{x}100$$

Equation 3-9: Between-Run Precision Calculation.

$$Between-Run\ CV\ (\%) = \left[\frac{Std\ Dev\ of\ mean\ for\ each\ conc.}{Grand\ mean\ for\ each\ concentration}\right] x100$$

Equation 3-10: Accuracy Calculation

$$Accuracy(\%) = \frac{Actual\ Value}{True\ Value} x100$$

3.3.13 Carryover

Analyte carryover was assessed for 2 different batches: NBOMes and the remaining NPSs. For the NPS carryover study 100 µL of each stock solution (100 µg/mL) was added to a culture tube, evaporated to dryness, derivatised with 50µL of PFPA:EtOAc (2:1), evaporated to dryness once more and reconstituted in 50 µl of EtOAc. This was then injected in triplicate followed by an EtOAc blank. This blank was then examined for the presence of analytes from the previous three injections. The same procedure was used to test for any potential NBOMe carry over using 100 µL of each stock solution (10µg/mL). This was also done in triplicate.

3.3.14 Selectivity

Selectivity was assessed to determine if there was any other potential source for positive results. Interferences can be either caused by exogenous compounds, i.e. other analytes present in the sample, or by endogenous compounds i.e. the matrix itself. In order to assess exogenous compounds 100 µL of 100 µg/mL solutions of each analyte listed in Table 3-5 was added to culture tubes and evaporated to dryness. Once dry, samples were derivatised with 50 µL of PFPA:EtOAc, evaporated to dryness again and reconstituted in 50 µl of EtOAc. The analytes were assessed in groups, rather than all together. The same procedure was carried out using blank matrix i.e. blood or urine. The purchased blood was pooled from 50 donors and the urine was collected from 10 different donors.

The selectivity of the method was then assessed by checking the data files from these samples and cross referencing these with the NPS SIM method, looking for peaks retention times close to the target analytes.

Table 3-5: Analytes used to assess method specificity.

Analytes			
6-MAM	Diazepam	Lidocaine	Oxycodone
7-aminoclonazepam	Diphenhydramine	Lorazepam	PCP
Acetominophen	DMAA	MDA	Pentobarbital
Alprazolam	Egonine	MDEA	Phenobarbital
Amitriptyline	EMDP	MDMA	Phentermine
Amobarbital	Ephedrine	Meperidine	Propofol
Amphetamine	Ethchlorvynol	Mescaline	Propoxyphene
Baclofen	Fentanyl	Mescaline	Protriptyline
Benzoylecgonine	Flunitrazepam	Methadone	Pseudoephedrine
Butalbital	Fluoxetamine	Methamphetamine	Secobarbital
Caffeine	Flurazepam	Nicotine	Secobarbital
Carisoprodool	GHB	Nitrazepam	Sibutramine
Chlordiazepoxide	Heroin	Norchlordiazopoxide	Temazepam
Chlorpheniramine	Hexobarbital	Nordiazepam	Thebaine
Citalopram	Hydrocodeine	Norfluoxetine	Tramadol
Clonazepam	Hydrocodone	Nortriptyline	Trazodone
Cocaethylene	Hydromorphone	Olanzapine	Triazolam
Cocaine	Ketamine	Oxazepam	αOH-alprazolam

3.3.15 Stability

Analyte stability was also investigated. Autosampler stability differs from other stability experiments in that the purpose is to assess whether analysing samples after a certain time period gives the same PAR and thus the same concentration. It therefore aims to answer the question that should instrument failure occur can you re-inject the sample and obtain the same result that would have been obtained had the sample been injected at t=0. The method analysed a large number of drugs, most of which did not have a commercially available deuterated I.S. and so the effects of analyte degradation may have significant impacts on the PAR calculated after each time point.

Having knowledge of autosampler stability is also crucial for this method as batch run times may range from hours to days depending on the number of samples being analysed, total run time is 30 minutes so a maximum of 48

injections can take place within a 24-hour time period. Should PAR's alter over time the resulting concentrations calculated using these would also vary.

In order to evaluate this, the 3 QC sample concentrations were analysed in triplicate to determine the concentration at time zero. The same samples were then left on the autosampler and re-injected at time 24, 48 and 72 hours.

The PAR of each QC at time 0, 24 and 48 hours was then calculated using Equation 3-2. The recovery of each analyte was then calculated using Equation 3-11. Analytes were identified as being unstable if their recovery fell out with the acceptable criteria of ±20%. As the vial contains both the analyte and its I.S. degradation will not have an effect on the PAR and thus the final concentration unless the analyte and its I.S. degrade at different rates.

Equation 3-11: Autosampler stability recovery equation

$$Recovery = \frac{Time\ Final\ PAR}{Time\ Initial\ PAR} X\ 100$$

The variation of analyte PA's within these autosampler results was also monitored to show whether the analytes and I.S's were themselves stable. This is particularly important for the NBOMe compounds as concentrations are low. Therefore, although the PAR may not change over the time, it may be that the analyte itself degraded to such a low concentration that it can no longer be detected.

The effect of 3 freeze-thaw and fridge cycles was also investigated, to determine whether this affected concentrations detected. Again this is an important parameter to consider for NPS methods as samples may not initially be screened for these drugs. Should the results of a retrospective analysis be regarded as valid then it is important to know any stability limitations.

Blank blood or urine samples (5 mL) were spiked with working solutions 1-4 as shown in Table 3-3, to produce 3 replicates of each QC concentration. All volumes added were multiplied by 5 to account for the larger blood or urine volume. This procedure was carried out twice for each matrix, giving a total of 18 blood samples and 18 urine samples as depicted in Figure 3-2.

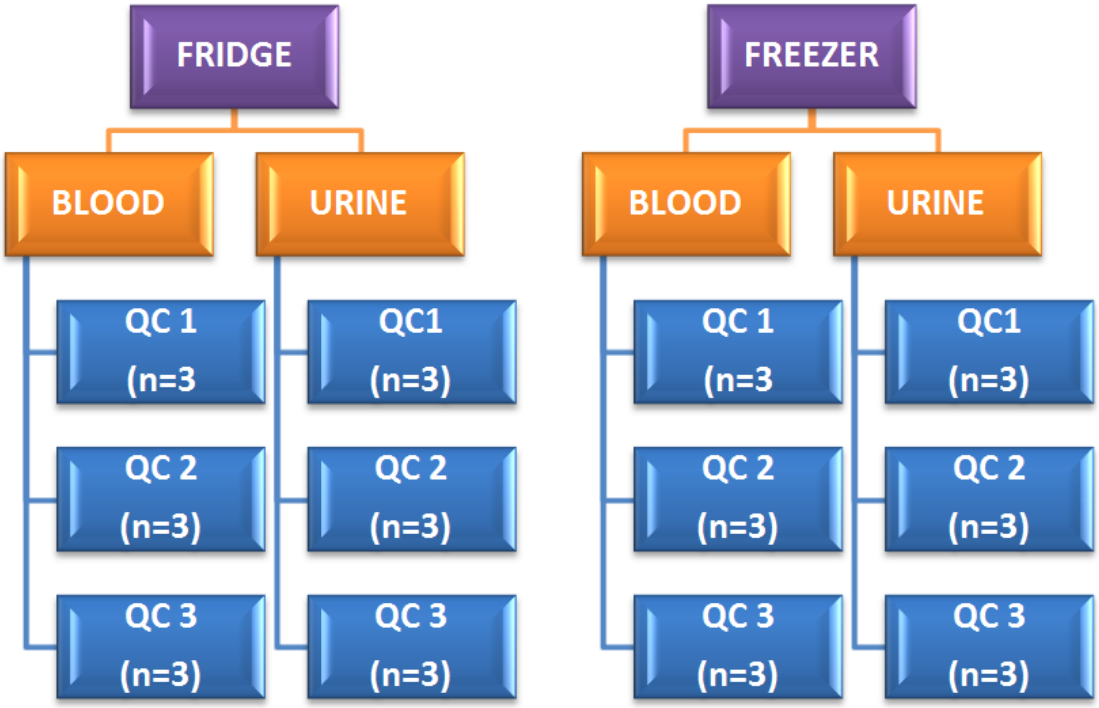


Figure 3-2: Schematic of blood and urine samples stored in the fridge and freezer

From each tube 1 mL of sample was removed, and analysed. The remaining freeze/fridge thaw samples were then capped and either stored in the freezer (-20ºC) or fridge (4ºC). Samples were then removed from the fridge and freezer, left to thaw and reanalysed after 24 hours. This was repeated twice with samples undergoing a total of 3 freeze or fridge thaw cycles. Again PARs were used to monitor the effect of freezer/ fridge thaw cycles on each analyte. Recovery was calculated in a similar manner to that of the autosampler stability, dividing the final PAR by the initial PAR and multiplying by 100.

3.3.16 Case samples

In order to verify whether the method was sufficiently robust for "real-life" samples, 12 blood samples were analysed, from a total of 8 different cases. Three blood samples were from a range of collection sites and only case 4 identified whether the sample was collected anti or post-mortem as shown in Table 3-6. Prior to analysis this was the only information provided.

Table 3-6: Collection site of each sample received from NMS labs.

Case Nº	Site of collection
1	Cardiac Blood
	Cardiac Blood
2	No info
	No info
3	No info
4	Post mortem Blood
5	Iliac Blood
6	Blood
7	Peripheral Blood
8	Cavity Blood
	No info
	Peripheral Blood

These samples were previously analysed using LC-MS/MS by NMS laboratories. Samples were extracted and analysed as per section 3.3.8 and 3.3.9. Calibrators and QCs were extracted and analysed alongside each batch of samples (same concentrations as those stated in section 3.3.7).

After analysis the concentrations and analytes detected were provided. No information was provided as to how the samples had been analysed. Two methanolic washes were run after the final QC and between each case sample to ensure carryover did not affect the results.

3.4 Results & Discussion

3.4.1 Limit of Detection (LOD) and Lower Limit of Quantitation (LLOQ)

The LOD and LLOQ for each drug is shown in Table 3-7. The LLOD for 25T2 and 25T7-NBOMe in blood was deemed too high and thus would not be applicable to forensic casework. These drugs were therefore excluded from the blood validation. They were however still quantifiable in urine at 0.5 µg/L and so were included for urine validation.

Table 3-7: LOD and LOQ for urine and blood.

Drug	URINE (µg/L)		BLOOD (µg/L)	
	LOD	LLOQ	LOD	LLOQ
METHIOPROPAMINE	0.5	5	0.5	10
Flephedrone	0.5	5	0.5	10
Mephedrone	1.0	10	1.0	10
5-APB	0.5	10	0.5	20
6-APB	0.5	10	0.5	20
Methylone	1.0	10	1.0	10
3-MeO-PCE	0.5	5	0.5	5
Butylone	0.5	5	0.5	5
Ethylone	1.0	10	1.0	20
Methoxetamine	0.5	5	0.5	5
Benzedrone	0.5	5	0.5	5
3-MeO-PCP	0.5	5	0.5	5
2-DPMP	0.5	10	0.5	10
MDPV	1.0	10	1.0	10
Naphyrone	1.0	20	1.0	50
25B-NBOMe	0.2	0.5	0.4	0.5
25C-NBOMe	0.2	0.5	0.4	0.5
25D-NBOMe	0.2	0.5	0.4	0.5
25E-NBOMe	0.3	0.5	0.4	0.5
25H-NBOMe	0.2	0.5	0.3	0.5
25I-NBOMe	0.2	0.5	0.4	0.5
Mescaline-NBOMe	0.3	0.5	0.3	0.5
25P-NBOMe	0.2	0.5	0.4	0.5
25T2-NBOMe	0.4	0.5	1.0	n/a
25T7-NBOMe	0.4	0.5	1.0	n/a

3.4.2 Linearity

All analytes followed an unweighted linear calibration model with the exception of flephedrone and methiopropamine which were quadratic. Both blood and urine methods produced correlation coefficients (R^2)> 0.99 for all analytes as shown in Figure 3-3 to Figure 3-14, with the exception of flephedrone and MXE in urine and 3-MeO-PCP in blood.

Urine Calibration Curves

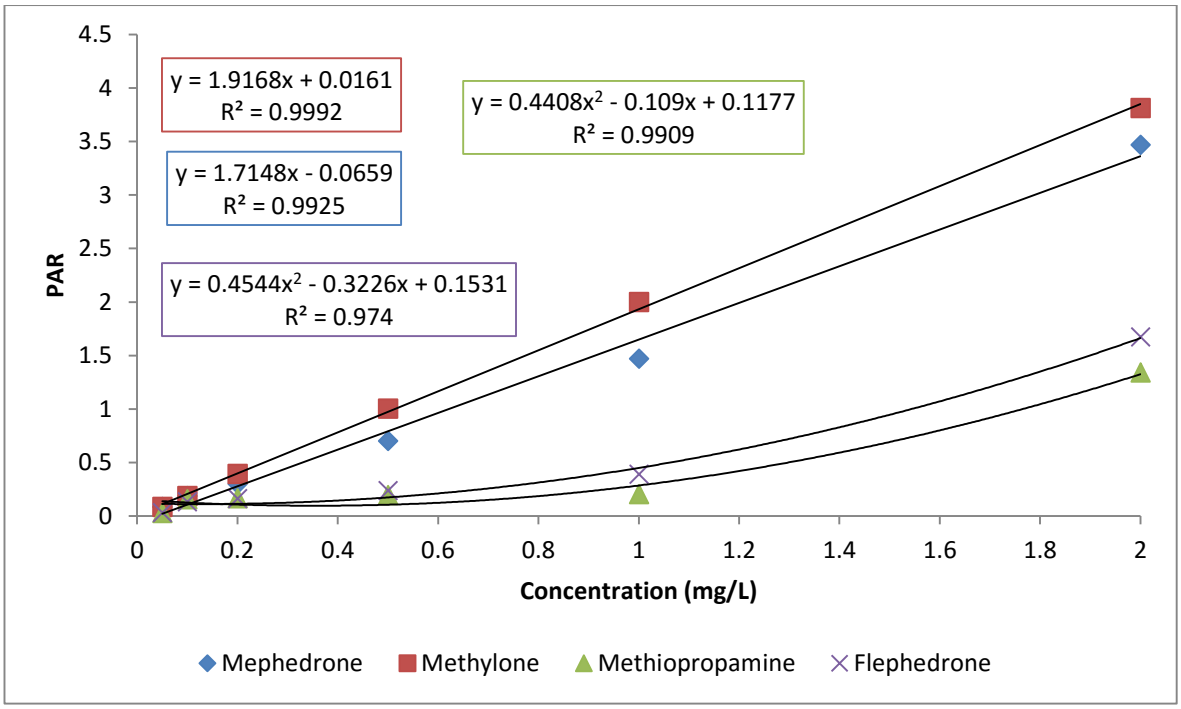


Figure 3-3: Example calibration curves for mephedrone, methylone, methiopropamine and flephedrone in blood.

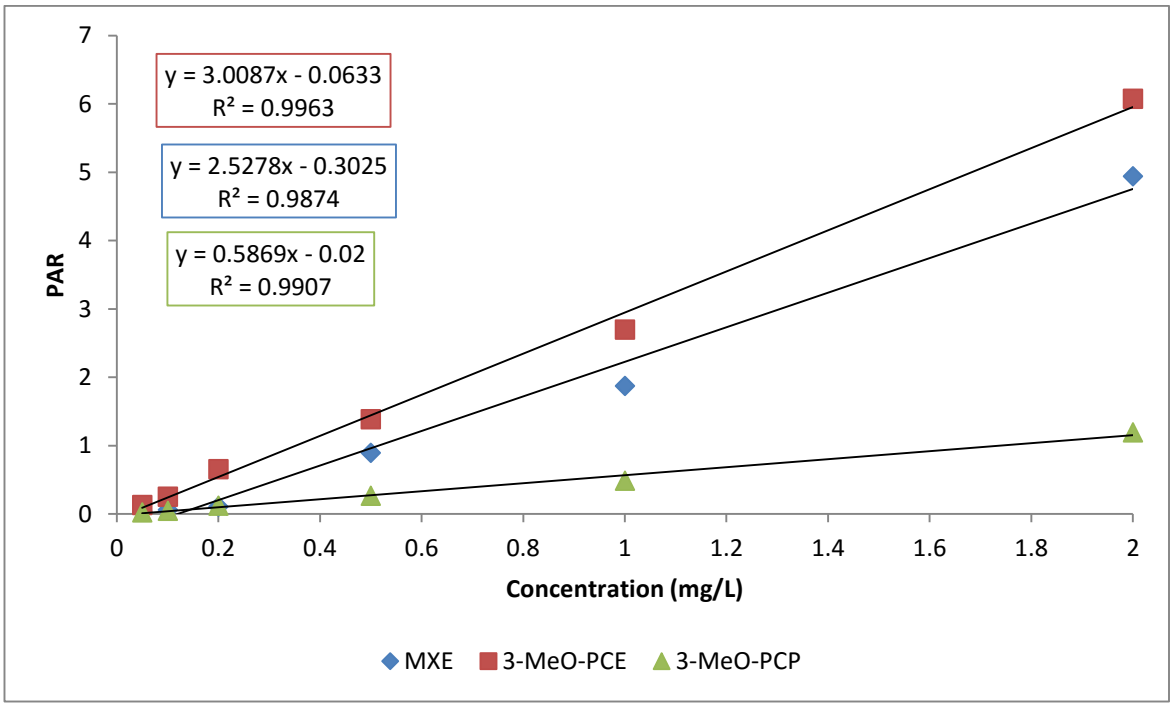


Figure 3-4: Example calibration curves for 3-MeO-PCE, 3-MeO-PCP and methoxetamine in urine.

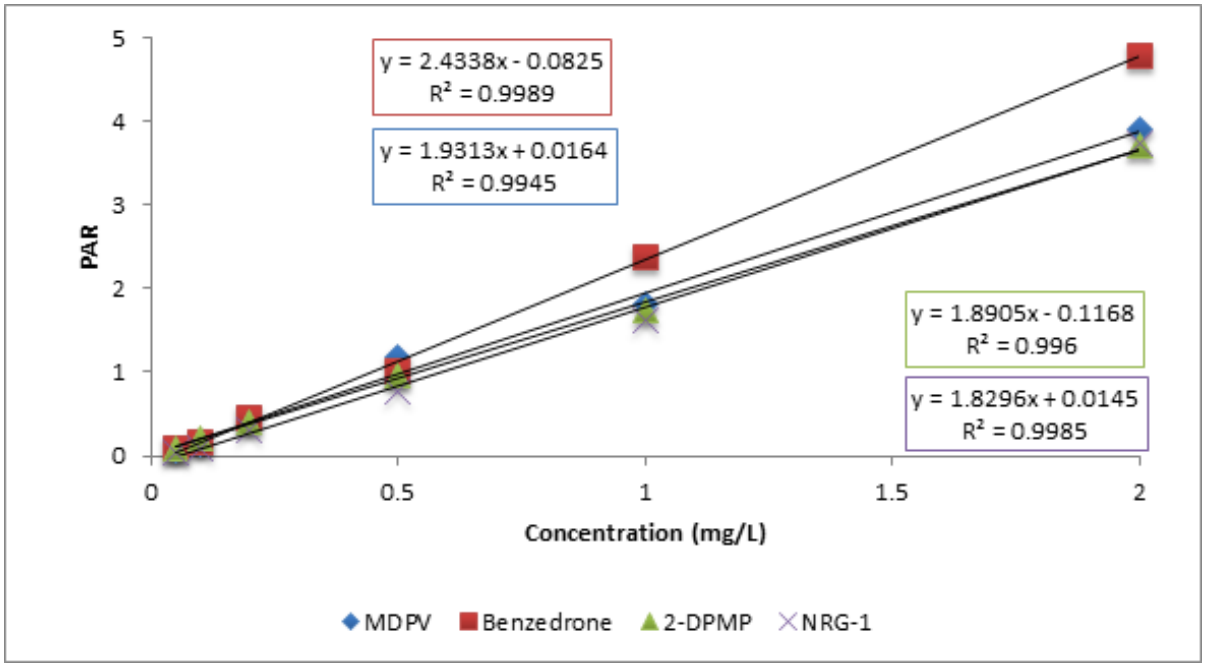


Figure 3-5: Example calibration curves for MDPV, benzedrone, 2-DPMP and naphyrone-1 in urine.

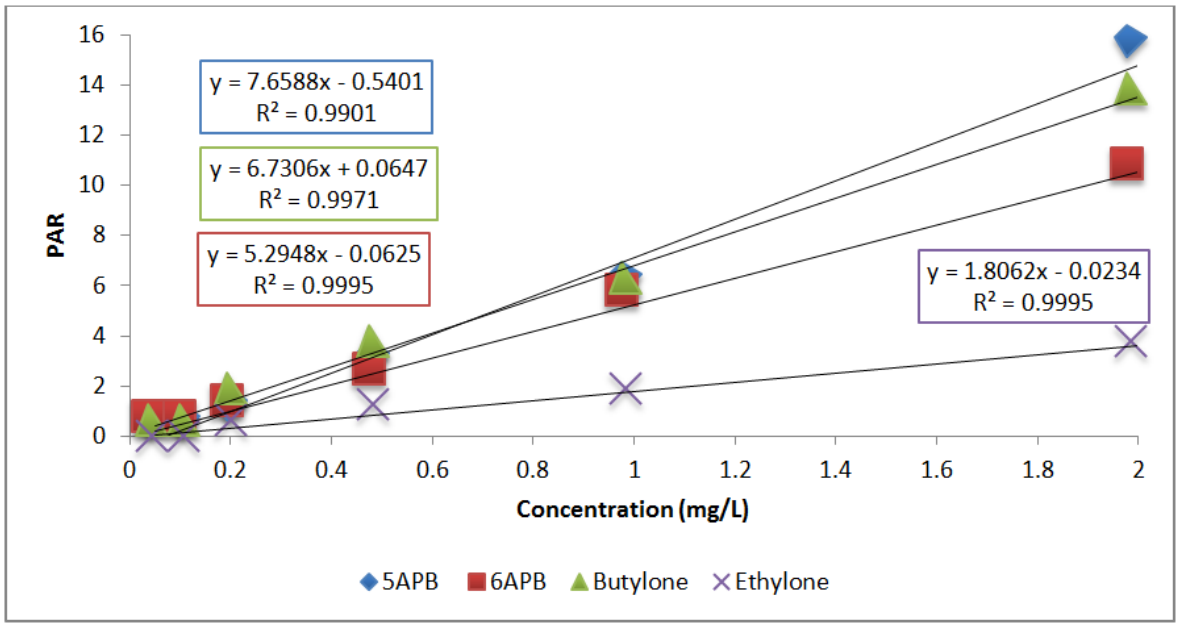


Figure 3-6: Example calibration curves for 5-APB, 6-APB, butylone and ethylone in urine.

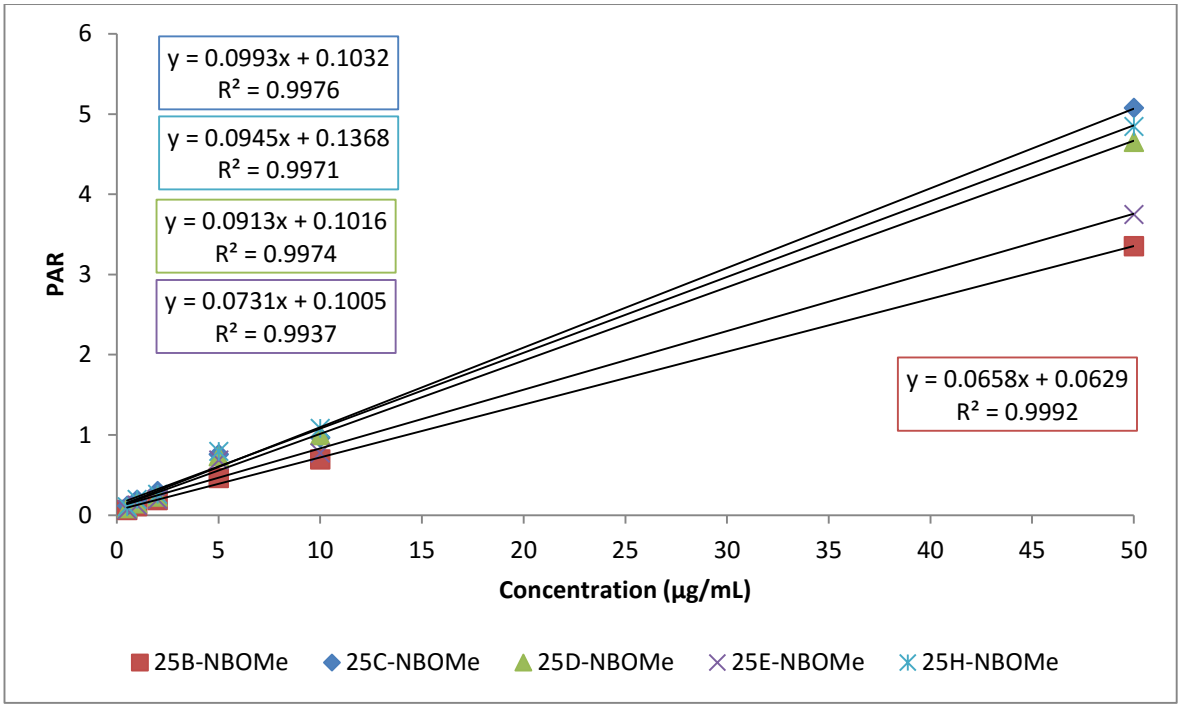


Figure 3-7: Example calibration curves for 25B, 25C, 25D, 25E and 25H-NBOMe in urine.

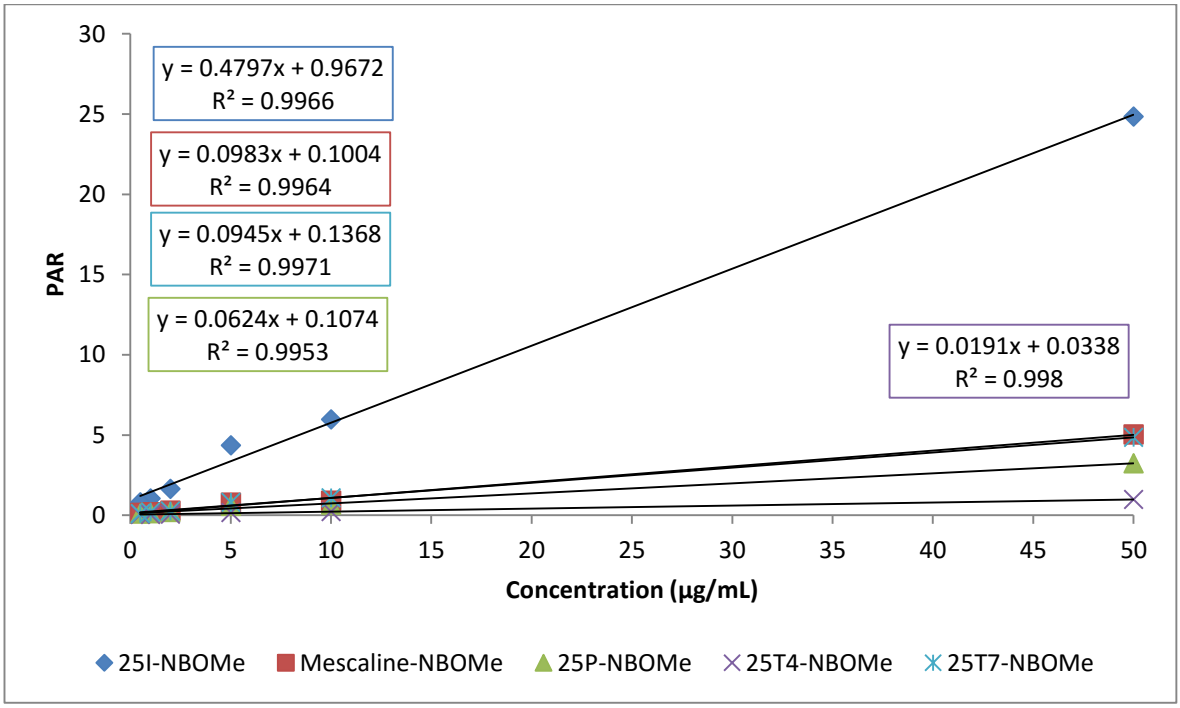


Figure 3-8: Example calibration curves for 25I, mescaline, 25P. 25T4 and 25T7-NBOMe in urine.

Blood Calibration Curves

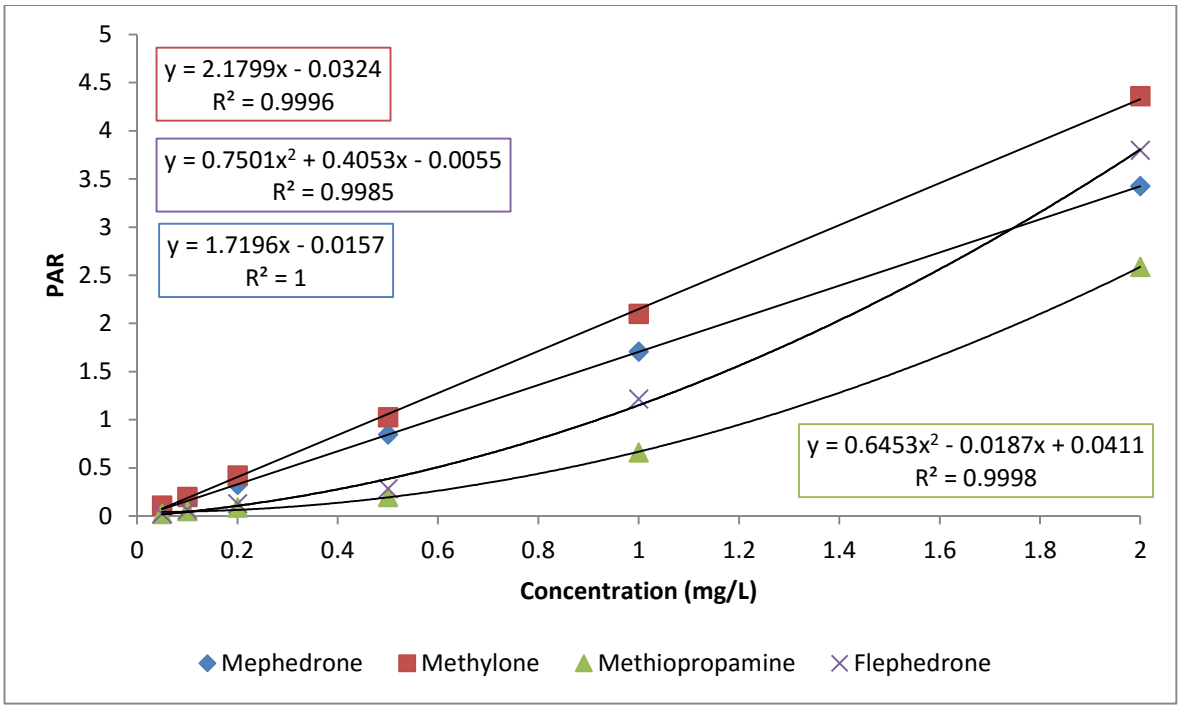


Figure 3-9: Example calibration curves for mephedrone, methylone, methiopropamine and flephedrone in blood.

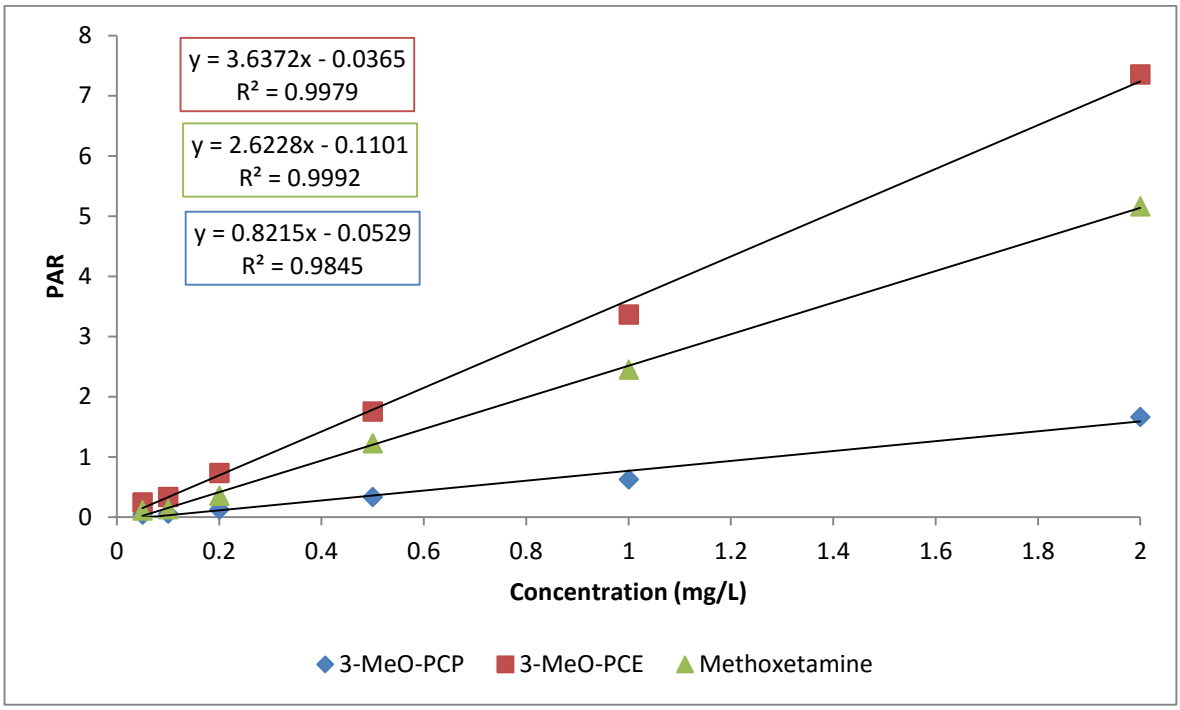


Figure 3-10: Example calibration curves for 3-MeO-PCE, 3-MeO-PCP and methoxetamine in blood.

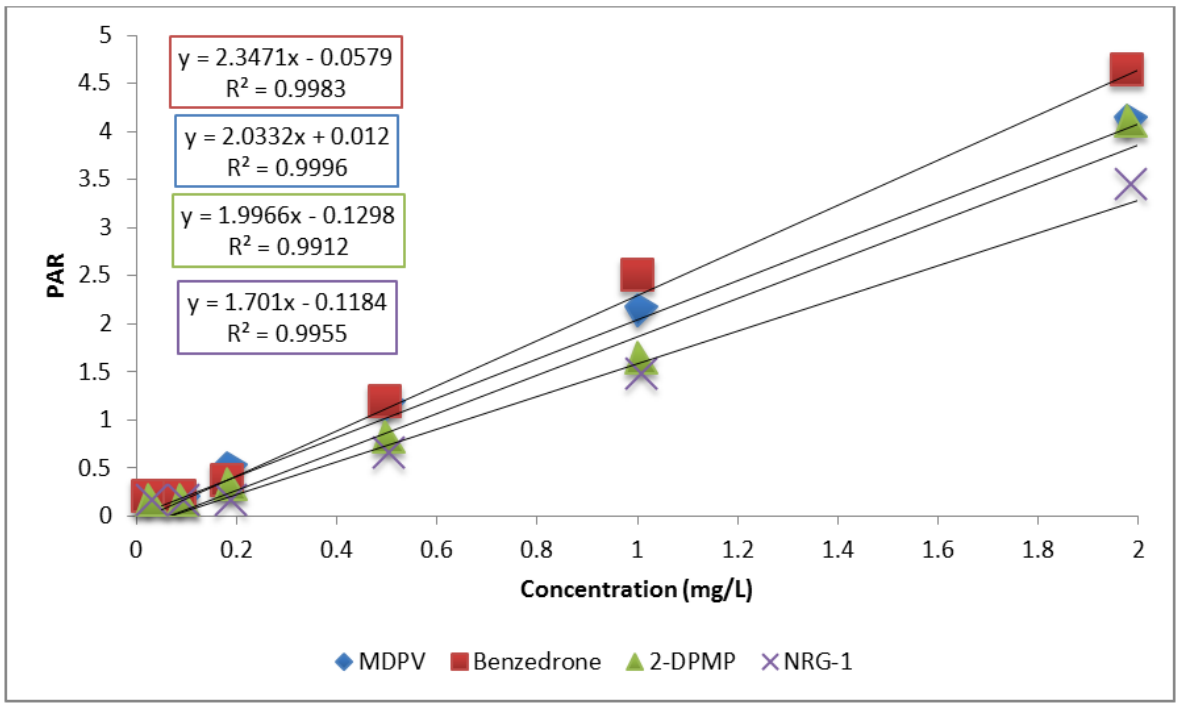


Figure 3-11: Example calibration curves for MDPV, benzedrone, 2-DPMP and naphyrone in blood.

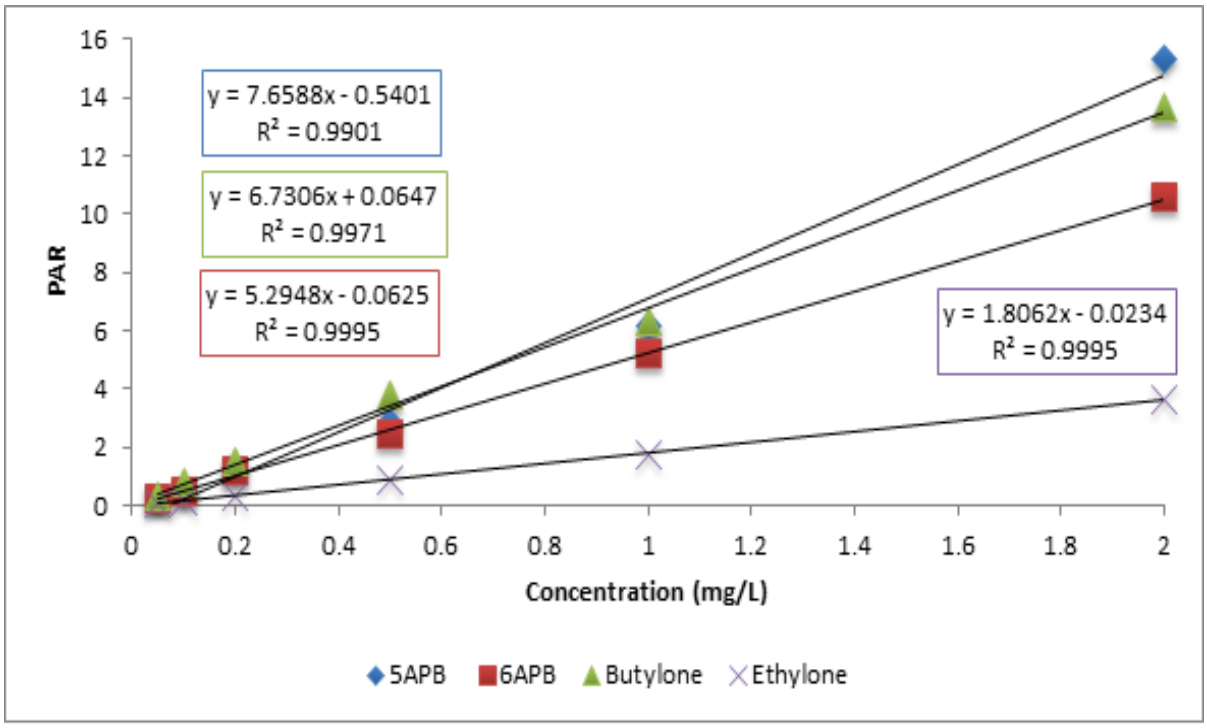


Figure 3-12: Example calibration curves for 5-APB, 6-APB, butylone and ethylone in blood.

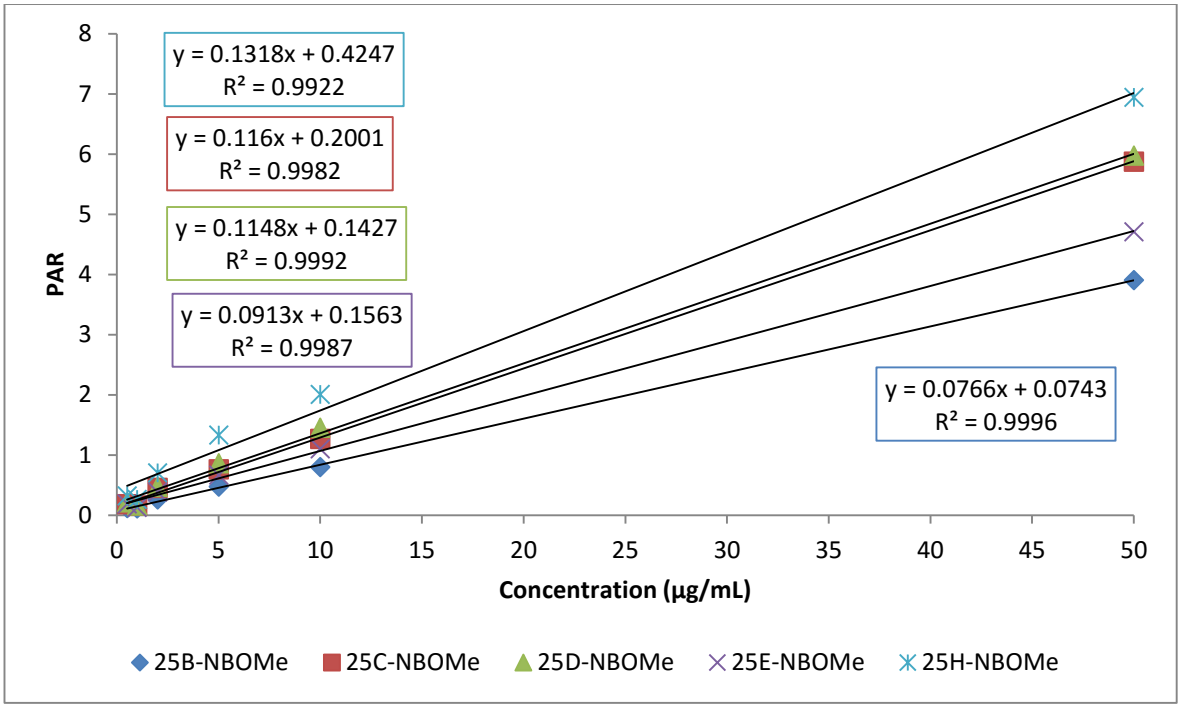


Figure 3-13: Example calibration curves for 25B, 25C, 25D, 25E and 25H-NBOMe in blood.

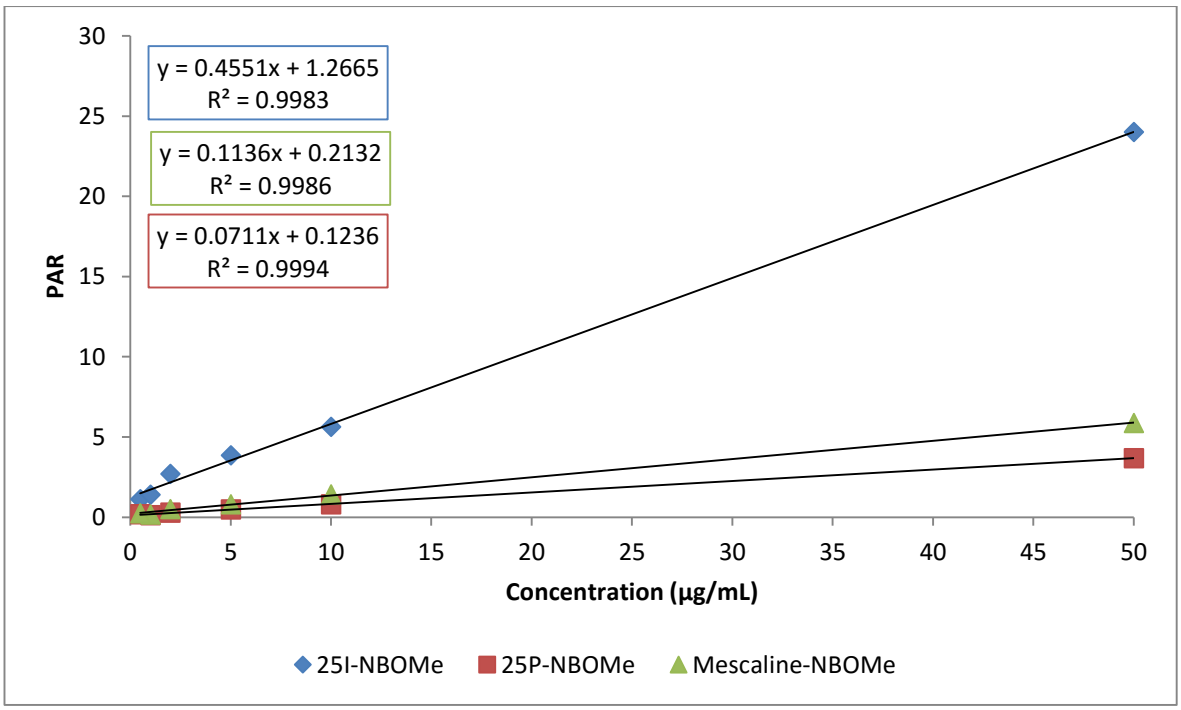


Figure 3-14: Example calibration curves for 25I, Mescaline and 25P-NBOMe in blood.

The calculated SIM ratios observed for each drug alongside their %CV are shown in Table 3-8. Ratios of the target ion to Q1, target ion to Q2 and Q1 to Q2 were monitored throughout the validation (n=6). Table 2-5 lists the target and qualifier ions assigned to each analyte. Average %CV values were within the acceptable range (<20%) for all analytes tested.

Table 3-8: TGT and Qualifier ion ratios for each analyte monitored (n=6).

Drug	TGT to Q1 Ratio	%CV	TGT to Q2 Ratio	%CV	Q1 to Q2 Ratio	%CV
METHIOPROPAMINE	0.9	10.2	1.6	4.5	1.7	6.2
FLEPHEDRONE	1.0	13.6	2.2	6.4	2.5	7.4
MEPHEDRONE-D_3	1.7	6.9	140.3	8.1	90.3	14.2
MEPHEDRONE	1.4	13.4	122.0	18.8	89.2	7.6
5-APB	2.1	4.7	8.9	7.4	4.3	6.5
6-APB	2.0	3.3	9.9	6.3	4.9	3.2
METHEDRONE	18.3	3.2	16.5	16.9	0.8	11.9
METHYLONE-D^3	1.3	5.9	6.2	1.7	4.2	2.1
METHYLONE	0.09	6.8	0.08	0.4	0.9	7.1
3-MeO-PCE	5.6	6.3	6.5	2.6	1.2	7.9
BUTYLONE	5.5	79	8.5	4.1	1.6	12.4
ETHYLONE-D_5	0.2	15.2	22.8	14.0	101.4	18.1
ETHYLONE	1.9	3.7	21.7	9.3	11.3	13.9
MXE	3.8	2.9	3.5	1.2	0.9	3.0
BENZEDRONE	1.8	4.1	6.7	8.2	3.6	4.5
3-MeO-PCP	0.3	5.9	4.7	16.6	15.4	11.1
2-DPMP	0.1	9.0	2.4	3.5	21.9	6.6
MDPV-D_8	13.1	15.5	10.7	0.7	0.8	19.9
MDPV	10.4	11.5	11.0	1.7	1.1	13.2
Naphyrone	5.8	11.9	48.5	14.0	8.4	13.8
25H-NBOMe	1.0	12.6	9.0	10.1	9.2	15.3
25D-NBOMe	1.9	17.9	7.1	11.8	4.0	18.9
25E-NBOMe	6.7	10.9	1.8	11.2	0.2	9.7
Mescaline-NBOMe	3.6	3.6	7.5	2.4	2.0	1.3
25P-NBOMe	1.7	17.5	4.3	11.1	8.9	8.4
25C-NBOMe	11.1	13.4	3.2	10.0	0.3	18.4
25B-NBOMe	3.0	10.4	26.2	7.3	8.0	13.9
25I-NBOMe-D_3	6.4	4.5	310.4	9.8	48.5	7.1
25I-NBOMe	12.4	6.9	16.5	3.8	1.3	3.0
25T4-NBOMe	3.2	9.3	1.3	11.3	0.4	12.7
25T7-NBOMe	2.7	15.9	9.0	10.5	3.4	5.6

3.4.3 Precision & Bias

The results for precision and bias for urine and blood methods are shown in Table 3-9 and Table 3-10 respectively.

3.4.3.1 Urine Results

The intra-day precision %CV results of all analytes extracted from urine were below the ≤20% limit. The intra-day urine results averaged at 8.9, 11.2 and 11.4% across all analytes for the low, medium, and high QCs respectively. Three of the analytes extracted from urine had intra-day precision values <5%, 3-MeO-PCE, 3-MeO-PCP and MDPV. All analytes met the criteria of precision ≤20% for all QCs.

All urine inter-day precision results fell within the SWGTOX <20% criteria. The inter-day results averaged at 11.9, 11.4 and 10.3% across all analytes for the low, medium and high QCs respectively. NBOMes had the poorest inter-day %CV results averaging at 13.8, 15.7, 13.4% across all QCs. This is unsurprising as these produced the smallest PARs and therefore nominal changes in values had a larger impact on %CV results. 25C-, 25D- and 25I-NBOMes performed particularly poorly and although all had inter-day precision results <20% they were all >15%. 5-APB, benzedrone, MDPV, and naphyrone all performed well with inter-day QC results all <10%.

The accuracy of each analyte in urine fell within the ±20% criteria although several QCs were at the bottom and top of this range. Methiopropamine and 25I-NBOMe were the only urine analytes to have an accuracy within ±10%. A further 5 analytes had an accuracy within ±15% of the expected value for all QCs (mephedrone, methoxetamine, benzedrone, 2-DPMP, 25E-NBOMe, and 25P-NBOMe).

Table 3-9: Accuracy and precision data for urine QCs.

	Intra day Precision (%CV) (n=18)			Inter-day Precision (%CV) (n=18)			Accuracy (%) (n=18)		
Drug	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
METHIOPROPAMINE	7.6	8.5	12.4	14.8	8.6	11.9	109.8	106.8	101.8
FLEPHEDRONE	10.6	6.2	12.4	11.5	9.5	17.5	102.3	118.9	115.4
MEPHEDRONE	15.4	6.8	7.9	11.6	11.3	18.6	112.3	93.7	93.2
5-APB	19.9	18.4	11.5	9.6	4.9	9.8	84.5	113.2	111.2
6-APB	2.3	12.8	10.3	15.3	3.4	7.8	121.4	118.4	118.5
METHYLONE	11.9	13.2	12.7	12.9	10.5	13.1	82.0	95.5	94.8
3-MEO-PCE	0.2	3.3	2.0	8.0	13.1	1.6	99.8	119.0	105.6
BUTYLONE	4.7	17.9	8.8	11.4	7.6	6.6	107.7	118.6	112.4
ETHYLONE	11.1	9.7	11.8	14.1	8.8	2.1	119.0	115.5	109.7
MXE	9.0	19.1	6.9	16.4	4.9	3.0	85.7	113.1	103.3
BENZEDRONE	10.9	11.4	7.1	6.6	8.0	4.2	99.8	113.4	109.6
3-MEO-PCP	1.6	3.9	3.1	9.3	9.1	11.7	93.9	120.0	114.5
2-DPMP	7.7	16.0	16.1	6.6	11.4	1.7	108.0	113.5	114.1
MDPV	2.8	4.5	3.3	7.6	9.6	8.1	100.2	120.0	109.6
NAPHYRONE	14.9	15.7	7.9	3.6	7.2	7.1	96.1	119.0	114.5
25B-NBOMe	14.2	6.9	12.3	11.3	7.9	12.3	85.2	105.6	115.7
25C-NBOMe	8.8	18.2	19.5	17.9	16.3	16.7	80.8	111.8	93.9
25D-NBOMe	10.4	16.3	16.4	17.8	18.7	19.7	87.5	119.3	86.2
25E-NBOMe	10.8	7.5	13.2	8.3	16.9	9.7	99.9	98.9	86.5
25H-NBOMe	8.9	15.8	18.7	17.5	19.8	14.7	83.5	92.5	84.8
25I-NBOMe	13.8	1.5	14.6	15.9	16.6	15.8	92.8	95.4	109.3
Mescaline-NBOMe	4.6	10.5	14.4	8.7	12.1	11.6	85.6	83.5	85.6
25P-NBOMe	10.4	18.8	16.8	10.8	17.8	11.5	114.0	96.8	97.4
25T4-NBOMe	4.1	13.5	15.1	19.5	19.3	6.4	88.9	92.1	80.0
25T7-NBOMe	6.5	3.8	10.4	10.7	11.2	15.3	88.8	89.0	80.0

3.4.3.2 Blood Results

The intra-day precision results of all analytes extracted from blood gave %CVs<20%. The intra-day blood precision results averaged at 10.7, 7.2 and 10.0% across all analytes for the low, medium and high QCs respectively. For blood only 3-MeO-PCE had intra-day precision <5%.

All blood inter-day precision results fell within the SWGTOX <20% criteria averaging at 11.5, 8.5 and 8.0% across all analytes for the low, medium and high QCs respectively. Similar to the urine results NBOMes had the poorest inter-day %CV results averaging at 11.8, 12.1 and 12.4% across all QCs. Benzedrone had the best inter-day variation (0.7-2.9%) whereas 25P-NBOMe gave the highest %CVs with all 3 QCs >15%.

The accuracy of each analyte in blood fell within the ±20% criteria with the exception of 25B-NBOMe (QC 2) and mescaline-NBOMe (QC 1). Since this method was validated additional deuterated NBOMe I.S. have become available which may produce better results. The average accuracy for the analytes across all QCs was 97.8% ranging from 80.5% -117.1%. Mephedrone was the most accurate compound with an accuracy ranging from 94.9% to 104.6%. Only 5 of the compounds had an average accuracy <±10%.

Table 3-10: Accuracy and precision data for blood QC's.

Blood	Intra-day Precision (%CV) (n=18)			Inter-day Precision (%CV) (n=18)			Accuracy (%) (n=18)		
Drug	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3
METHIOPROPAMINE	17.2	12.2	10.6	17.7	10.9	5.3	87.8	113.7	119.0
FLEPHEDRONE	7.8	13.2	10.8	15.6	12.5	6.5	88.1	102.7	106.6
MEPHEDRONE	12.9	1.9	6.8	10.5	10.5	9.5	94.9	100.5	104.6
5-APB	14.8	6.1	14.1	14.8	6.1	3.4	110.3	93.2	83.0
6-APB	11.9	8.9	5.3	13.1	1.2	7.4	101.0	89.4	102.4
METHYLONE	9.6	1.4	6.7	8.8	12.9	13.5	92.3	112.9	119.3
3-MeO-PCE	3.7	1.8	0.5	5.7	4.9	1.6	116.9	119.1	115.3
BUTYLONE	8.5	12.9	2.9	14.7	2.2	6.6	83.7	93.2	82.4
ETHYLONE	8.3	6.4	13.7	14.1	0.2	0.3	84.4	95.1	97.8
MXE	10.6	10.7	7.2	13.1	11.3	9.9	102.5	90.1	83.9
BENZEDRONE	7.9	9.4	11.3	1.5	2.9	0.7	99.6	82.4	93.7
3-MeO-PCP	8.2	2.2	10.6	5.6	8.3	3.6	114.3	84.9	87.6
2-DPMP	11.7	15.1	11.1	15.0	5.2	11.8	84.0	80.5	91.9
MDPV	4.1	12.4	14.4	3.4	5.6	2.1	106.1	84.5	91.8
NAPHYRONE	15.8	10.2	14.6	6.2	3.6	3.1	117.8	115.5	85.5
25B-NBOMe	19.8	4.6	9.9	16.5	14.1	10.2	85.1	75.6	80.8
25C-NBOMe	15.5	4.8	13.7	14.7	13.6	12.0	102.2	108.0	92.2
25D-NBOMe	5.8	2.0	8.4	11.5	9.2	12.4	98.5	97.7	92.6
25E-NBOMe	12.8	2.7	10.5	14.9	13.4	19.5	93.6	107.2	107.3
25H-NBOMe	8.7	1.5	8.6	15.7	6.0	15.4	116.9	112.5	102.9
25I-NBOMe	3.4	3.2	10.3	4.2	7.7	8.3	110.1	90.0	81.1
Mescaline-NBOMe	13.8	1.8	13.5	11.6	15.4	2.7	77.2	113.9	99.9
25P-NBOMe	14.5	18.4	14.6	15.1	17.2	18.4	81.2	104.6	110.1

3.4.4 Carryover

No carryover was seen using the method at the concentrations tested as shown in Figure 3-15 and Figure 3-16. Due to the low dose levels of NBOMe compounds, doses higher than those tested would not occur in “real life” cases. To date the highest NBOMe concentration detected is 7.5 ng/mL, which is well below the concentrations tested in this carryover study.(*126*)

Synthetic cathinones on the other hand have the potential to be seen in higher concentrations than those tested and therefore carryover may be possible with these compounds.

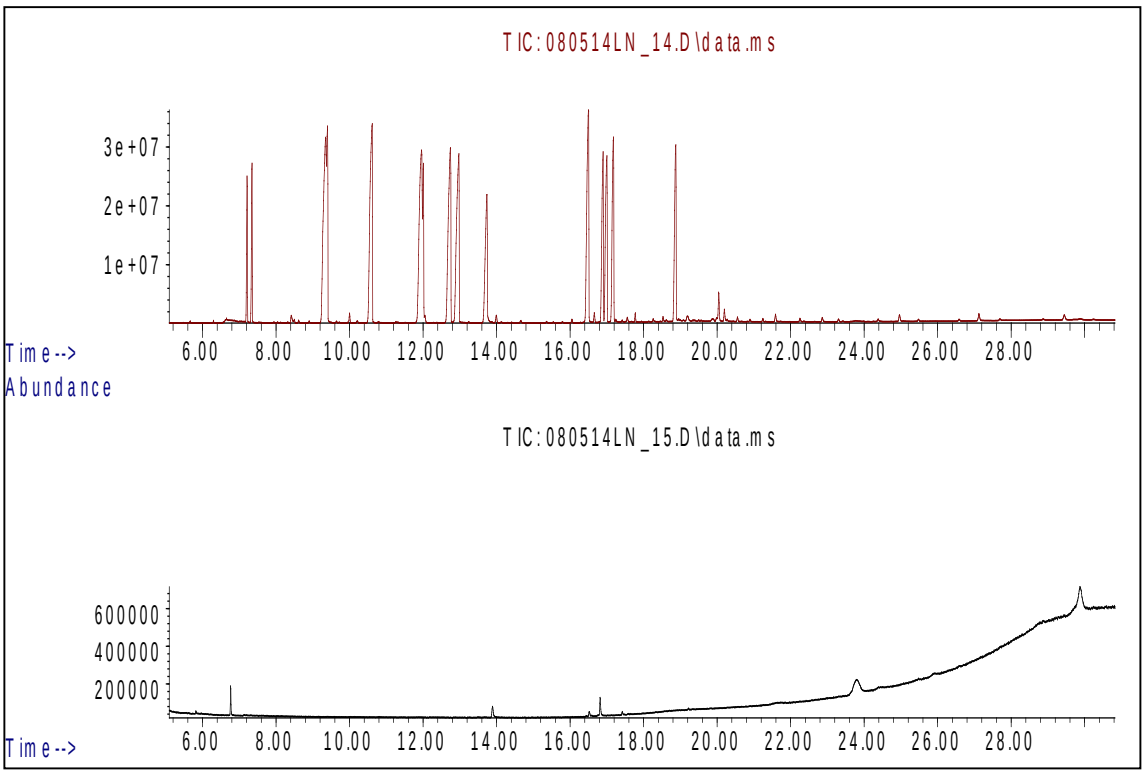


Figure 3-15: Example carryover chromatogram.
Top chromatogram corresponds to NPS analytes and chromatogram below shows the subsequent EtOAc blank.

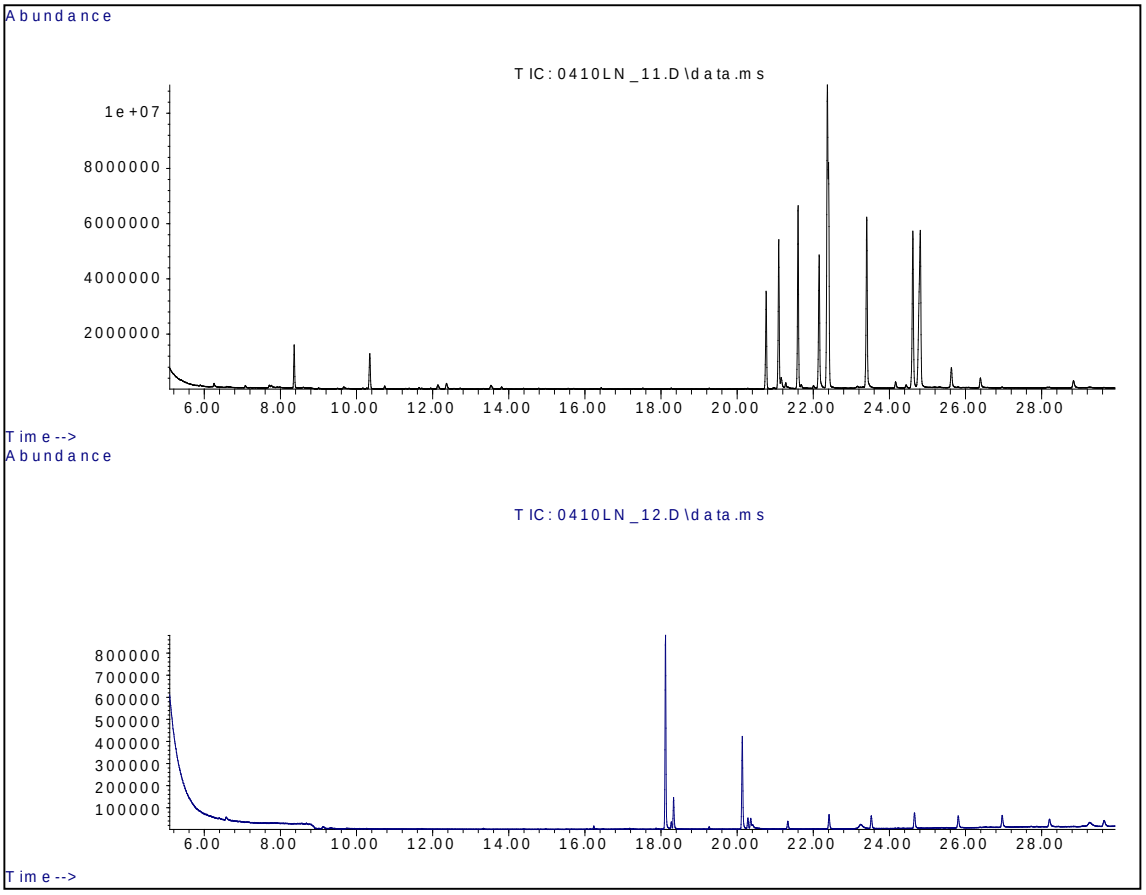


Figure 3-16: Example carryover chromatogram.
Top chromatogram corresponds to NBOMe analytes and chromatogram below shows the subsequent EtOAc blank.

3.4.5 Selectivity

No interferences were observed for any of the analytes tested (Figure 3-17). Although peaks were observed using the method these were at differing retention times with different mass spectra and so would not affect the identification and quantification of the 28 NPSs being analysed. No matrix interferences were observed. The I.S. were successfully separated using both retention time and selected ions and thus would have no impact on the peak areas of their non-deuterated counterparts and vice versa.

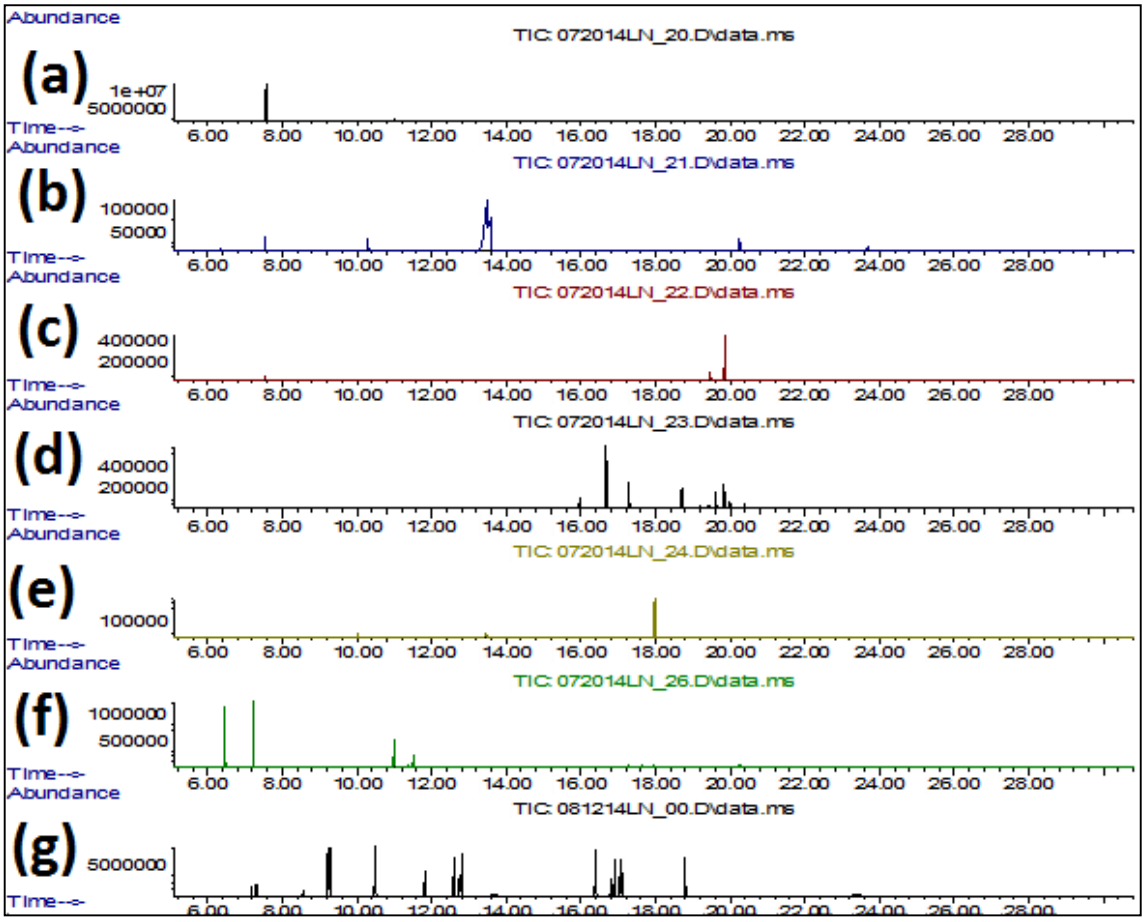


Figure 3-17: Selectivity chromatograms.
Chromatograms (a-f) correspond to the analytes listed in Table 3-5. Chromatogram (g) shows the chromatogram produced from a level 1 urine calibrator. Peaks present in chromatograms (a-f) do not related to any shown in (g) thus there was no observed cross over between method analytes and the analytes tested for during specificity testing.

3.4.6 Stability

3.4.6.1 Autosampler Urine and Blood Stability

Autosampler urine results for each of the 3 QCs are shown in Table 3-11 through Table 3-16. All analytes tested had % recoveries within the acceptable criteria of ±20% with the exception of 3-MeO-PCE, 25B-NBOMe, 25P-NBOMe, 25T4-NBOMe and 25T7-NBOMe. The lack of deuterated I.S's may have affected these results as the PAR for these compounds was extremely low especially for QC1. As a result of this any small differences observed in the PARs highly impacted on the % recovery of these compounds. No analyte fell out with ±20% for all 3 QC's in either matrix. This study does not also take into account the stability of the I.S's themselves, which could also have impacted the results observed.

Table 3-11: Urine QC1 autosampler stability

Analyte	PAR (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.05	0.06	0.05	**100.0**
FLEPHEDRONE	0.14	0.15	0.13	**92.8**
MEPHEDRONE	0.12	0.10	0.11	**86.7**
5-APB	0.30	0.25	0.31	**103.3**
6-APB	0.49	0.44	0.47	**95.9**
METHYLONE	0.15	0.13	0.13	**86.7**
3-MEO-PCE	0.04	0.04	0.03	**75.0**
BUTYLONE	0.41	0.35	0.38	**92.7**
ETHYLONE	0.13	0.13	0.12	**92.3**
MXE	0.12	0.13	0.12	**100.0**
BENZEDRONE	0.11	0.10	0.12	**109.1**
3-MEO-PCP	0.04	0.05	0.04	**100.0**
2-DPMP	0.14	0.15	0.13	**92.9**
MDPV	0.13	0.10	0.11	**84.6**
NAPHYRONE	0.08	0.08	0.07	**87.5**
25B-NBOMe	0.04	0.02	0.03	**75.0**
25C-NBOMe	0.08	0.05	0.07	**87.5**
25D-NBOMe	0.12	0.07	0.11	**91.7**
25E-NBOMe	0.10	0.08	0.09	**90.0**
25H-NBOMe	0.13	0.07	0.14	**107.7**
25I-NBOMe	0.19	0.21	0.17	**89.5**
Mescaline-NBOMe	0.08	0.06	0.08	**100.0**
25P-NBOMe	0.05	0.03	0.04	**80.0**
25T4-NBOMe	0.01	0.01	0.01	**100.0**
25T7-NBOMe	0.01	0.02	0.01	**100.0**

Table 3-12: Urine QC2 autosampler stability.

Analyte	PAR (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.24	0.21	0.27	**112.5**
FLEPHEDRONE	0.79	0.92	0.76	**96.2**
MEPHEDRONE	0.58	0.48	0.59	**101.7**
5-APB	1.80	1.50	1.50	**83.3**
6-APB	2.50	2.30	2.30	**92.0**
METHYLONE	1.78	1.65	1.72	**96.6**
3-MEO-PCE	1.77	1.83	1.74	**98.3**
BUTYLONE	2.30	2.10	2.00	**87.0**
ETHYLONE	0.60	0.70	0.60	**100.0**
MXE	0.93	0.99	0.96	**103.2**
BENZEDRONE	1.10	1.01	1.02	**92.7**
3-MEO-PCP	0.26	0.29	0.27	**103.9**
2-DPMP	0.66	0.57	0.58	**87.9**
MDPV	0.65	0.49	0.57	**87.7**
NAPHYRONE	0.80	0.74	0.88	**110.0**
25B-NBOMe	0.15	0.11	0.13	**86.7**
25C-NBOMe	0.37	0.23	0.33	**89.2**
25D-NBOMe	0.39	0.29	0.35	**89.7**
25E-NBOMe	0.34	0.21	0.29	**85.3**
25H-NBOMe	0.47	0.39	0.41	**87.2**
25I-NBOMe	1.01	0.93	1.05	**104.0**
Mescaline-NBOMe	0.30	0.29	0.33	**110.0**
25P-NBOMe	0.27	0.17	0.22	**81.5**
25T4-NBOMe	0.03	0.04	0.04	**133.3**
25T7-NBOMe	0.04	0.04	0.03	**75.0**

Table 3-13: Urine QC3 autosampler stability

Analyte	PAR (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.50	0.54	0.47	94.0
FLEPHEDRONE	1.87	1.78	1.70	90.9
MEPHEDRONE	3.26	3.59	3.47	106.4
5-APB	6.27	5.90	5.70	90.9
6-APB	7.70	7.20	8.10	105.2
METHYLONE	2.59	2.69	2.64	98.1
3-MEO-PCE	5.58	5.10	5.10	91.4
BUTYLONE	8.62	7.10	7.80	90.5
ETHYLONE	2.48	2.00	2.00	80.7
MXE	3.26	3.00	3.10	95.1
BENZEDRONE	3.56	3.47	3.59	100.8
3-MEO-PCP	0.80	0.88	0.76	95.0
2-DPMP	2.99	2.91	3.16	105.7
MDPV	3.12	2.64	3.05	97.8
NAPHYRONE	2.95	2.54	2.61	88.5
25B-NBOMe	1.17	1.10	1.05	89.7
25C-NBOMe	1.37	1.69	1.16	84.7
25D-NBOMe	1.15	1.20	0.94	81.7
25E-NBOMe	1.21	1.20	1.01	83.5
25H-NBOMe	1.43	1.70	1.46	102.1
25I-NBOMe	1.19	1.03	1.09	91.6
Mescaline-NBOMe	0.98	1.23	0.83	84.7
25P-NBOMe	1.17	1.31	1.07	91.4
25T4-NBOMe	0.46	0.44	0.40	87.0
25T7-NBOMe	0.45	0.36	0.33	73.3

Table 3-14: Blood QC1 autosampler stability

Analyte	Par (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.05	0.07	0.06	**120.0**
FLEPHEDRONE	0.11	0.09	0.12	**109.1**
MEPHEDRONE	0.14	0.13	0.15	**107.1**
5-APB	0.27	0.25	0.27	**100.0**
6-APB	0.56	0.50	0.54	**96.4**
METHYLONE	0.11	0.14	0.13	**118.2**
3-MEO-PCE	0.04	0.04	0.04	**100**
BUTYLONE	0.51	0.46	0.49	**96.0**
ETHYLONE	0.14	0.14	0.14	**100.0**
MXE	0.16	0.14	0.15	**93.4**
BENZEDRONE	0.11	0.10	0.10	**90.9**
3-MEO-PCP	0.05	0.05	0.05	**100.0**
2-DPMP	0.13	0.13	0.14	**107.7**
MDPV	0.12	0.14	0.13	**108.3**
NAPHYRONE	0.08	0.07	0.07	**87.5**
25B-NBOMe	0.04	0.05	0.05	**125.0**
25C-NBOMe	0.08	0.06	0.07	**87.5**
25D-NBOMe	0.12	0.13	0.10	**83.3**
25E-NBOMe	0.10	0.12	0.11	**110.0**
25H-NBOMe	0.13	0.17	0.12	**92.3**
25I-NBOMe	0.19	0.23	0.21	**110.5**
Mescaline-NBOMe	0.08	0.10	0.09	**112.5**
25P-NBOMe	0.05	0.06	0.05	**100.0**

Table 3-15: Blood QC2 autosampler stability

Analyte	PAR (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.25	0.19	0.23	**92.0**
FLEPHEDRONE	0.74	0.69	0.60	**81.1**
MEPHEDRONE	0.64	0.58	0.57	**89.1**
5-APB	1.58	1.43	1.52	**96.2**
6-APB	2.57	2.32	2.45	**95.3**
METHYLONE	1.81	1.88	1.78	**98.3**
3-MEO-PCE	1.84	2.17	2.15	**116.8**
BUTYLONE	2.57	2.32	2.50	**97.3**
ETHYLONE	0.72	0.79	0.76	**105.6**
MXE	1.42	1.52	1.55	**109.2**
BENZEDRONE	2.76	2.45	2.50	**90.6**
3-MEO-PCP	0.26	0.26	0.24	**92.3**
2-DPMP	0.75	0.69	0.63	**84.0**
MDPV	0.71	0.59	0.60	**84.5**
NAPHYRONE	0.85	0.98	0.92	**108.2**
25B-NBOMe	0.15	0.20	0.18	**120.0**
25C-NBOMe	0.37	0.45	0.42	**113.5**
25D-NBOMe	0.39	0.32	0.35	**89.7**
25E-NBOMe	0.34	0.25	0.32	**94.1**
25H-NBOMe	0.47	0.39	0.45	**95.7**
25I-NBOMe	0.90	0.99	0.87	**96.7**
Mescaline-NBOMe	0.30	0.32	0.28	**93.3**
25P-NBOMe	0.27	0.36	0.30	**111.1**

Table 3-16: Blood QC3 autosampler stability

Analyte	PAR (n=3)			Recovery %
	T=0	T=24	T=48	
METHIOPROPAMINE	0.63	0.67	0.53	**84.1**
FLEPHEDRONE	1.65	1.90	1.93	**115.8**
MEPHEDRONE	2.87	3.07	2.89	**100.7**
5-APB	5.54	5.32	5.09	**91.9**
6-APB	6.59	6.36	6.80	**103.2**
METHYLONE	2.52	2.48	2.55	**101.2**
3-MEO-PCE	4.02	4.18	4.05	**100.7**
BUTYLONE	7.49	6.89	7.20	**96.1**
ETHYLONE	2.88	2.22	2.48	**86.1**
MXE	3.46	2.60	2.87	**82.9**
BENZEDRONE	2.98	2.99	2.72	**91.3**
3-MEO-PCP	0.82	0.72	0.68	**82.9**
2-DPMP	3.05	2.78	2.71	**88.9**
MDPV	2.47	3.14	2.55	**88.9**
NAPHYRONE	3.09	3.05	3.07	**99.4**
25B-NBOMe	1.17	1.26	1.37	**117.1**
25C-NBOMe	1.37	1.35	1.27	**92.7**
25D-NBOMe	1.15	1.08	1.11	**96.5**
25E-NBOMe	1.21	1.32	1.45	**119.8**
25H-NBOMe	1.43	1.34	1.40	**97.9**
25I-NBOMe	1.19	1.26	1.23	**103.4**
Mescaline-NBOMe	0.98	1.12	0.86	**87.8**
25P-NBOMe	1.17	1.05	0.93	**79.5**

Blood samples showed slightly better autosampler stability averaging at 98.8% across all 3 QCs (ranging 79.5%-120.0%). Urine averaged at 93.6% across all QCs (ranging 75.0-133.3%).

This is slightly misleading however, as it does not take into account the accuracy of each QC, and as blood has a higher number of % recovery rates greater than 100% this inflates this value. This is illustrated by Figure 3-18 and Figure 3-19. From this we can see that more of the urine QC's fell out with the ±20% cut-off point, 10%, in comparison to blood which only saw 1 QC (4%) fall out with this limit. However, 50% of the blood QC's % recovery results fell within ±20% whereas only 40% of the urine QC's did. This is further illustrated by the number of QC's which had recovery results ranging ±5%, accounting for 30% of urine results versus only 25% of blood results.

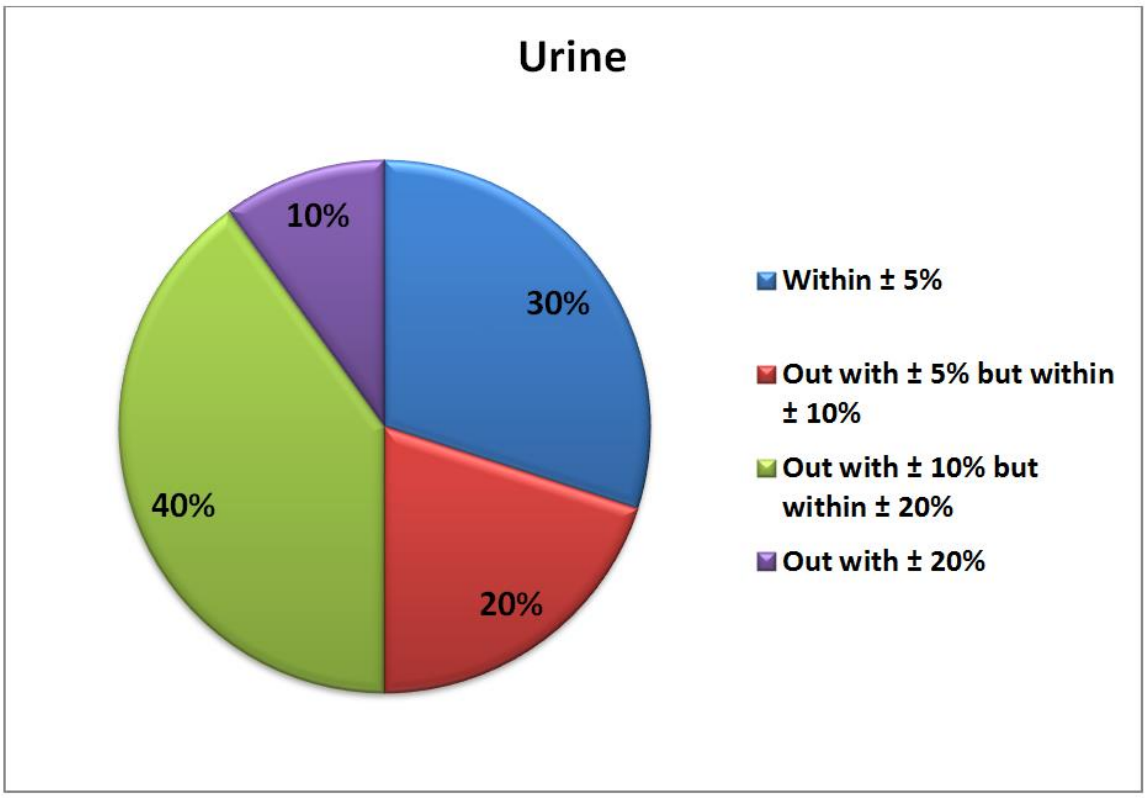


Figure 3-18: Accuracy of all urine QCs.

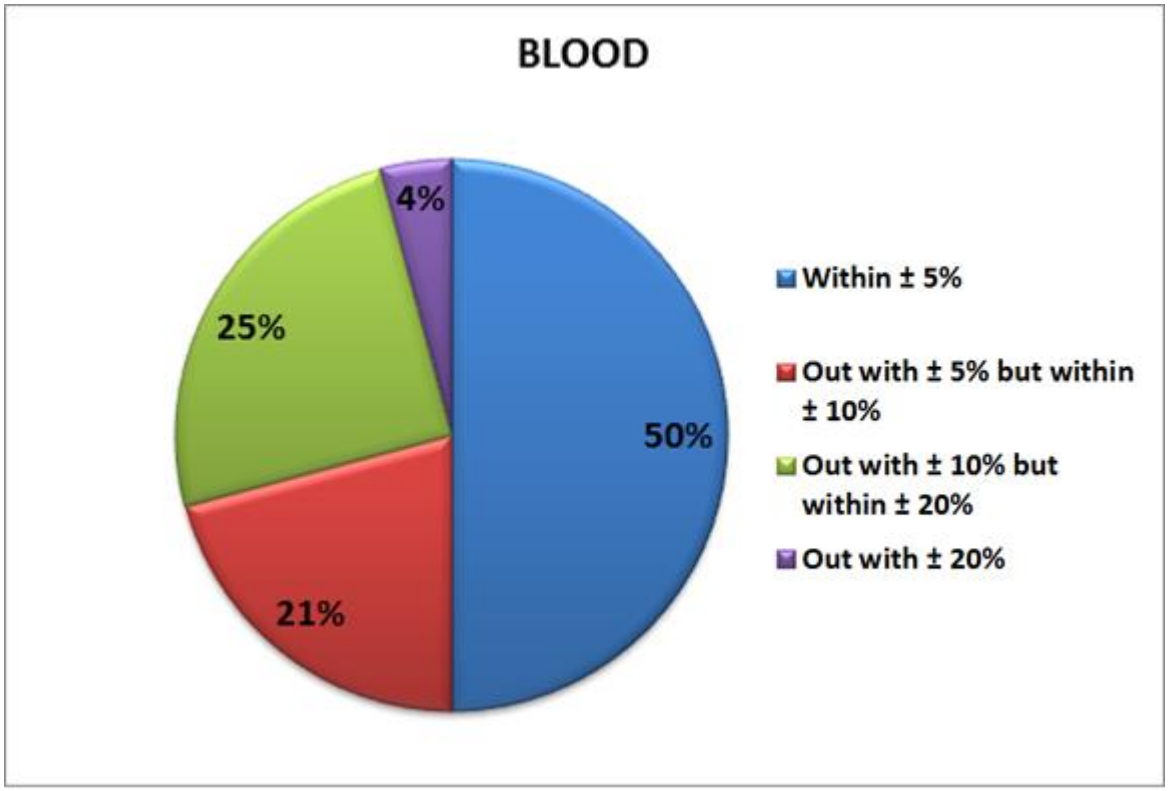


Figure 3-19: Pie chart showing the accuracy of all blood QCs.

These results indicate that autosampler stability is not an issue with these compounds up to 48 hrs. As the reconstitution volume for these samples is so small (50 µl) it is much more likely that the sample will have evaporated before this point in time should re-injection be required. These results correlate with previously published validations which showed no significant degradation to samples up to 72 hrs stored on the autosampler. (*201, 202*)

The peak area % recovery for each analyte QC in both blood and urine is shown in Table 3-17, full results can be found in Appendix 8. From Table 3-17 it is clear that degradation was seen for all compounds over the 48 hour period. Overall mephedrone-D3 was the most affected analyte in urine with an average loss of 31% across all 3 QCs. Ethylone-D_5 was the most affected analyte in blood with an average loss of 34% across all 3 QCs. This was in contrast to methylone-D_3 which showed the least degradation in urine over the 48 hour period (-9%), and 25B-NBOMe which showed the least degradation in blood (8%).

Table 3-17:Autosampler P.A. recovery for each drug and I.S. in each matrix.

	% Recovery					
	Urine			Blood		
	QC1	QC2	QC3	QC1	QC2	QC3
METHIOPROPAMINE	-18.4	-5.3	-22.7	-1.0	-24.0	-34.8
FLEPHEDRONE	-24.3	-19.0	-25.2	-10.0	-33.1	-9.3
MEPHEDRONE	-25.2	-14.3	-12.4	-11.6	-26.5	-21.9
5-APB	-3.4	-25.0	-18.6	-10.3	-21.6	-25.1
6-APB	-10.3	-17.2	-5.8	-13.5	-22.3	-15.9
METHYLONE	-30.4	-15.4	-11.0	-21.8	-34.2	-35.3
3-MeO-PCE	-29.9	-11.6	-18.1	-10.3	-4.8	-17.9
BUTYLONE	-13.3	-21.8	-18.9	-13.9	-20.7	-21.7
ETHYLONE	-13.7	-10.0	-27.8	-10.3	-14.0	-29.8
MXE	-6.5	-7.1	-14.8	-15.9	-11.1	-32.4
BENZEDRONE	-7.7	-23.7	-16.4	-20.2	-21.8	-17.2
3-MeO-PCP	-6.5	-6.6	-14.9	-10.3	-24.8	-32.4
2-DPMP	-21.4	-27.7	-12.4	-5.5	-27.5	-19.4
MDPV	-28.4	-27.9	-19.0	-4.9	-27.1	-6.4
NAPHYRONE	-26.0	-9.5	-26.7	-23.2	-6.6	-9.9
25B-NBOMe	-36.5	-28.3	-26.5	-4.4	-10.0	-8.9
25C-NBOMe	-25.9	-26.2	-30.7	-33.1	-14.8	-27.9
25D-NBOMe	-22.4	-25.7	-33.1	-36.3	-32.7	-24.9
25E-NBOMe	-23.8	-29.4	-31.6	-15.9	-29.4	-6.8
25H-NBOMe	-8.8	-27.8	-16.4	-29.4	-28.2	-23.9
25I-NBOMe	-24.2	-14.0	-25.0	-15.5	-27.5	-19.6
Mescaline-NBOMe	-15.3	-9.0	-30.6	-14.0	-30.0	-31.8
25P-NBOMe	-32.3	-32.6	-25.1	-23.5	-16.6	-38.2
25T4-NBOMe	-15.3	10.3	-28.8	-17.5	-17.4	-22.5
Mephedrone-D_3	-15.3	-37.9	-39.9	-10.3	-18.5	-18.5
Ethylone-D_5	-18.4	-15.8	-18.1	-33.8	-33.0	-36.1
Methylone-D_3	-6.5	-10.0	-10.4	-12.2	-13.7	-9.3
MDPV-D_8	-19.7	-12.4	-12.7	-23.5	-25.0	-22.2
NBOMe-D_3	-15.4	-17.8	-17.1	-1.0	-24.0	-34.8

3.4.6.2 Fridge and Freezer Stability

The fridge and freezer stability results were collected over a 7-day period. The results of these samples will differ from that of the autosampler stability to some degree at their will be more matrix present which may hence this process.

The results for blood and urine QCs after 2 cool/warm and freeze/thaw cycles are shown in Table 3-18 to Table 3-21. These results show that analytes stored in the fridge underwent the largest degradation for both matrices. This is unsurprising as freezer storage has been shown to be more effective in reducing sample degradation. (*203*)

A total of 8 analytes fell out with the acceptable criteria (±20%) after 2 cool/warm fridge cycles for both QCs in urine: mephedrone, methylone, MXE, 2-DPMP, 25E-NBOMe, 25I-NBOMe 25T2-NBOMe and 25T4-NBOMe. Only 25P-NBOMe fell out with the ±20% recovery required after 2 freeze/thaw cycles for both QC's in urine.

Only 5 analytes fell out with the acceptable criteria (±20%) after 2 cool/warm fridge cycles for both QCs in blood: mephedrone, 25C-NBOMe, 25I-NBOMe, mescaline-NBOMe and 25P-NBOMe. No analytes fell out with this range for both QCs tested after 2 freeze/thaw cycles in blood.

Mephedrone and methylone were the least stable analytes after 2 cool/warm cycles with average recoveries of only 61.3% and 64.5% respectively. Previous studies looking at the stability of methylone have been inconclusive with prior work showing little to no degradation to samples after 72 h.(*201, 204*) The majority of these studies utilized some sort of preservative for the storage of their samples which was not the case here. This is important when comparing any of the results here to other previously published work as the use of preservatives may significantly reduce the degradation of these substances. The blood used in this work was diluted with saline solution and this may too have impacted on the results seen.

Table 3-18: Freeze thaw cycles on analyte stability in urine

Urine	Fridge Cool/Warm Cycle PAR (n=3)				Freezer Thaw PAR (n=3)			
Drug	0	1	2	Recovery %	0	1	2	Recovery %
METHIOPROPAMINE	0.24	0.21	0.16	**66.7**	0.24	0.26	0.20	**83.3**
FLEPHEDRONE	0.79	0.60	0.69	**87.3**	0.79	0.75	0.76	**96.2**
MEPHEDRONE	0.58	0.49	0.33	**56.9**	0.58	0.43	0.65	**112.1**
5-APB	1.80	1.67	1.38	**76.7**	1.80	1.92	1.75	**97.2**
6-APB	2.50	2.23	2.42	**96.8**	2.50	2.87	2.69	**107.6**
METHYLONE	1.78	1.52	1.12	**62.9**	1.78	1.88	1.52	**85.4**
3-MEO-PCE	1.77	2.22	1.55	**87.6**	1.77	2.46	2.05	**115.8**
BUTYLONE	2.30	2.96	2.09	**90.9**	2.30	2.96	2.56	**111.3**
ETHYLONE	0.60	0.90	0.57	**95.0**	0.60	0.68	0.73	**121.7**
MXE	0.93	0.82	0.66	**71.0**	0.93	0.90	0.87	**93.5**
BENZEDRONE	1.10	1.06	1.12	**101.8**	1.10	1.00	1.23	**111.8**
3-MEO-PCP	0.26	0.29	0.27	**103.8**	0.26	0.31	0.32	**123.1**
2-DPMP	0.66	0.31	0.30	**45.5**	0.66	0.76	0.78	**118.2**
MDPV	0.65	0.68	0.66	**98.5**	0.65	0.75	0.62	**95.4**
NAPHYRONE	0.80	0.88	0.76	**95.0**	0.80	1.10	0.94	**117.5**
25B-NBOMe	0.15	0.12	0.18	**120.0**	0.15	0.15	0.16	**106.7**
25C-NBOMe	0.37	0.32	0.38	**102.7**	0.37	0.30	0.35	**94.6**
25D-NBOMe	0.39	0.38	0.34	**87.2**	0.39	0.34	0.37	**94.9**
25E-NBOMe	0.34	0.33	0.27	**79.4**	0.34	0.27	0.29	**85.3**
25H-NBOMe	0.47	0.44	0.38	**80.9**	0.47	0.38	0.41	**87.2**
25I-NBOMe	1.01	0.87	0.70	**69.3**	1.01	0.96	0.92	**91.1**
Mescaline-NBOMe	0.30	0.32	0.29	**96.7**	0.30	0.32	0.33	**110.0**
25P-NBOMe	0.27	0.20	0.25	**92.6**	0.27	0.24	0.21	**77.8**
25T4-NBOMe	0.03	0.03	0.02	**66.7**	0.03	0.04	0.04	**133.3**
25T7-NBOMe	0.04	0.05	0.03	**75.0**	0.04	0.06	0.04	**100.0**

Table 3-19: Effects of fridge cool/warm and freeze thaw cycles on analyte stability in urine (QC3)

Urine	Fridge Cool/Warm Cycle PAR (n=3)				Freezer Thaw PAR (n=3)			
Drug	0	1	2	Recovery %	0	1	2	Recovery %
METHIOPROPAMINE	0.50	0.49	0.41	**82.0**	0.50	0.47	0.43	**86.0**
FLEPHEDRONE	1.87	1.43	1.23	**65.8**	1.87	1.68	1.75	**93.6**
MEPHEDRONE	3.26	2.59	2.37	**72.7**	3.26	3.67	3.33	**102.1**
5-APB	6.27	5.00	5.30	**84.5**	6.27	6.31	6.09	**97.1**
6-APB	7.70	7.23	6.51	**84.6**	7.70	8.22	8.04	**104.4**
METHYLONE	2.59	1.95	1.46	**56.4**	2.59	2.34	2.60	**100.4**
3-MEO-PCE	5.58	4.20	4.67	**83.7**	5.58	6.43	6.22	**111.5**
BUTYLONE	8.62	8.58	8.31	**96.4**	8.62	9.62	9.70	**112.5**
ETHYLONE	2.48	2.16	2.15	**86.7**	2.48	2.34	2.86	**115.3**
MXE	3.26	2.92	2.56	**78.5**	3.26	3.15	2.91	**89.3**
BENZEDRONE	3.56	3.45	3.10	**87.1**	3.56	3.30	3.40	**95.5**
3-MEO-PCP	0.80	0.53	0.82	**102.5**	0.80	0.87	0.88	**110.0**
2-DPMP	2.99	2.12	2.19	**73.2**	2.99	3.10	2.50	**83.6**
MDPV	3.12	3.57	3.28	**105.1**	3.12	3.06	3.42	**109.6**
NAPHYRONE	2.95	2.74	2.54	**86.1**	2.95	3.10	2.89	**98.0**
25B-NBOMe	1.17	1.13	1.08	**92.3**	1.17	1.15	1.16	**99.1**
25C-NBOMe	1.37	1.19	0.98	**71.5**	1.37	1.21	1.41	**102.9**
25D-NBOMe	1.15	1.01	1.07	**93.0**	1.15	1.21	1.19	**103.5**
25E-NBOMe	1.21	1.35	1.15	**71.4**	1.21	1.15	1.09	**90.1**
25H-NBOMe	1.43	1.10	1.22	**85.3**	1.43	1.42	1.35	**94.4**
25I-NBOMe	1.19	0.96	0.94	**79.0**	1.19	1.02	0.88	**73.9**
Mescaline-NBOMe	0.98	0.68	0.63	**64.3**	0.98	111.00	0.91	**92.9**
25P-NBOMe	1.17	1.15	0.85	**72.6**	1.17	1.20	0.93	**79.5**
25T4-NBOMe	0.46	0.36	0.27	**58.7**	0.46	0.39	0.41	**89.1**
25T7-NBOMe	0.45	0.40	0.25	**55.6**	0.45	0.37	0.44	**97.8**

Table 3-20: Effects of fridge cool/warm and freeze thaw cycles on analyte stability in blood (QC2)

Blood	Fridge Cool/Warm Cycle PAR (n=3)				Freezer Thaw PAR (n=3)			
Drug	0	1	2	Recovery %	0	1	2	Recovery %
METHIOPROPAMINE	0.25	0.33	0.26	**104.0**	0.25	0.19	0.27	**108.0**
FLEPHEDRONE	0.74	0.76	0.65	**87.8**	0.74	0.88	0.79	**106.8**
MEPHEDRONE	0.64	0.49	0.33	**51.6**	0.64	0.71	0.65	**101.6**
5-APB	1.58	1.65	1.52	**96.2**	1.58	2.9	2.5	**108.6**
6-APB	2.57	2.17	2.55	**99.2**	2.57	3.09	2.7	**105.1**
METHYLONE	1.81	1.22	0.89	**49.2**	1.81	1.68	1.78	**98.3**
3-MEO-PCE	1.84	1.63	1.77	**96.2**	1.84	2.36	2.24	**121.7**
BUTYLONE	2.57	2.38	2.02	**78.6**	2.57	2.87	2.8	**108.9**
ETHYLONE	0.72	0.99	0.86	**119.4**	0.72	0.74	0.87	**120.8**
MXE	1.42	1.41	1.32	**93.0**	1.42	1.66	1.6	**112.7**
BENZEDRONE	2.76	2.62	2.33	**84.4**	2.76	2.23	2.39	**86.6**
3-MEO-PCP	0.26	0.21	0.15	**57.7**	0.26	0.29	0.23	**88.5**
2-DPMP	0.75	0.7	0.65	**86.7**	0.75	0.92	0.88	**117.3**
MDPV	0.71	0.63	0.6	**84.5**	0.71	0.77	0.69	**97.2**
NAPHYRONE	0.85	0.59	0.62	**72.9**	0.85	0.78	0.76	**89.4**
25B-NBOMe	0.15	0.14	0.14	**93.3**	0.15	0.19	0.11	**73.3**
25C-NBOMe	0.37	0.4	0.28	**75.7**	0.37	0.32	0.34	**91.9**
25D-NBOMe	0.39	0.32	0.33	**84.6**	0.39	0.35	0.47	**120.5**
25E-NBOMe	0.34	0.37	0.27	**79.4**	0.34	0.26	0.28	**82.4**
25H-NBOMe	0.47	0.34	0.4	**85.1**	0.47	0.51	0.41	**87.2**
25I-NBOMe	0.9	0.88	0.64	**71.1**	0.9	0.87	0.92	**102.2**
Mescaline-NBOMe	0.3	0.29	0.21	**70.0**	0.3	0.26	0.33	**110.0**
25P-NBOMe	0.27	0.31	0.23	**85.2**	0.27	0.22	0.27	**100.0**

Table 3-21: Effects of fridge cool/warm and freeze thaw cycles on analyte stability in blood (QC3)

Blood	Fridge Cool/Warm Cycle PAR (n=3)				Freezer Thaw PAR (n=3)			
Drug	0	1	2	Recovery %	0	1	2	Recovery %
METHIOPROPAMINE	0.63	0.56	0.57	**90.5**	0.63	0.58	0.53	**84.1**
FLEPHEDRONE	1.65	1.55	1.43	**86.7**	1.65	1.51	1.78	**107.9**
MEPHEDRONE	2.87	2.74	1.83	**63.8**	2.87	2.65	2.77	**96.5**
5-APB	5.54	5.6	4.32	**78.0**	5.54	5.55	5.39	**97.3**
6-APB	6.59	5.79	6.98	**105.9**	6.59	6.72	6.09	**92.4**
METHYLONE	2.52	2.84	2.25	**89.3**	2.52	2.68	2.21	**87.7**
3-MEO-PCE	4.02	5.3	4.32	**107.5**	4.02	4.22	3.92	**97.5**
BUTYLONE	7.49	7.24	6.81	**90.9**	7.49	6.49	7.11	**94.9**
ETHYLONE	2.88	2.23	2.79	**96.9**	2.88	1.85	3.09	**107.3**
MXE	3.46	2.39	2.55	**73.7**	3.46	3.30	3.00	**86.7**
BENZEDRONE	2.98	2.12	2.32	**77.9**	2.98	2.39	2.81	**94.3**
3-MEO-PCP	0.82	0.78	0.8	**97.6**	0.82	0.86	0.93	**113.4**
2-DPMP	3.05	2.77	2.68	**87.9**	**3.05**	3.4	3.55	**116.4**
MDPV	3.19	2.55	3.4	**106.6**	2.47	2.8	2.49	**100.8**
NAPHYRONE	3.09	2.38	2.78	**90.0**	2.99	2.3	2.58	**86.3**
25B-NBOMe	1.17	1.25	0.8	**68.4**	1.17	1.21	1.06	**90.6**
25C-NBOMe	1.37	1.28	1.02	**74.5**	1.37	1.09	1.4	**102.2**
25D-NBOMe	1.15	0.97	0.81	**70.4**	1.15	1.1	1.15	**100.0**
25E-NBOMe	1.21	1.05	1.02	**84.3**	1.21	1.13	1.22	**100.8**
25H-NBOMe	1.43	1.27	1.14	**79.7**	1.43	1.54	1.38	**96.5**
25I-NBOMe	1.19	0.91	0.89	**74.8**	1.19	1.01	0.96	**80.7**
Mescaline-NBOMe	0.98	1.07	0.73	**74.5**	0.98	1.1	0.81	**82.7**
25P-NBOMe	1.17	0.82	0.87	**74.4**	1.17	1.09	0.82	**70.1**

Previously published NBOMe methods have shown this class of compound to be unaffected by freeze/thaw cycles and this was corroborated by this research, with these compounds having an average recovery rate of 94%, (70.1-133.3%).(*162*) Of the stability data previously published it has been shown that 25T2-NBOMe was the most unstable compound. Although this method does not contain this compound it does contain 25T4 and 25T7-NBOMe, which were shown here to be susceptible to degradation in the fridge, with only 64% of the analyte recovered after 2 cool/warm cycles (ranging 55.6-75.0%).(*162*)

Again the lack of deuterated I.Ss may also have impacted on these results as the PAR for some compounds was extremely low, e.g. methiopropamine and 3-MeO-PCP. As a result of this, any differences observed upon the PARs affected the recovery of these compounds. This work should therefore be repeated once deuterated reference standards become available to better show the stability of these compounds in these matrices.

3.4.7 Case Samples

Case 1 contained 25I-NBOMe at concentrations of 1.77 µg/L and 1.32 µg/L in the two blood samples received. This was the only NBOMe identified in this case. These results were within 10% of the results obtained by NMS laboratories which were 1.81 µg/L and 1.44 µg/L respectively. A chromatogram and mass spectra are shown in Figure 3-20.

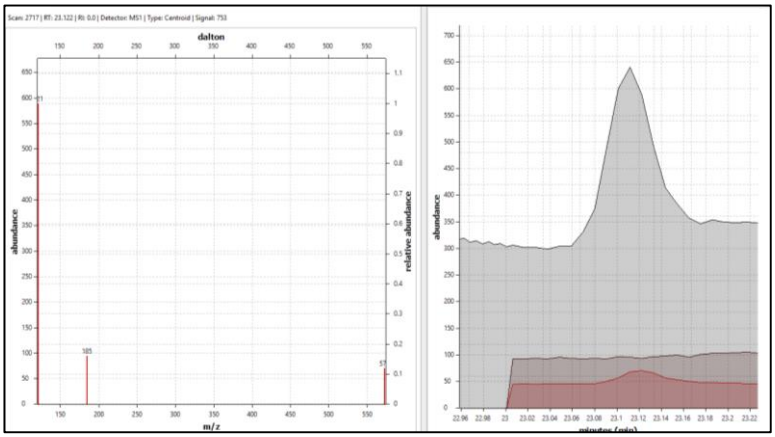


Figure 3-20: Extracted chromatogram of case 1 with the mass spectrum showing the monitored ions.

Case 2 contained only 25C-NBOMe at concentrations of 1.69 µg/L and 2.37 µg/L for the two blood samples. NMS found concentrations of 2.17 µg/L and 2.16 µg/L respectively. There was no information provided as to the collection sites of these blood samples; however, it may be that they were from the same site given the similarity of the two NMS results. The results obtained using this method differ slightly from those of NMS laboratories with concentrations lower (-22%) for the first sample and higher (+10%) for the second. In order to interpret these changes better further information would be required as to how each sample was stored, for example whether one was closer to the back of the freezer than the other. It is also not known whether they are from different sites where components in the blood may have affected the 25C-NBOMe concentration. It is also unknown whether samples underwent additional testing and as a result more freeze-thaw cycles. All these variables could have affected the concentrations of 25C-NBOMe in the samples and thus affected the concentrations detected. It is possible that these results are within the realms of "normal" experimental error as the concentrations are so low. The chromatogram and mass spectra for case 2 is shown in Figure 3-21.

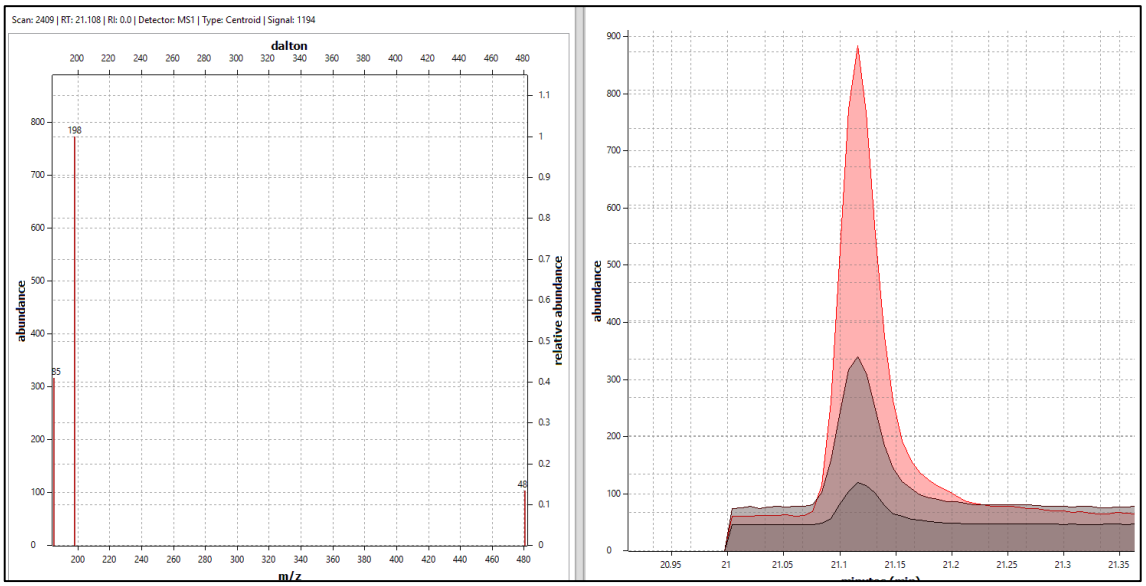


Figure 3-21: Extracted chromatogram of case 2 with the mass spectrum showing the monitored ions

Case 3 - no analytes were detected. NMS were able to identify 25I-NBOMe at a concentration of 0.32 µg/L which is below the LOD (0.4 µg/L) for this method. It therefore follows that it was not detected by GC-MS.

Case 4 – no analytes were detected. NMS were able to detect 25I-NBOMe at a concentration of 1.55 µg/L. This case involved post-mortem blood and is the only sample where it is known whether the blood was drawn ante- or post-mortem. This sample therefore raises the question of post-mortem stability of these compounds as it appears there has been a significant reduction in the concentration. As the history of the storage conditions of the sample is not known, no conclusions can be drawn as to why the reduction in concentration has taken place. It is possible that components within the post-mortem blood have resulted in its degradation. In order to assess this, it would be interesting to analyse this sample for any metabolites which may be present.

Case 5 contained the highest concentration of NBOMes detected for all samples testing positive for 25C-NBOMe (4.04 µg/L). Although there is no information available, the high concentration detected may indicate that this is a post-mortem sample, however without additional information this cannot be confirmed. The concentration detected was 11% lower than that by NMS, again the time interval between sample analysis will have contributed to this.

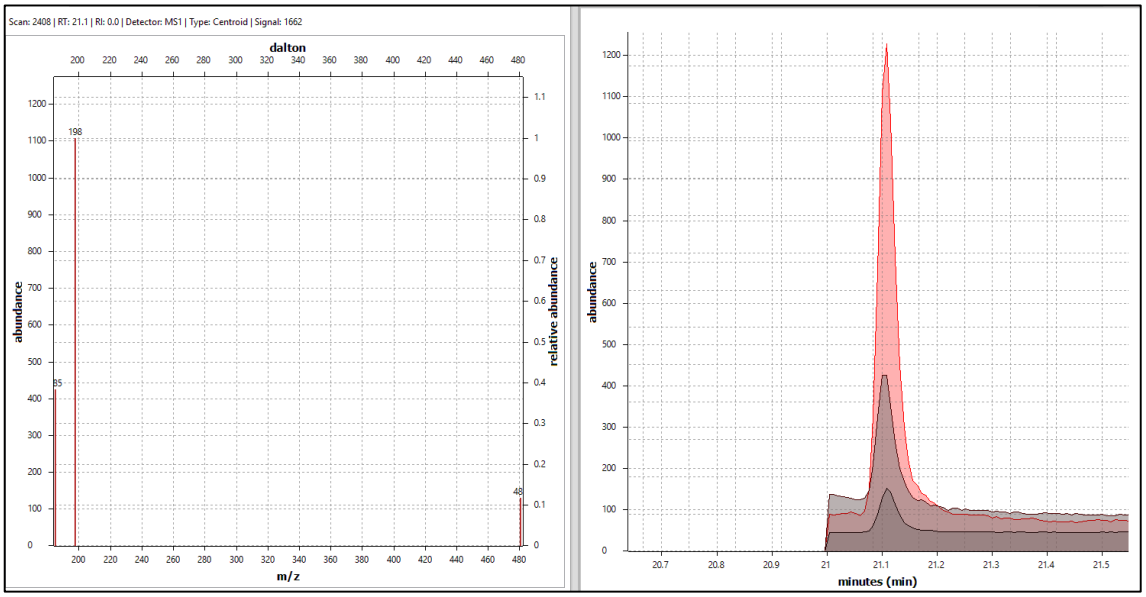


Figure 3-22: Extracted chromatogram of case 5 with the mass spectrum showing the monitored ions.

Case 6 - no analytes were detected. NMS detected 25I-NBOMe at a concentration of 0.73 µg/L. Similar to case 4 it is possible that concentrations may have degraded and now fall below the LOD of this method.

Case 7 contained less than 1 mL of blood; therefore, only 250 µl of sample was extracted as in section 3.3.8. All other steps remained the same. The results of this sample showed that it was clearly positive for methoxetamine only as shown in Figure 3-23. Case 7 was quantified as having a methoxetamine concentration of 7.6 mg/L. Although this falls out with the calibration range of the method the reduced volume concentration of 1.9 mg/250 mL was within the calibration range.

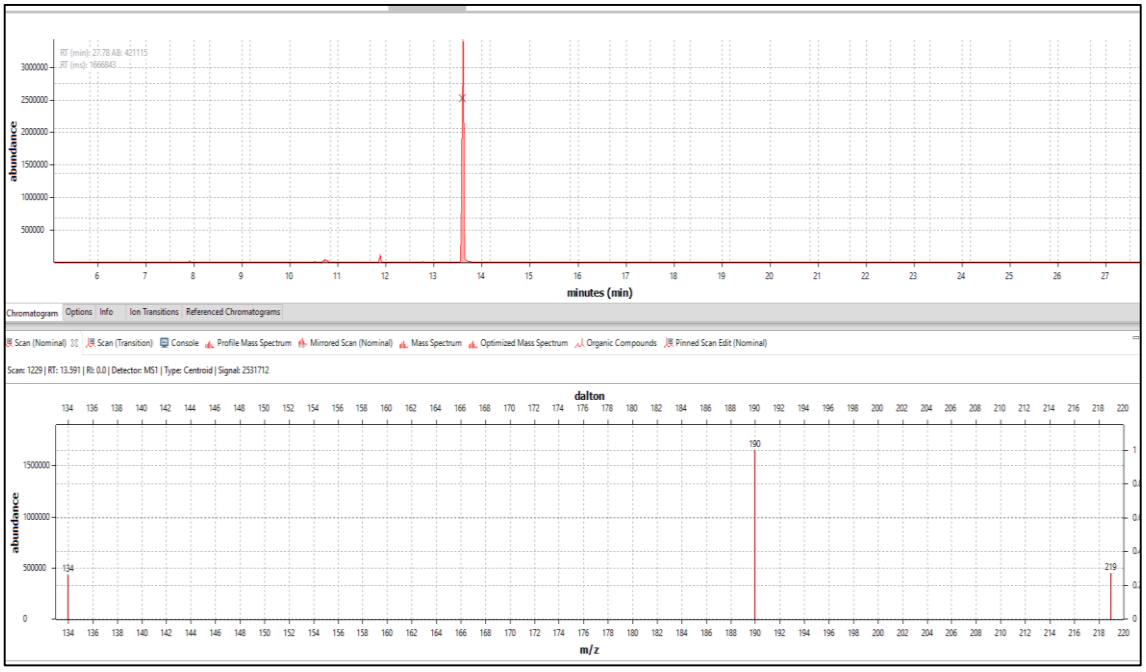


Figure 3-23: Chromatogram of case 7 the mass spectrum showing the monitored ions.

Case 8 tested positive for methylone (Figure 3-24) at concentrations of 0.11, 0.08 and 10.6 mg/L. NMS were only able to quantify the 1st and 3rd sample relating to this case, identifying concentrations of 0.17 and 10 mg/L. This case highlights the importance of knowing where blood samples were collected as the methylone concentrations vary hugely between cavity and peripheral blood sites.

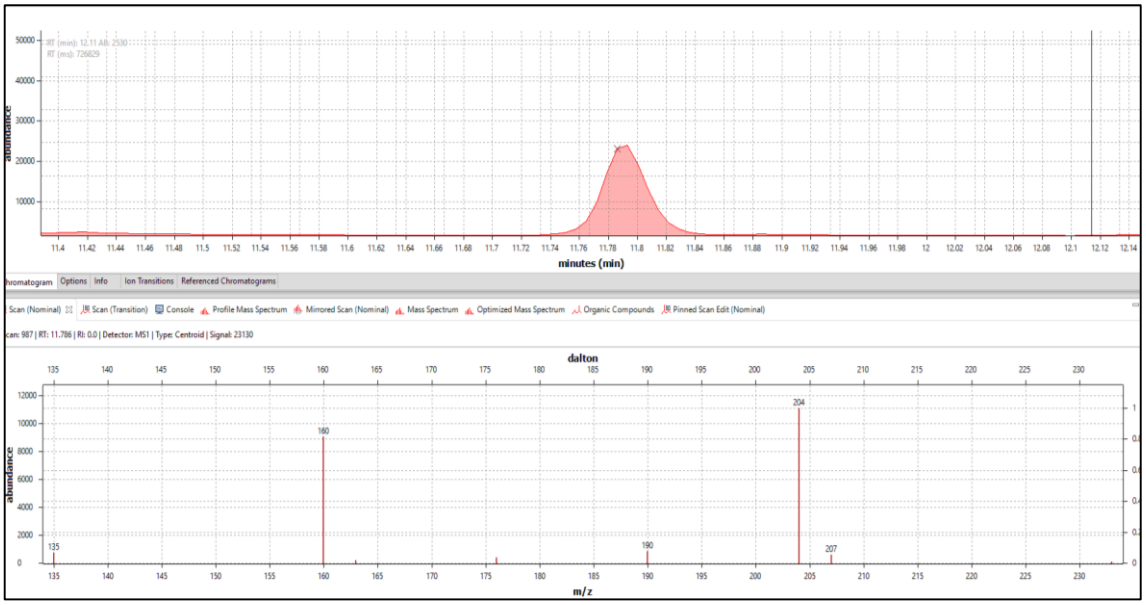


Figure 3-24: Chromatogram and mass spectra of Case 8 sample1.

A summary of all results can been seen in Table 3-22.

Table 3-22: Summary of case results and comparison with NMS results.

Case Nº	Site of collection	GC-MS Method		NMS Results		%Deviation
		Analyte Identified	Concentration Detected	Analytes Identified	Concentrations Detected	
1	Cardiac Blood	25I-NBOMe	1.77 µg/L	25I-NBOMe	1.81 µg/L	(2.2%)
	Cardiac Blood	25I-NBOMe	1.32 µg/L	25I-NBOMe	1.44 µg/L	(8.3)
2	No info	25C-NBOMe	1.69 µg/L	25C-NBOMe	2.17 µg/L	(22.1)
	No info	25C-NBOMe	2.37 µg/L	25C-NBOMe	2.16 µg/L	9.7
3	No info	N.D.		25I-NBOMe	0.32 µg/L	N/A
4	Post mortem Blood	N.D.		25I-NBOMe	1.55 µg/L	N/A
5	Iliac Blood	25C-NBOMe	4.04 µg/L	25C-NBOMe	4.53 µg/L	(10.8)
6	Blood	N.D.		25I-NBOMe	0.78 µg/L	N/A
7	Peripheral Blood	Methoxetamine	7.60 mg/L	Methoxetamine	8.2 mg/L	(10.8)
8	Cavity Blood	Methylone	0.11 mg/L	Methylone	0.17 mg/L	(7.3)
	No info	Methylone	0.08 mg/L	Methylone	Not Quantitated	N/A
	Peripheral Blood	Methylone	10.60 mg/L	Methylone	10 mg/L	(35.3)

*ND- Not detected

Due to the compounds analysed by this GC-MS method, case samples were more difficult to come by than more commonly abused drugs such as heroin, cocaine or amphetamine. As a result, only 12 samples were currently available from NMS laboratories containing substances detected by this method. Unfortunately, these samples were all blood and no case samples were available to test the validity of the urine method.

At the time of analysis, NMS only screened for flephedrone, mephedrone, methylone, ethylone, butylone methoxetamine, MDPV, naphyrone, 25B-NBOMe, 25C-NBOMe and 25I-NBOMe and thus only held records relating to samples which were positive for these substances. Unfortunately, the samples tested did not test positive for any of the other compounds in this method.

To date, this is the first known method for the identification of NBOMes in blood using GC-MS. Due to the low concentrations detected, all previously published methods for biological matrices have used LC-MS/MS. The method was unable to detect 25I-NBOMe in 3 of the 5 NBOMe samples due to low concentrations and although in some instances it was possible to detect and quantify NBOMes using this instrumentation it is much more challenging. Due to the small peak areas involved manual integration was required for the majority of the calibrators, QCs and samples which is much more time intensive. Although the integration parameters of ChemStation can be altered there is a trade-off between detecting peaks of interest and detecting everything on the chromatogram.

The method itself was also relatively long taking 30 minutes per sample resulting in a maximum of 48 injections per day. It is therefore suggested that this method would be used for targeted analysis when NPS use was suspected, rather than a quantitative screening method.

Instrument maintenance was also important when developing this method due to the low concentrations detected, making it important that the instrument remained clean throughout analysis. This may be a limitation if instrumentation is shared between different forensic disciplines.

3.5 Conclusion

A robust and sensitive method for the simultaneous analysis of various NPSs in urine and blood was validated. The method was successfully able to identify and quantitate NBOMes in 5/8 samples, and thus was not able to compete alongside more sensitive techniques such as LC-MS/MS.

The majority of analytes were separated both chromatographically and by their mass spectra. The method could also be easily updated to incorporate new NPSs as and when they become available.

This method is of particular use for laboratories who do not possess an LC-MS/MS for the detection of NBOMes in acute fatalities. The method may be more applicable to urine cases as concentrations may be higher, although this would need further investigation using “real life” samples, especially since metabolites may be more relevant targets.

3.6 Future Work

Due to the long run-time of the GC-MS method it was not possible to complete all additional validation parameters listed in the SWGTOX guidelines.

SWGTOX recommends that 3 freeze/thaw cycles are performed this was not possible due to time constraints. It is therefore suggested that this work is repeated to meet these standards. Further stability work should be carried out where spiked matrix is aliquoted into individual vials prior to fridge and freezer storage, thus removing the cool/warm, freeze thaw variable. This will give a better understanding as to whether it was the storage conditions themselves, or the effects of these cycles which caused fluctuations in the amount of analyte detected.

SWGTOX does not provided a specific time period for assessing autosampler stability, however, the majority of publications evaluate this parameter up to 72 hours. This was the initial plan for this validation, however, due to instrument failure the 72 hour samples were unable to be analysed.

Chapter 4: Stability of Mephedrone in Bovine Blood

4.1 Cathinone Stability

Knowing the stability of a drug is extremely important in forensic toxicology as it can affect the concentrations detected and alter any interpretations made from these concentrations.(*205*) It is therefore important for toxicologists to be aware of the time period between sample collection and analysis, the conditions the sample was transported under and additional factors such as degradation in post mortem cases as all will affect the concentrations detected and reported. Samples may be called into question in court and the defence may opt to have samples re-analysed at a later date. Samples may be re-analysed as detection methods within the laboratory improve, or if initial analysis was not confirmatory, i.e. the QC's in a batch failed to pass the methods acceptable criteria. Knowledge of drug stability is therefore vital in all these instances. More recently with the appearance of NPSs, many laboratories performed retrospective analysis on samples where NPS use was suspected (*189*). Again, the stability of these drugs plays an important role when carrying out this analysis and interpreting results as the concentrations detected may differ significantly from when the sample was initially received.

Previous work has shown that cathinone is unstable in its natural plant form (Khat) and that the fresh leaves must be consumed quickly in order to extract the drug (*206*). This instability is one of the many factors as to why it took over 100 years of experimental work before cathinone was isolated from the plant (*207*). Cathinone stability in biological matrices is equally poor with various studies showing rapid loss of the target analyte over a short time period (*165, 208, 209*).

The stability of mephedrone has also been noted as being poor in various publications validating analytical methods (SWGTOX guidelines state that analyte stability should be investigated as part of method validation).(*200*) This is unsurprising as mephedrone is an analogue of cathinone, which is well documented as unstable.(*207*) Previous work has shown that mephedrone stability is affected by storage temperature and the use of preservatives;

however, the majority of this research only focuses on one variable at a time over a short period of time. (*210, 211*)

4.2 Aims

The aim of this study was to assess the stability of mephedrone and the effects of storage temperature and preservatives upon its stability. In order to ascertain the effect of preservatives upon blood samples unpreserved blood will need to be used as a control. When blood is drawn from individuals it goes directly into a bag which contains anti-coagulants already. As a result blood collected from blood banks cannot be used. Ethical issues surrounding the use of blood also prevents blood collection specifically for this project and hence unpreserved bovine blood was used, as this avoids these issues.

4.3 Materials & Methods

4.3.1 Chemicals

Mephedrone and mephedrone-D_3 reference standards (1 mg/mL) were purchased from Cerilliant (Dorset, England). Unpreserved bovine blood was donated from Sandyford Abattoir (Paisley, UK). Citric acid powder was from Sigma Aldrich (Dorset, England) and Fluoride/Oxalate tubes were from Teklab (Durham). SPE Cleanscreen® columns were purchased from Presearch (Hampshire, England). All other chemicals were of analytical grade and purchased from Sigma Aldrich (Dorset, England).

4.3.2 Solution Preparations

4.3.2.1 Citric Acid Solution

A 0.8% citric acid solution was made by weighing 8 g of citric acid and dissolving this in 100 mL dH_2O in a small beaker. This solution was then transferred to a 1 L volumetric flask, the empty beaker rinsed with more dH_2O and these washes added to the 1 L volumetric flask. This was repeated several times until the 1 L flask had been made up to the mark. The volumetric flask was inverted several times to ensure thorough mixing.

4.3.2.2 Phosphate Buffer (pH6)

Phosphate buffer (pH 6) was prepared by adding 12.14 g sodium dihydrogen orthophosphate and 1.62 g of potassium dihydrogen to a 1 L volumetric flask. This flask was then made up to the mark using dH_2O before being pH adjusted to pH 6.

4.3.2.3 Mephedrone and mephedrone-D_3 solutions (10 µg/mL)

To produce a 10 µg/mL solution of mephedrone, 1 mL of a 1 mg/mL reference solution was transferred to a 10 mL volumetric flask and made up to the mark using MeOH. This solution was inverted several times to ensure thorough mixing before being transferred to a labelled amber bottle for storage throughout the project. The same procedure was used to make a 10 µg/mL solution of mephedrone-D_3 I.S. solution. A second 10 µg/mL mephedrone solution was made by a different analyst on a different day and used for the preparation of QCs.

4.3.3 Blood preparation and storage

Fresh, preservative free, bovine blood (1.25 L) was collected from the abattoir. Mephedrone (1 mg/mL) was transferred to a 1 L volumetric flask and made up to the mark using bovine blood, producing a final mephedrone concentration of 1 mg/L. This solution was inverted several times and shaken to ensure the drug was thoroughly mixed throughout the blood solution. Aliquots of 1 mL were added to 120 individual 2.5 mL plastic vials to provide information on stability when no preservative has been added. A 250 mL aliquot of the mephedrone spiked blood was transferred to a separate beaker and spiked with 3.75 mL citric acid solution (0.8%). As with the unpreserved vials, 1 mL of the citric acid mephedrone spiked blood was added to 120 individual 2.5 mL plastic vials. The effect of sodium fluoride (1.67%)/potassium oxalate (0.20%) preservative was investigated by adding 1 mL of mephedrone spiked bovine blood to 120 commercially manufactured preservative vials.

Samples were then stored at 3 different temperatures (-20°C, 4°C and 20°C) as shown in Figure 4-1. Samples were stored individually to avoid the effects of freeze-thaw cycles and 20°C samples were also stored in the dark to avoid the

additional variable of light affecting stability. The remaining 250 mL bovine blood which was not spiked with mephedrone was stored at 4ºC for use when preparing QCs and calibration standards.

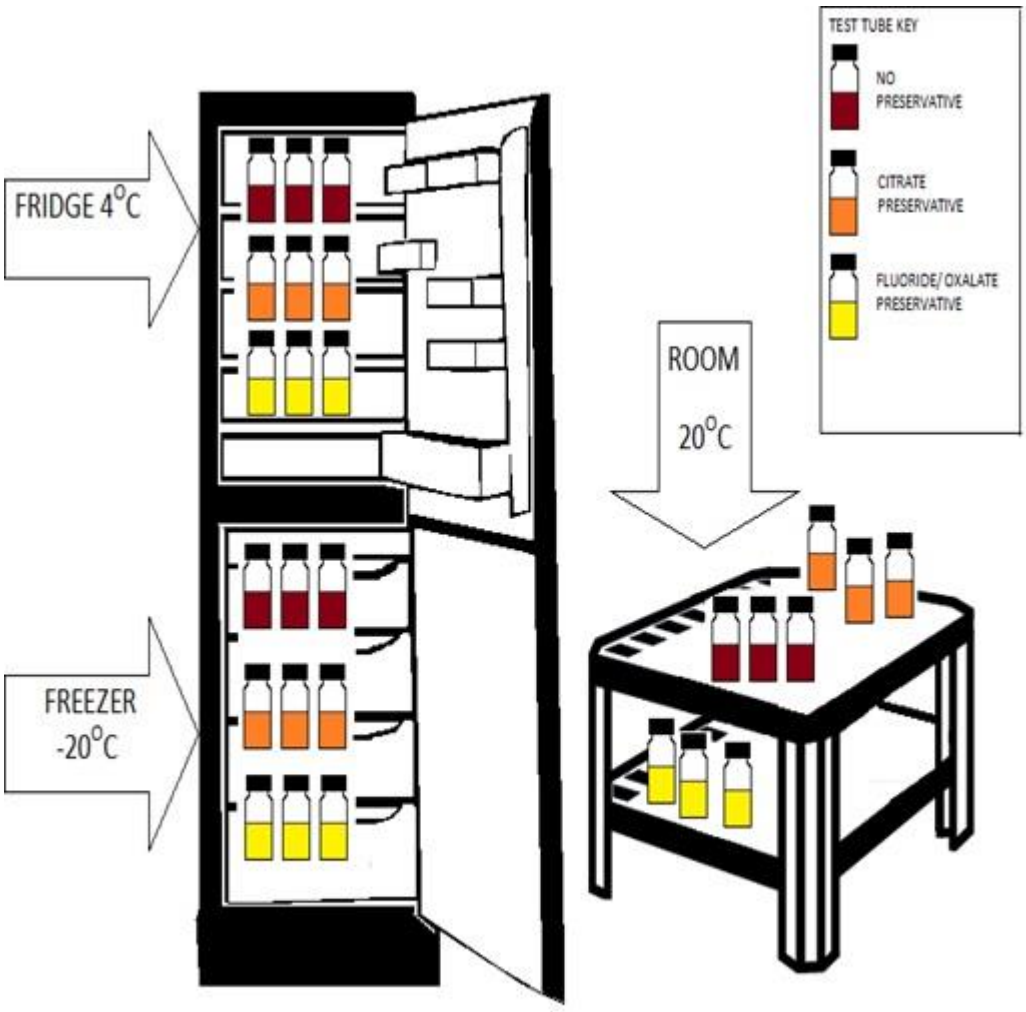


Figure 4-1: Storage conditions of mephedrone spiked bovine blood samples stored without preservative and with two types of preservative at different temperatures.

4.4 Sample Analysis

Samples were analysed daily for the first 7 days and weekly for a period of 10 weeks. They were analysed in triplicate with freshly prepared calibrators and QCs run prior to each analysis.

4.4.1 Calibrators, QCs and Sample Preparation

Calibrators and QCs were prepared using the FMS amphetamine method. To all calibrator, QC and sample test tubes 5 mL of pH 6 phosphate buffer was added,

as well as I.S. (50 µl of 10 µg/mL mephedrone-D_3). To the calibrator test tubes, mephedrone solution (10 µg/mL) was added in volumes from 0-200 µl and to the QC test tube 42 µl of mephedrone (10 µg/mL) was added as shown in Table 4-1. QC solutions were made using the QC mephedrone solution. Blank bovine blood (1 mL) was then added to the calibrators and QC. Calibrators, QCs and samples were vortex mixed for 30 seconds, prior to being centrifuged for 10 minutes at 3500 rpm.

Table 4-1: Volume of mephedrone solution (10µg/mL) added to each test tube to produce calibrators and QC.

	Mephedrone Solution (10µg/mL) volume (µL).	Mephedrone Concentration (mg/L)
Blank	0	0
Calibration 1	10	0.1
Calibration 2	25	0.25
Calibration 3	50	0.5
Calibration 5	100	1.0
Calibration 6	200	2.0
QC	42	0.42

4.4.2 Extraction

Extraction was carried out using SPE. Cleanscreen® columns were conditioned using 3 mL MeOH, 3 mL dH_2O and 1 mL pH 6 phosphate buffer. The samples, calibrators and QCs were then loaded onto the columns before washing with 3 mL dH_2O, 1 mL of 1 M acetic acid and 3 mL MeOH. The columns were then left to dry under full vacuum for 5 minutes. Elution was carried out using 3 mL of CH_2Cl_2/IPA/NH_3 (78/20/2). The samples, calibrators and QCs were then evaporated to dryness under a stream of nitrogen with no additional heat before derivatization with 50 µl of PFPA:EtOAc (2:1) for 20 mins at 60^{O}C. This derivatization solution was then evaporated off again using a stream of nitrogen before the samples, calibrators and QCs were reconstituted in 250 µl EtOAc and transferred to GC vials for analysis.

4.4.3 Instrumentation

Analysis was carried out on a Bruker GC-MS/MS fitted with a DB5 column (30 m x 0.25 mm, 0.25 µm). Helium was used as carrier gas at a flow rate of 1.5 mL/min.

The temperatures of the injection port, ion source and interface were 225ºC, 250ºC and 200ºC respectively. The injection port was operated in splitless mode. The initial oven temperature was 80ºC, (held for 2 minutes), then increased to 170ºC at a rate of 25ºC/min. The temperature was then ramped to 200ºC at a rate of 5ºC/min before a final ramp of 25ºC/min to 300ºC (hold time 2 minutes). The total analysis time was 23 minutes. The mass spectrometer was operated in single quadrupole EI mode (full scan mode from m/z 40 to m/z 450 amu). Data was processed using Xcaliber 1.4 software.

4.5 Results

Mephedrone was identified using m/z ions 204, 160 and 119 with 204 being used for quantitative analysis. Mephedrone-D_3 was identified using ions 207, 163 and 119 with 207 being used for quantitative analysis. Mephedrone and mephedrone-D_3 eluted at 13.1 minutes and 13.4 minutes respectively as shown in Figure 4-2.

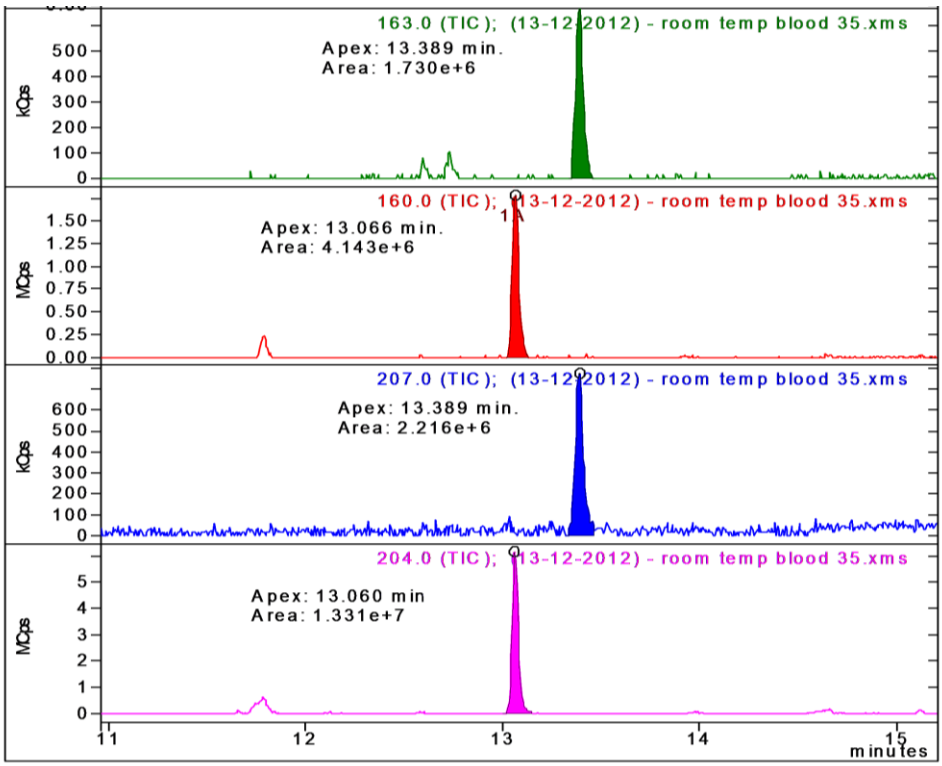


Figure 4-2: Example chromatogram showing an unpreserved blood sample stored at room temperature on day 35.

The effect of preservatives and storage temperatures are shown in Figure 4-3 to Figure 4-5. Mephedrone concentrations were least stable when stored with no preservative, with mephedrone becoming undetectable in unpreserved samples

stored at room temperature after a period of 21 days as shown in Figure 4-3. After 1 day, unpreserved samples stored at room temperature decreased on average by 19%. Unpreserved samples stored in the fridge and freezer did not show any significant decrease in mephedrone concentrations until day 36 and 43 respectively.

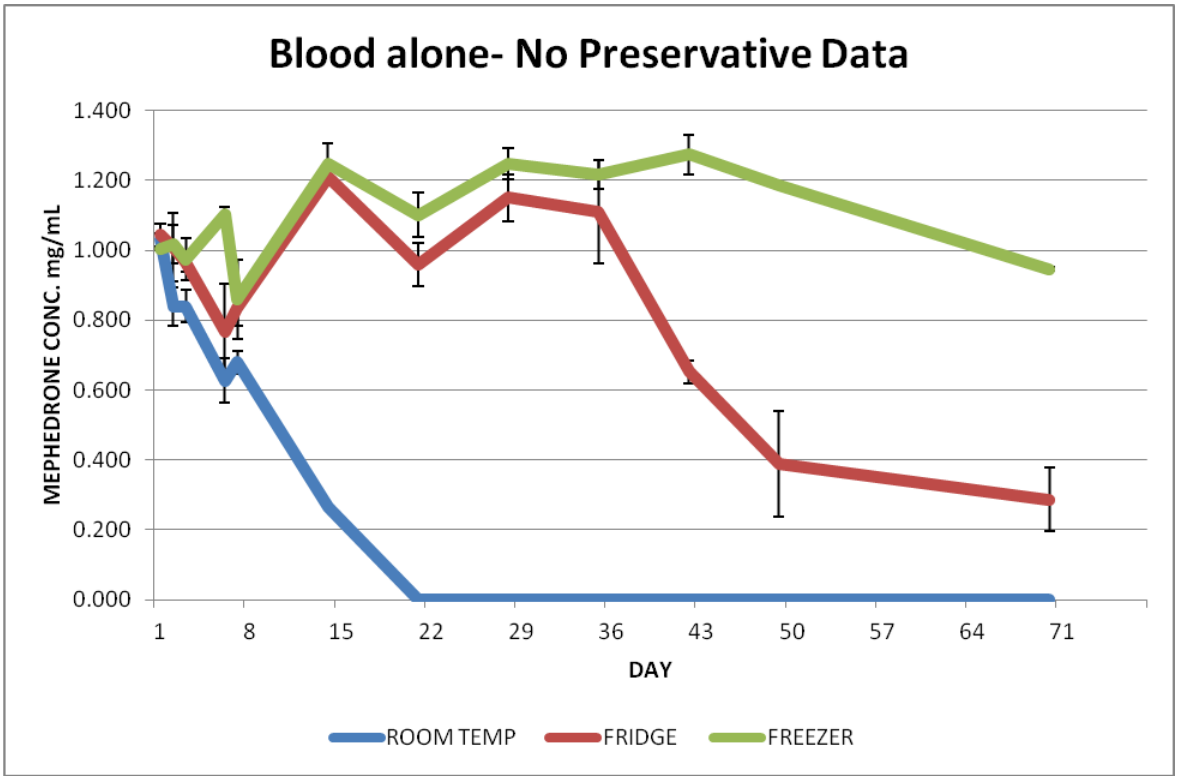


Figure 4-3: Mephedrone concentrations of samples stored with no preservative over 10 weeks at 3 different temperatures.

Samples stored with citric acid preservative as shown in Figure 4-4 were the most stable over the 70-day period. The addition of citric acid to samples stored at room temperature had little effect on mephedrone preservation with samples seeing an average decrease of 17% after the 1st day. Mephedrone concentrations were again undetectable in room temperature stored samples after 21 days. Samples stored in fridge and freezer conditions using citric acid preservative remained relatively stable throughout the 10-week period.

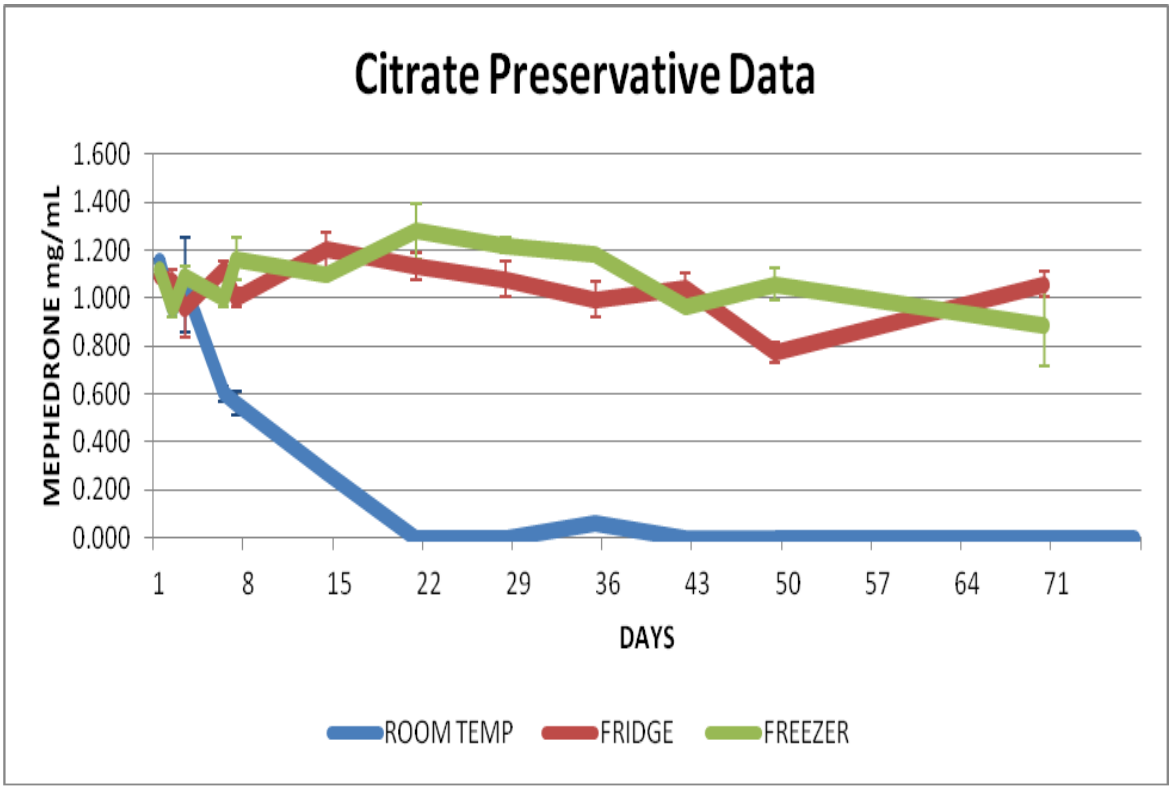


Figure 4-4: Mephedrone concentrations of samples stored with citric acid preservative over 10 weeks at 3 different temperatures.

Fluoride/oxalate samples were stable when stored in the freezer as shown in Figure 4-5. Fridge samples were initially stable however concentrations rapidly decreased after 35 days becoming undetectable after 49 days. Room temperature samples preserved with fluoride/oxalate were stable for slightly longer than unpreserved or citrate preserved samples at room temperature. These became undetectable after 28 days; however mephedrone concentrations decreased by 23% on average after the first day.

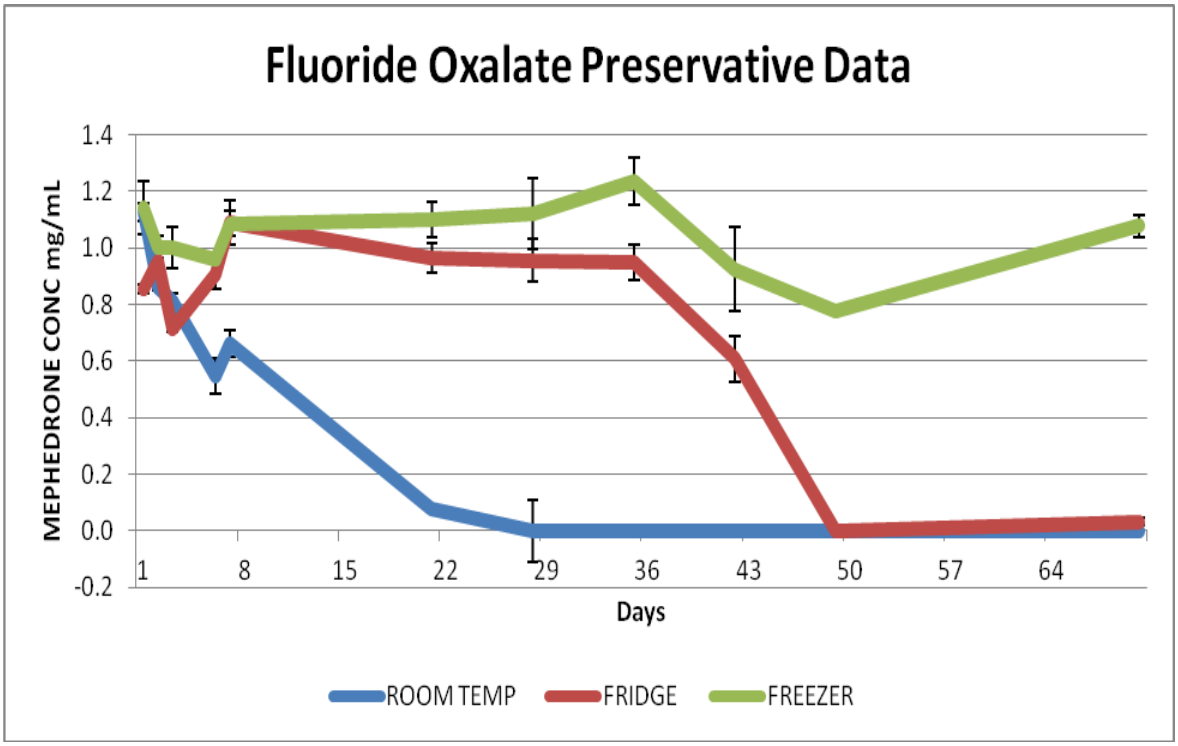


Figure 4-5: Mephedrone concentrations of samples stored with fluoride/oxalate preservative over 10 weeks at 3 different temperatures.

4.6 Discussion

The data produced in this study correlate with several previous studies in that mephedrone is unstable at room temperature and to a lesser degree when stored in refrigerators.

Johnson *et al.* investigated the effect of storage temperature on MDPV, mephedrone, BZP and TFMPP in three biological matrices (whole blood, plasma and urine) over a period of 14 days (*211*). They found that mephedrone rapidly degraded when stored at room temperature, unable to detect it by day 7. This differs greatly from this research which was able to find mephedrone at small concentrations up to day 21. Johnson *et al.* carried out their analysis using LC-MS/MS with a lower LOD (2 ng/mL) than the method used here and their room temperature was maintained by a thermostat at 22°C. It is therefore unlikely that either of these variables is responsible for the elongation in detection time between these studies. The source of human whole blood was not provided in this publication, and therefore any preservatives and anti-coagulants used at the point of collection are unknown, although it is likely that they would have slowed degradation of mephedrone in samples. Therefore, it falls that a

component of human blood not present in bovine blood may cause the accelerated degradation seen in previous research.

Maskell *et al*. investigated the stability of 4 NPSs including mephedrone and MDMA when stored in solutions of varying formalin concentration and pH at 4°C (*212*). In this study mephedrone degradation occurred at a faster rate with increased formalin concentration and with increasing pH. As a result, sample pH may be another reason that Johnson *et al*. were unable to detect mephedrone after 7 days in room temperature, as this information was not provided.

This work was carried out prior to the GC-MS method validation described in Chapter 3. This was due to the on-going development of this method adding additional NPSs when they became available.

The GC-MS was also operated in full scan mode opposed to SIM. This was to enable retrospective data mining to determine if any breakdown products could be identified. Unfortunately, this was not possible to do as the baseline of the GC-MS/MS was sufficiently high to mask peaks, and these were only identifiable when specific ions were extracted.

4.7 Conclusion

To maximise the stability of mephedrone, samples should be stored at -20°C and preserved using citric acid solution. Although fluoride/ oxalate was shown to preserve samples when stored at -20°C, degradation was still problematic at 4°C with a loss of 36% after 35 days. Regarding retrospective analysis, care should be taken when interpreting negative results in cases where the history supports mephedrone use. The time between death and post mortem investigation should also be taken into consideration as should the storage conditions of the sample in the lead up to toxicological analysis and screening.

4.8 Further Work

Cerilliant's certificate of analysis states that they did not observe a decrease in the purity of methanolic mephedrone reference material over the course of a week when stored at -15°C to 40°C, and that methanolic mephedrone solutions

are stable for up to a year when refrigerated. This work suggests that this might not be the case. Future work on this topic would examine whether methanolic solutions of mephedrone are as unstable at room temperature as when the drug is in blood. Until this work is completed it is recommended that methanolic mephedrone solutions are stored in the freezer to prevent solution degradation.

This study is also limited by the number of variables investigated. Future work should also look into the stability of mephedrone in additional biological matrices, the effect of light and additional preservatives which are commonly encountered by forensic laboratories. The effect of pH may also be a factor and was not investigated in this short study. Although the pH of blood is regulated to fall between 7.35 and 7.45 it may fall outside this in cases of ketoacidosis, and this in turn could affect the findings of this study. The pH of urine however varies much more and this may be a more important variable to consider when investigating the stability of mephedrone in urine samples.

Chapter 5: Immunoassay screening: cross-reactivity

5.1 Enzyme-linked Immunosorbent Assay

Prior to confirmatory analysis, samples typically undergo presumptive testing for a range of drug classifications. This allows forensic laboratories to have targeted analysis, reducing sample run time, overhead costs and the amount of sample used during testing. The most common presumptive tests for drugs of abuse are enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA). These commercially produced assays allow samples to be screened either for specific drugs and their metabolites or for a specific drug group, indicating what drugs may be present within the sample. EIA and ELISA are commonly used due to the speed at which positive samples can be identified and because they have the potential for full and or semi-automation.

All EIA and ELISA tests are based on the principle that each antigen has a corresponding antibody. ELISA and EIA kits are typically found in a 96 well plate format with anti-drug antibodies coated onto the inside of each well. Controls, a blank and samples are then added to individual wells before the addition of an enzyme conjugate. The drug and enzyme conjugate compete for antibody binding sites, hence this type of ELISA/EIA is termed competitive immunoassay.(*213*) Excess conjugate is removed through wash steps before the addition of a substrate. This substrate then produces a colour change indicating the amount of enzyme conjugate bound to the antibodies. The ELISA kits, which were purchased from Immunalysis, used in this project turn from yellow to blue to indicate the presence of bound enzyme conjugate, therefore the more intense the blue colour, the more enzyme conjugate has been able to bind to the antibodies contained within the well. The amount of enzyme conjugate able to bind inversely relates to the amount of drug present in the sample, as the drug competes with the enzyme conjugate and prevents binding occurring. This is illustrated further in Figure 5-1.

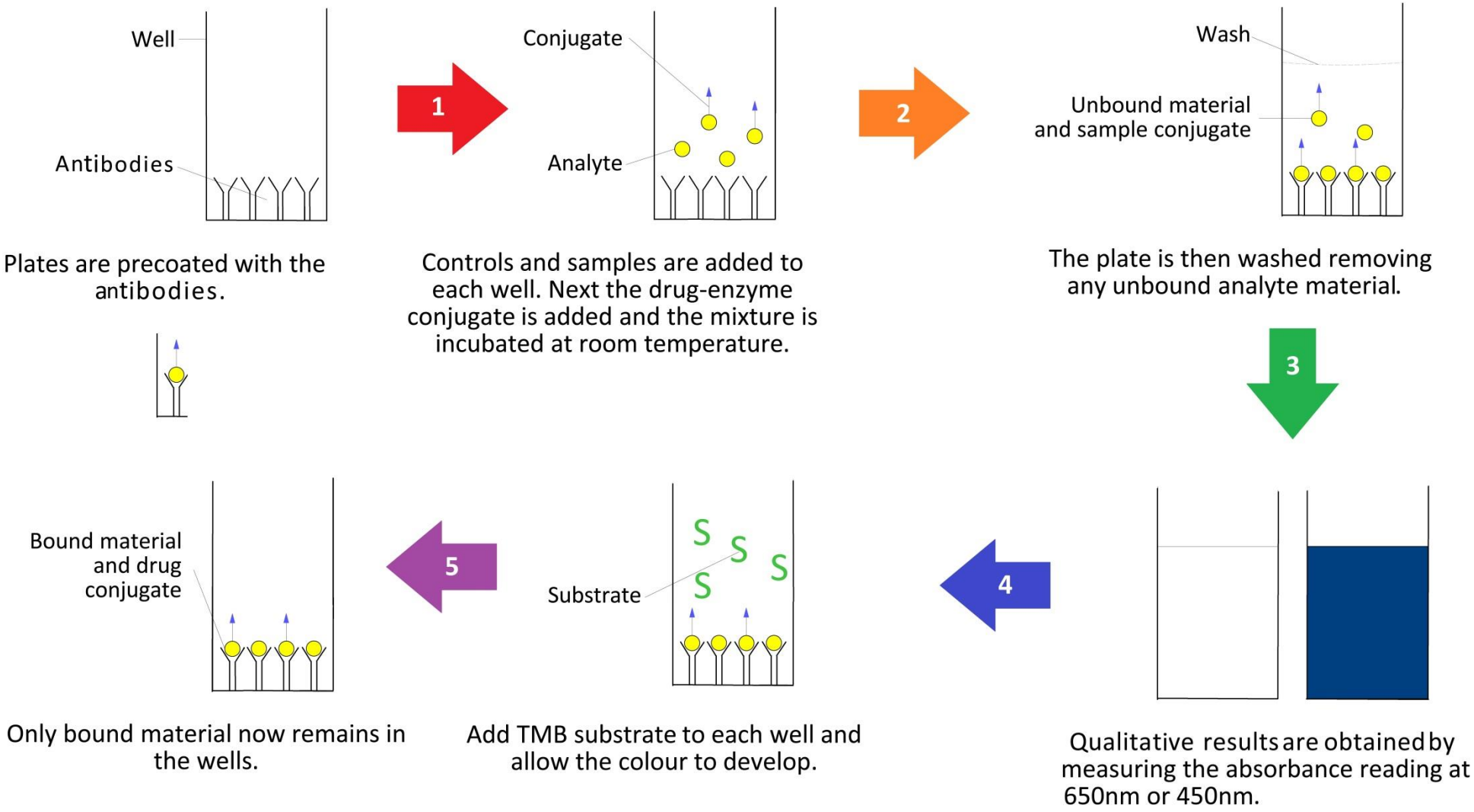


Figure 5-1: Immunalysis ELISA protocol.

5.1.1 ELISA Cross Reactivity

In order to determine whether an assay is suitable for the detection of a particular drug or for a particular drug class, its cross reactivity is calculated as shown in Equation 5-1.

Equation 5-1: Percentage Cross Reactivity.(*214*)

$$Cross\ Reactivity\ (\%) = \frac{Apparent\ conc.of\ target\ drug}{Conc.\ of\ added\ drug} x100$$

Cross reactivity relates to the level at which the target drug molecules bind to the antibody coated wells. This is important when dealing with different biological matrices, for example urine samples when metabolites are used to establish the presence of a drug. Cross reactivity can also be used to determine how the assay interacts with other substances which are not the specific drug it was designed to cross react with. Kits can be designed to be drug specific or drug group specific. For example, morphine will react with both an opiates kit and a morphine specific kit, the only difference being that there will be lower cross reactivity with the morphine kit as it should only test specifically for this opiate. However, this is not always the case, and with the sudden influx of new drugs onto the market, all with similar structures to drugs already identified by ELISA kits, it is likely that some of these will produce positive ELISA results.

Mephedrone cross reactivity with methamphetamine ELISA kits has been shown, although no cut off levels were provided in this brief publication.(*189*) This publication also noted that mephedrone failed to cross-react with their amphetamine ELISA kits. MDPV cross reactivity with a phencyclidine immunoassay kit has also recently been published with a cut-off concentration of 6.2mg/L.(*215*)

5.2 Point of Care Testing

Point of care testing (POCT) is similar to ELISA, providing presumptive results as to whether a drug is present in a biological sample. Since these results are presumptive a follow up analysis should be carried out using alternative analytical methods for confirmation.

POCT is predominantly used in hospitals and work place testing and is sometimes referred to as near patient testing as it takes place away from the laboratory. Its main benefits are that it is non-evasive, simple to use and quick to perform.

POCT uses lateral flow technology first commercially applied in 1985 to Unipaths Clearview home pregnancy test.(*216*) Lateral (or vertical) flow assays consist of an absorbent pad to which the sample (in this case urine) is applied to. The sample then travels along the pad which contains antibodies specific to the target analyte or class of analytes being tested for. These antibodies are conjugated to coloured particles, typically colloidal gold nanoparticles or latex microspheres.

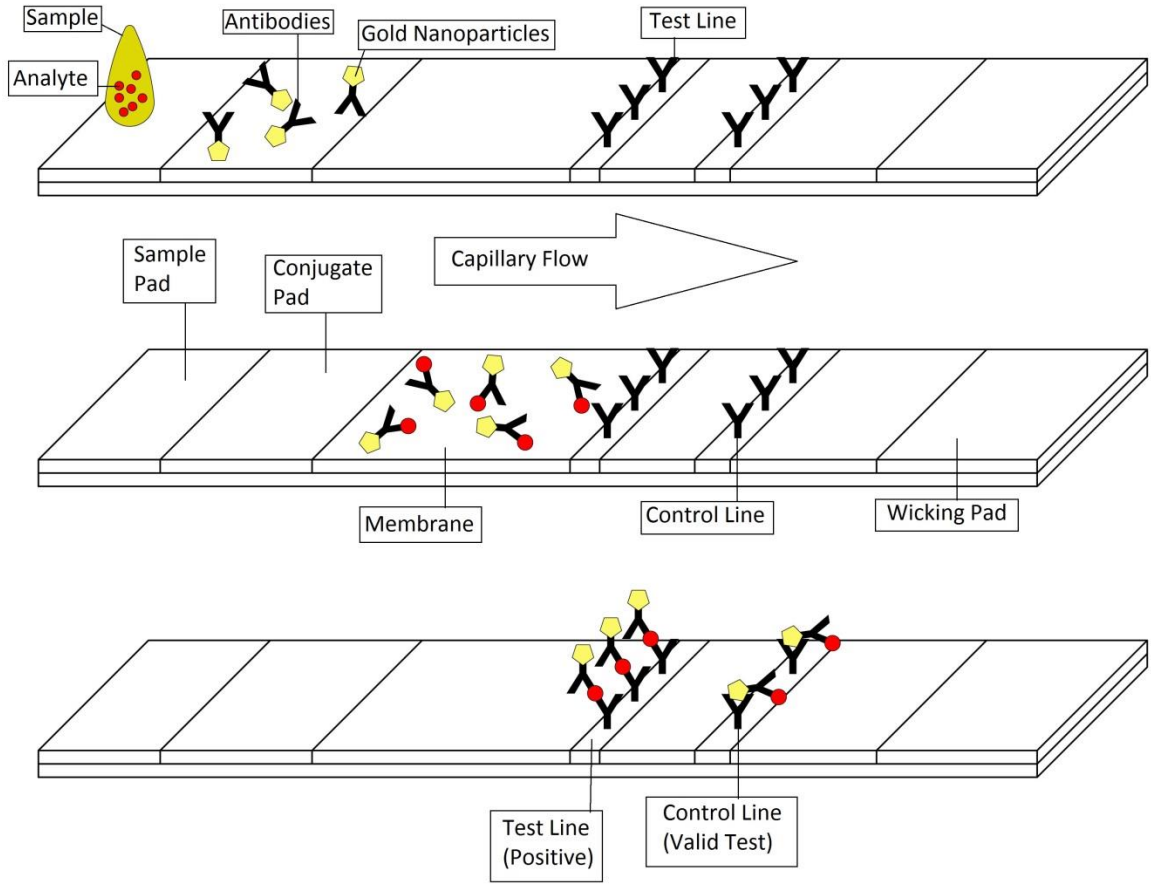


Figure 5-2: Diagram of a lateral flow POCT.

Any target analytes present in the sample bind to these colour conjugated antibodies, travelling further down the sample pad by capillary flow. The analytes and antibodies then reach the reaction membrane line (usually nitrocellulose or cellulose acetate) to which “anti-target” antibodies are bound.

In traditional lateral flow tests the sample and its conjugated antibodies travel over this area, and are immobilized by these “anti-target” antibodies. This creates a colour change resulting in a positive sample line appearing. This is shown in Figure 5-2.

In this research the lateral flow tests used competitive binding, thus analyte contained within the sample bound to the “anti-target” antibodies preventing a colour change. After the test line unbound conjugate antibodies continue to travel until they reach a second membrane line. Here they become bound by another set of antibodies, again, causing a coloured line to appear known as the control line.

Absence of this control line on a test shows that capillary flow of the sample has not occurred and therefore any negative results cannot be relied upon as the test has failed to work.

5.3 Aims

The aim of this work was to establish if any cross-reactivity was observed using amphetamine, methamphetamine and ketamine ELISA kits with various NPSs. The cross-reactivity of ketamine substitutes, methoxetamine, 3-MeO-PCE and 3-MeO-PCP for various ketamine POCT was also investigated.

5.4 Materials & Methods

5.4.1 Chemicals & Reagents

Amphetamine and methamphetamine Immunoassay® ELISA kits were purchased from AgriYork (Pocklington, UK). Ketamine Immunoassay® ELISA kits were donated from AgriYork (Pocklington, UK). The ELISA screening protocols used were accredited to ISO17025 for forensic purposes. WorldCassette™ Rapid Test (Japan), Nal von Minden (Germany) and DRUGCHECK® Dip Drug Test (The Netherlands) for ketamine were also supplied by AgriYork (UK).

Cerilliant Mephedrone, ethylone and methylone reference standards were purchased from Sigma-Aldrich (Dorset, UK). Naphyrone, 5-IAI, MDAI, 2-DPMP, butylone, 5-APB, 6-APB, methedrone, ketamine, 3-MeO-PCE, 3-MeO-PCP and MXE

were purchased from LGC Standards (Luckenwalde, Germany). MDPV and flephedrone were purchased from Toronto Research Company (Canada). PBS buffer was from Agriyork (Pocklington, UK).

Expired red blood cell pouches were supplied by the Western Infirmary Hospital (Glasgow, UK). Urine was supplied by a willing donor. ELISA plates were washed using a Dynex plate washer and read with a Dynex MRX plate reader.

5.4.2 Blank Blood Preparation

These red blood cells had been collected according to the UK Blood Services guidelines (166:167) and were frozen upon arrival into the FMS department. The volume of red blood cells was measured once the contents of the pouch had fully defrosted and diluted 1:1 with 1% saline solution. Saline solution was prepared as per section 2.4.1.2.

5.4.3 ELISA Methodology

5.4.3.1 Amphetamine and Methamphetamine Kits

A 100 µg/mL amphetamine solution was made by transferring 500 µL of a 1 mg/mL reference standard to a 5 mL volumetric flask. It was then made up to the mark using MeOH. Using this solution, a 10 µg/mL amphetamine solution was then prepared by transferring 500 µl of the 10 µg/mL solution to a 5 mL volumetric flask. This was then made up to the mark using MeOH. This procedure was repeated using methamphetamine to give methamphetamine solutions of 100 µg/mL and 10 µg/mL. Using these 2 solutions amphetamine and methamphetamine calibrators (levels 1-4) and QC's (±50% the 25 ng/mL cut-off value) were prepared using the volumes specified in Table 5-1. Blood calibrators and QC's and spiked samples were diluted 1:10.

Table 5-1: Solutions and volumes used to produce blood and urine amphetamine / methamphetamine levels and QC's along with their final concentrations.

	Level Nº	Solution Used (µg/mL)	Volume Used (µl)	Concentration prior to dilution (ng/mL)	Final Concentration (ng/mL)
Blood	1	N/A	0	0	0
	2	10	25	250	25
	3	10	100	1000	100
	4	100	50	5000	500
	-50%	10	13	130	13
	+50%	10	38	380	38

To produce the spiked blood samples 1 mL aliquots of blank blood was transferred to clean glass culture tubes. These were then spiked with 100 µl of each drug solution (100 µg/mL) listed in section 5.4.1. These were then diluted 1:10 using PBS buffer giving a final concentration of 1000 ng/mL. A 10 µL aliquot of calibrator (level), QC or drug was added to the wells in duplicate as shown in Figure 5-3. The plates were then left to incubate in the dark for 1 hour and washed before stop solution was added and the plates were read. This protocol was used for both amphetamine and methamphetamine ELISA kits.

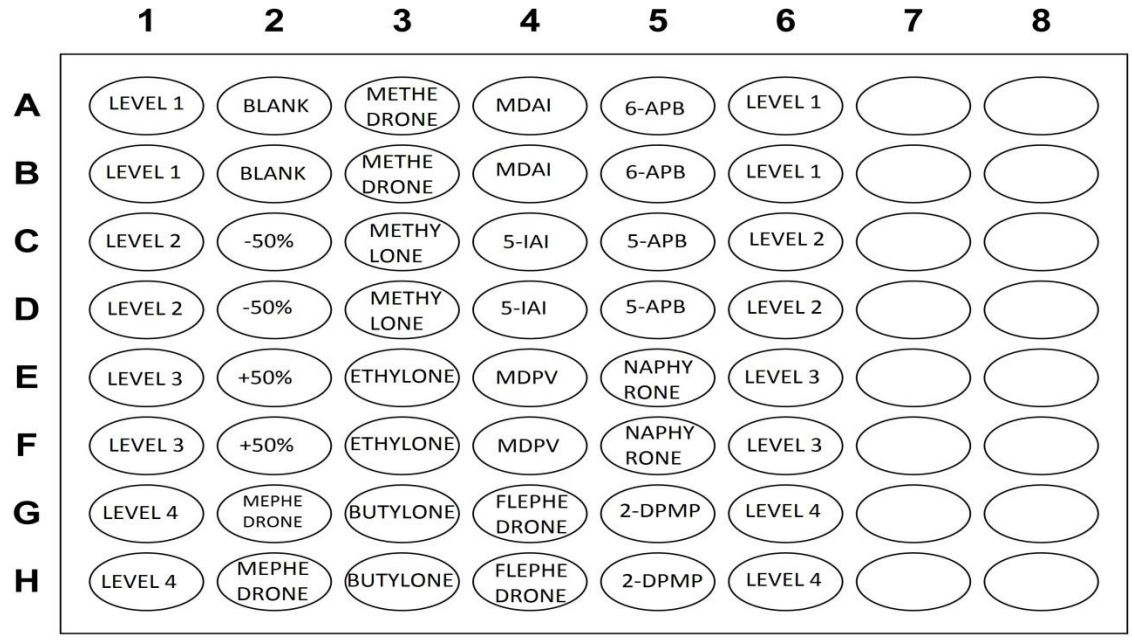


Figure 5-3: Diagram of amphetamine/ methamphetamine ELISA kits

5.4.3.2 Ketamine Kits Methodology

A 100 µg/mL ketamine solution was made by transferring 500 µL of a 1 mg/mL reference standard to a 5 mL volumetric flask. It was then made up to the mark using MeOH. A 500 µL aliquot of this 100 µg/mL solution was then transferred to a fresh clean 5 mL volumetric flask and made up to the mark again with MeOH, giving a final concentration of 10 µg/mL. From the 10 µg/mL solution 500 µL was removed and transferred to another clean 5 mL volumetric flask, made up to the mark using MeOH, giving a final concentration of 1 ng/mL.

The ketamine 1 and 10 µg/mL solutions were then used to spike 1 mL of blood or urine to produce calibrators. Controls were also made in blood and urine (±50% of the manufacturers cut off point). Blood calibrators and QC's were diluted 1:10 in PBS buffer whereas urine QC's, calibrators and samples were diluted 1:20 as per manufacturer's instructions. The solutions and volumes used to spike each 1 mL of matrix are shown in Table 5-2.

Table 5-2:Solutions and volumes used to produce blood and urine levels and QC's along with their final concentrations.

	Level Nº	Solution Used (µg/mL)	Volume Used (µl)	Concentration prior to dilution (ng/mL)	Final Concentration (ng/mL)
Blood	1	N/A	0	0	0
	2	1	100	100	10
	3	10	60	600	60
	4	10	300	3000	300
	-50%	1	50	50	5
	+50%	1	150	150	15
Urine	1	N/A	0	0	0
	2	10	20	200	10
	3	10	120	1200	60
	4	10	600	6000	300
	-50%	10	50	500	25
	+50%	10	150	1500	75

As the 3 ELISA plates used contained 96 wells it was decided that a total of 8 different analyte concentrations could be tested for each matrix, with each matrix being tested on a separate plate. The MXE, 3-MeO-PCE and 3-MeO-PCE

were prepared using 4 different working solutions, 0.01-5 mg/mL. The 5 mg/mL solution was prepared by weighing 5 mg of drug powders and adding 1 mL of MeOH. Using this solution a 1 mg/mL solution was prepared by transferring100µl to a fresh vial and adding 900 µl of MeOH. The 0.1 mL solution was prepared by transferring 100 µl of the 1 mg/mL solution to another fresh vial and again adding 900 µl of MeOH. The final solution of 0.01 mg/L was then made by transferring 100 µl of the 0.1 µl/mL solution to a fresh vial and adding 900 µl MeOH.

This procedure was carried out for each of the 3 analytes. The volumes of each of these solutions used to spike 1 mL of each matrix are shown in Table 5-3. The resulting matrix concentration is also shown. As with the calibrators and QC's blood and PBS spikes were diluted 1:10 and the urine spikes were diluted 1:20, the resulting concentrations after these dilutions are also shown in Table 5-3.

Table 5-3: Concentrations of test sample after 1:10 and 1:20 dilutions.

Sample Nº.	Working Solution Conc. (mg/mL)	Volume Used (µL)	Resulting Conc (µg/mL)	Conc. 1:10 dilution (µg/mL)	Conc 1:20 dilution (µg/mL)
1	5	100	1000	100	50
2	5	100	500	50	25
3	5	20	100	10	5
4	1	50	50	5	2.5
5	0.1	100	10	1	0.5
6	0.1	50	5	0.5	0.25
7	0.01	100	1	0.1	0.05
8	0.01	10	0.1	0.01	0.005

A 10 µL aliquot of each calibrator and QC was added to the wells in duplicate. A further 10 µL of MXE, 3-MeO-PCP, and 3-MeO-PCE at the concentrations listed in Table 5-3 were also added to wells in duplicate. This was carried out for drug samples in blood, urine and PBS solution. A summary of this is shown in Figure 5-4. The numbers following the analyte name correspond to those listed as the sample Nº in Table 5-3.

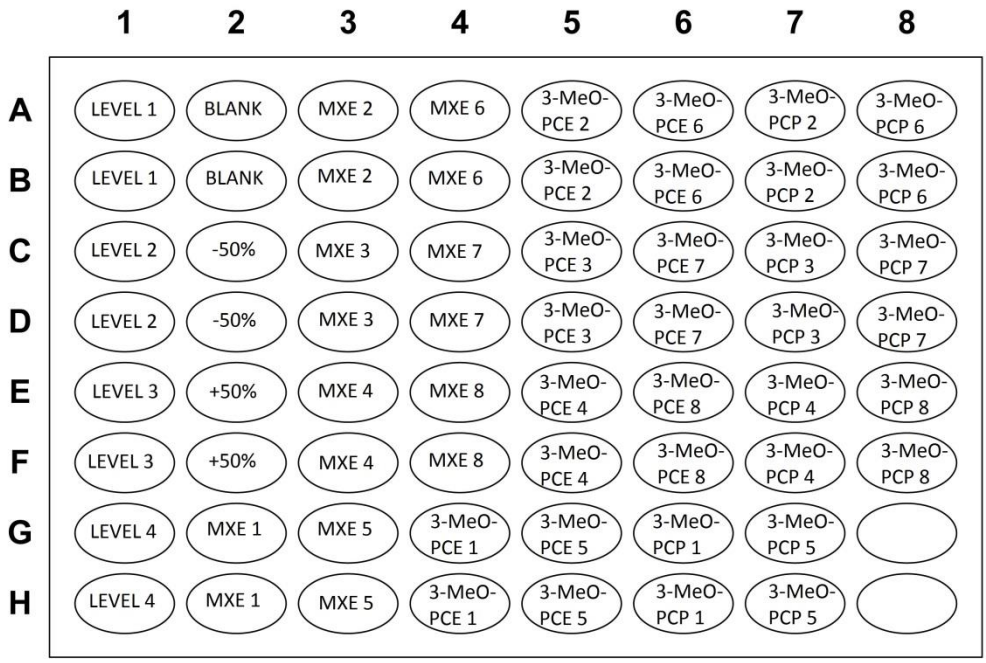


Figure 5-4: Diagram of ketamine ELISA plate

5.4.4 POCT Methodology

Blank human urine samples were spiked with MXE at different concentrations (100, 250, 500 and 1000 µg/mL). POCT was then carried out on each of these samples using both the DRUGCHECK® Dip Drug Test and the WorldCassette™ Rapid Test as per the manufacturer's instructions. To the WorldCassette™ Rapid Test 1-2 drops of spiked urine was added by Pasteur pipette to the sample hole. The kits were kept flat on the bench and only read once the control line had appeared. Nal von Minden and DRUGCHECK® Dip Drug Test use vertical flow and each test was directly placed into a small beaker containing 1-2 mL of spiked urine. Again readings were only taken after the appearance of the control line. Nal von Minden tests were used to test MXE urine samples at concentrations of 50 µg/mL and 500 µg/mL. Positive and negative controls for each POCT were also carried out using blank urine and urine spiked at 10 µg/mL.

5.5 Results & Discussion

5.5.1 ELISA

5.5.1.1 Amphetamine & methamphetamine

The percent cross reactivity's of each drug at 0.1 mg/L with both plates is shown in Table 5-4. Those which gave positive results and successfully cross reacted are highlighted in pale green. Those which did not cross react to an extent to give a positive response are highlighted in pale red. From the Table 5-4 it is clear that ethylone cross reacted to the highest extent with both the amphetamine kit and the methamphetamine kit. The cut-offs for these drugs were not investigated due to the high price of the kits.

Table 5-4: Percent cross reactivity for Immunalysis amphetamine and methamphetamine ELISA kits spiked with various new psychoactive substances

DRUG	AMPHETAMINE % CROSS REACTIVITY	METHAMPHETAMINE % CROSS REACTIVITY
2-DPMP	0.1	0.3
5-IAI	0.1	0.4
MDAI	0.7	0.6
MDPV	0.1	0.3
Naphyrone	0.1	0.3
Butylone	0.6	1.0
Flephedrone	0.8	1.3
5-APB	1.2	1.8
6-APB	1.3	1.8
Ethylone	2.7	3.2
Mephedrone	1.5	2.0
Methedrone	1.3	1.8
Methylone	1.2	1.8

The results from this study correlated with previous research that tested a variety of NPSs against various different amphetamine ELISA plates including Immunalysis.(*217*) During this study MDPV, Mephedrone, flephedrone and butylone were all found to have cross reactivities below 0.25% which was also the case in this short study. This previous study did not test any of the additional

NPSs listed in Table 5-4, nor did it examine cross-reactivity with methamphetamine ELISA kits.

Although some of the NPSs tested did cross react sufficiently to produce positive results all were tested at 10 µg/mL. Therefore it is unlikely that blood false positives would routinely be seen due to the high concentrations needed. Urine false positives however may occur as concentrations can be much greater depending on the circumstances surrounding the case.

5.5.1.2 Ketamine

MXE, 3-MeO-PCE and 3-MeO-PCP all produced positive results using the Immunalysis ELISA kits in blood, urine and PBS solution at different concentrations. Concentrations at which each drug gave positive results (ketamine cut-off = 50 ng/mL) are shown below in Table 5-5. These concentrations are those of the original samples and not that of the samples after 1:10 or 1:20 dilution.

Table 5-5: Sample concentrations at which ELISA provided positive results

MATRIX	MXE (µg/mL)	3-MeO-PCE (µg/mL)	3-MeO-PCP (µg/mL)
BLOOD	10	100	50
URINE	5	50	50
PBS	5	10	10

Cross reactivity was established by comparing measured ketamine concentrations with the actual concentrations of each drug added as shown in Table 5-6.

Table 5-6: Cross reactivity of each drug in each matrix

MATRIX	MXE (%)	3-MeO-PCE (%)	3-MeO-PCP (%)
Blood	0.1	0.01	0.02
Urine	0.2	0.02	0.02
PBS	0.3	0.02	0.07

False positive results for MXE blood samples are unlikely to be seen in case work due to the high concentrations needed (above 10µg/mL) before cross reactivity is observed. This is far higher than reported blood and serum concentrations for MXE toxicity which range between approximately 0.1µg/mL and 0.4µg/mL (*95, 96*). In one reported fatality a femoral blood concentration of approximately 8.5µg/mL was detected, which is still below the 10µg/mL cut off point, was found (*98*).

Urine samples, however, could result in false positive results since a lower cut off value of 5µg/mL was observed. In one paper, urine samples were found to have MXE concentrations ranging from 2µg/mL to 165.3µg/mL in patients (n=6) suffering from acute toxicity.(*97*) It is likely that post mortem samples could have concentrations far greater than these. Had ketamine ELISA analysis been carried out on these 6 urine samples, 66% would have cross reacted sufficiently to produce a positive response.

To date there is very little published toxicological information for either 3-MeO-PCE or 3-MeO-PCP. Anecdotal evidence suggests that the recreational dose for both of these substances are much lower than that of ketamine or MXE and as a result toxicological concentrations are expected to be lower. 3-MeO-PCE and 3-MEO-PCP only showed cross reactivity at much higher levels (10-100µg/mL) and therefore cross reactivity is unlikely to be seen with these substances and ketamine ELISA plates, unless in instances of acute toxicity. Higher cross reactivity may be a result of steric hindrance in the case of 3-MeO-PCP and due to the lack of oxygen double bond on the cyclohexane ring in both 3-Meo-PCP and 3-MeO-PCE as shown in Figure 5-5.

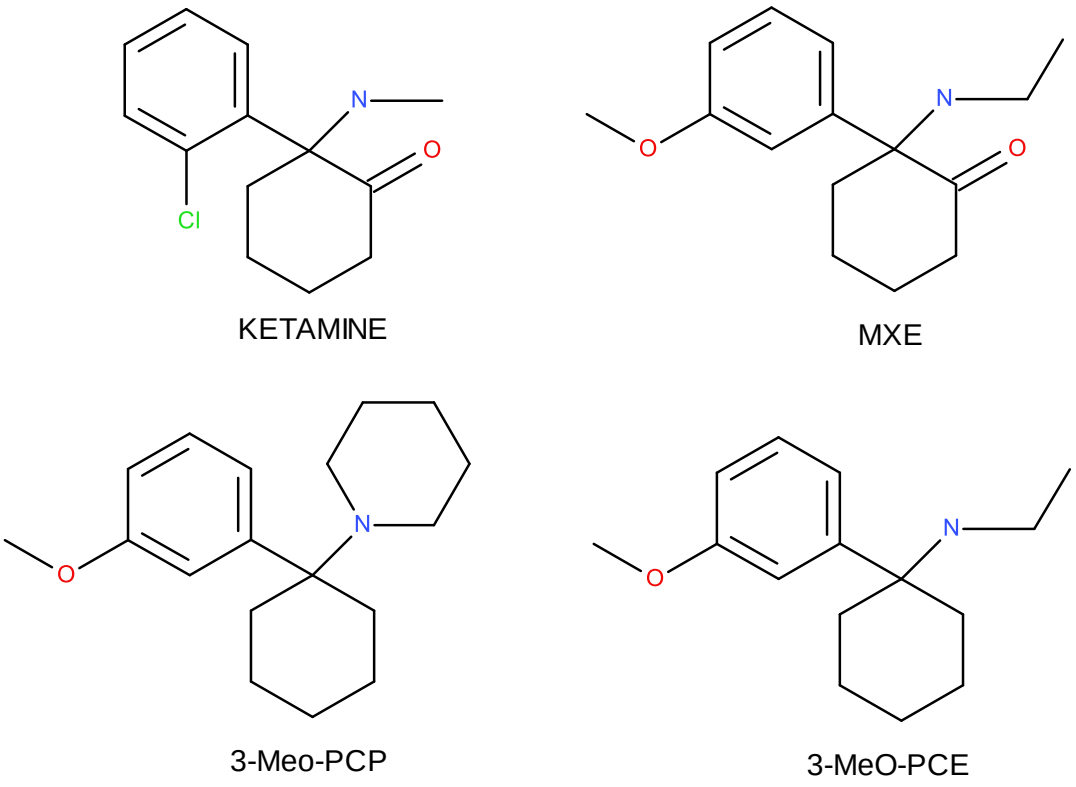


Figure 5-5: Chemical structure of ketamine, MXE, 3-MeO-PCE and 3-MeO-PCP

5.5.2 POCT

Tests were positive if the analyte line failed to appear and negative if it did. Any faint lines at the analyte area were deemed negative regardless of how faint the line was as per manufacturer's instructions. Cut off values for each POCT are shown below in Table 5-7.

Table 5-7: Cut off values for ketamine POCT

COMPOUND	WorldCassette™	DRUGCHECK®	Nal von Minden
Ketamine	500ng/mL	1,000ng/mL	1,000ng/mL
Norketamine	50,000ng/mL	4,000ng/mL	1,000ng/mL

WorldCassette™ Rapid Test, DRUGCHECK® Dip Drug Test and the Nal von Minden Test showed no cross reactivity with MXE. The WorldCassette™ Rapid Test produced a very faint line at concentrations 500µg/mL and 1mg/mL. Although this line was extremely faint these tests were deemed negative as per manufacturer's instructions which note that any test producing any lines, no matter how faint should be treated as negative. These concentrations are much higher than would ever be seen in practise and therefore would not allow for the

detection of MXE on site. Test results for both POCT's are shown in Figure 5-6, Figure 5-7 and Figure 5-8.

Figure 5-6: DRUGCHECK® Dip Drug POCT results

Figure 5-7: WorldCassette™ Rapid Check POCT Results

Figure 5-8: Nal von Minden POCT results

From this data it is clear that cross reactivity would not been seen in "real life" cases as no cross reactivity was observed with any POCT examined up to 1 mg/mL. As previously discussed in chapter 1, section 1.3.5, typical MXE urine concentrations range from 2µg/mL to 165µg/mL, far lower than 1mg/mL.

5.6 Conclusion

This work shows that cross-reactivity may be observed using amphetamine, methamphetamine and ketamine Immunalysis ELISA kits in cases of severe NPS toxicity. Cross reactivity was seen using the amphetamine and methamphetamine kits for 5-APB, 6-APB, ethylone, mephedrone, methedrone and methylone. In addition to these substances, methamphetamine kits also cross-reacted with butylone and flephedrone.

Cross reactivity was observed for MXE, 3-MeO-PCE and 3-MeO-PCP using the ketamine Immunalysis ELISA kit at concentrations ranging from 5-100 µg/mL. Cross-reactivity was seen for urine samples more so than blood, and therefore it is unlikely that cross-reactivity would be seen in "real-life" blood samples. Cross-reactivity may however be observed in urine samples.

These results indicate that care should be taken when confirming ELISA results, ensuring that any false positives are fully investigated and have not been caused by one of these NPSs which was not included on the confirmatory analysis panel.

POCT's did not show cross reactivity for methoxetamine, 3-MeO-PCE and 3-MeO-PCP up to concentrations of 1 mg/mL. Care should be taken when interpreting ketamine POCT's as these substances will not test positive. Therefore, a negative ketamine result does not mean that an individual has not used another ketamine type substance.

Chapter 6: Method Validation of 25B, 25C and 25I-NBOMe in Urine and Hair using LC-MS/MS

6.1 Introduction

6.1.1 LC-MS/MS

Liquid chromatography mass spectrometry (LC-MS) is a chromatographic technique, combining high performance liquid chromatography (HPLC) and mass spectrometry (MS). The LC column is packed with a sorbent (stationary phase) and a liquid (mobile phase) is pumped through under high pressure. The sample is injected and travels through the column separating different analytes depending on their interaction with the stationary phase versus their affinity with the mobile phase. If a molecule has a greater affinity for the stationary phase than the mobile phase more interactions will take place between that molecule and the stationary phase. This in turn increases the time it takes to travel through the column and reach the detector. If the molecule has a greater affinity for the mobile phase than that of the stationary phase fewer interactions between the molecule and the stationary phase will occur, reducing the time the molecule will take to travel through the instrument and reach the detector.

The analyst is therefore able to control the retention times of analytes by altering the chosen column (i.e. selecting a different stationary phase) or by altering the mobile phase composition.(*218*) The LC mobile phase can be kept constant (isocratic) or altered over the run (gradient), in a similar way to how oven temperatures are controlled in GC.

Before any molecules reach the mass analyser they must undergo ionisation. This can be achieved using a number of different techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionisation (APPI). The instrumentation used in this study utilised ESI and so this will be the only ionization method discussed briefly here. (*219*)

In ESI the source generates analyte ions in solution prior to the mass analyser. Electrical energy is applied to the sample as it flows through a capillary

(typically stainless steel or quartz silica) which is sustained at a high voltage (2.5-6.0 kV) relative to its surrounding walls. The capillary can be operated in either positive or negative modes. In positive ESI the capillary is positively charged and thus attacks the negatively charged ions within the sample repelling the positive. In negative ESI the opposite occurs. The resulting droplet spray is then either predominantly positive (positive ESI) or negative (negative ESI) depending on the charges applied to the capillary. The use of a nebulising gas at this stage such as nitrogen will increase the sample flow rate. (*220*)

Droplet formation is dependent on several factors such as the diameter of the capillary, the applied potential inference (V_0), the flow rate of the sample and the flow rate and temperature of any nebulising gas.

Once a droplet is formed and expelled from the capillary it enters the desolvation region of the source, where aerosol formation takes place. A drying gas such as nitrogen can be used to assist in aerosol formation. As the droplets travel through this region of the source the solvent is evaporated, reducing the size of the droplets until the ions contained within the droplet are expelled into the gaseous phase. The resulting ions are then sampled by the sampling skimmer cone before proceeding to the mass analyser.(*220*) This is shown in Figure 6-1.

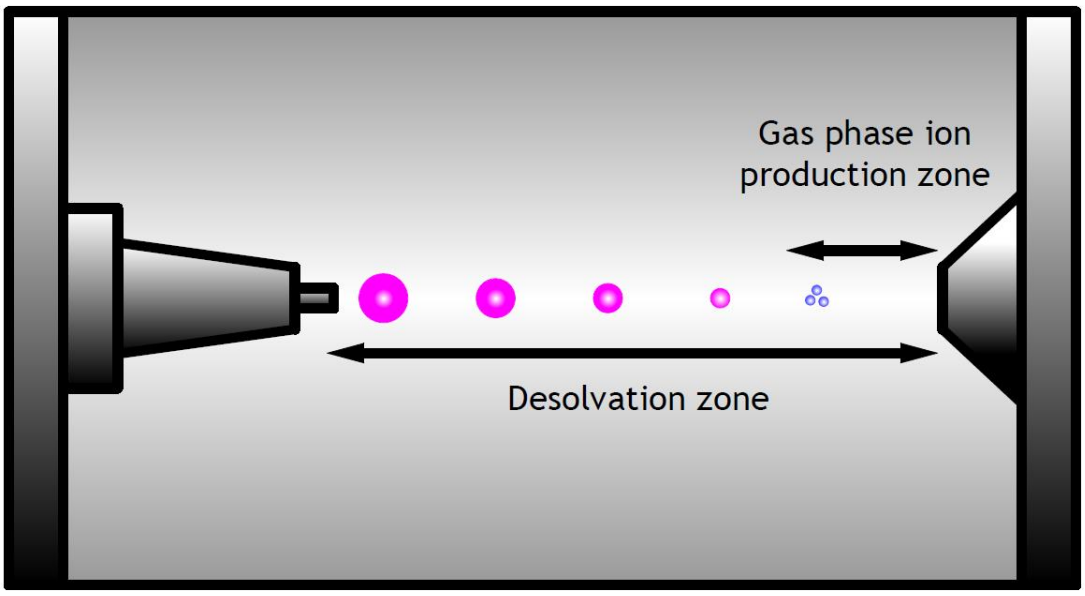


Figure 6-1: Schematic of aerosol formation.

This fragmentation is known as a soft ionisation technique due to the limited number of fragments that are formed, thus retaining the molecular ion. As this is

a soft ionisation technique the resulting mass spectra produced can be very simple, making unique identification more difficult. The use of tandem MS however can overcome this limitation.(*221*)

MS involves the ionisation of the analytes once they have left the column, and this can be achieved using a wide variety of mass analysers such as iontrap, quadrupole or time-of-flight. MS/MS is the combination of more than one MS mass analyser in the same instrument. The first mass analyser filters for the precursor ions, typically the molecular ion for the analyte of interest. The precursor ion is then fragmented to produce product ions, typically achieved by the use of high energy nitrogen gas. The second mass analyser then filters for the specific product ions. This achieves a highly sensitive and targeted analysis where the precursor ion and resulting product ions are monitored, ensuring that it is the analyte of interest. A schematic of an LC-MS/MS instrument is shown in Figure 6-2.

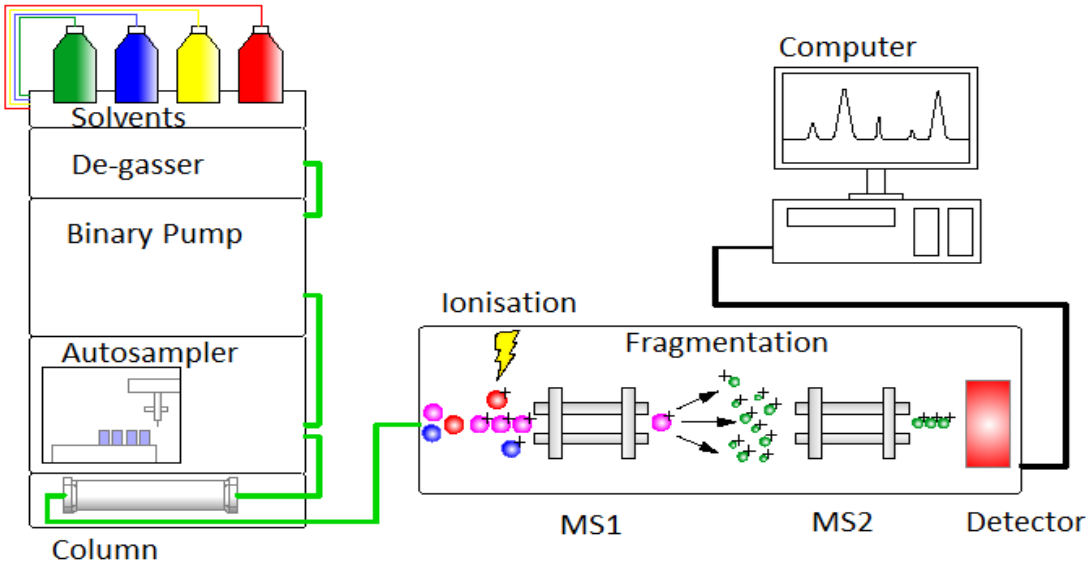


Figure 6-2: Schematic of a LC-MS/MS instrument.
Samples travel through the LC column to the MS/MS where they undergo ESI ionisation (at the interface between the LC and the MS/MS), separation, fragmentation, further separation and then detection.

The use of LC-MS/MS in forensic toxicology laboratories has grown rapidly in the last 10 years, as more papers are published and instrumental costs fall. LC-MS/MS is a highly specific technique allowing for the rapid analysis of multiple analytes simultaneously.(222) The relatively short run-times in comparison to

traditional GC-MS systems also make it appealing, coupled with the need for fewer sample clean up steps. GC-MS analysis is dependent on the analyte in question becoming volatile in the injection port, and thus the use of derivatising agents is common. This is not required in LC-MS analysis and so sample preparation time is also reduced.(*223*)

6.1.2 Hair Analysis

The analysis of hair poses significant benefits in forensic toxicology. Not only is it utilised when more conventional matrices such as blood and urine are not available, but it also provides an approximate timeline of drug use. This is particularly useful in cases of child custody, work place drug testing or in DFSA cases.(*224-226*)

Each time drugs are administered to the body they are carried through the blood and are distributed to various tissues and fluids before being eliminated. As illustrated in Figure 6-3 drugs are deposited into the hair follicle via the blood and are trapped there permanently. The drug then grows out with the hair. This is a simplistic model of how drugs are incorporated into hair and in reality other factors such as incorporation through sweat may play a part.(*227*) Many factors affect the rate at which drugs will be incorporated into the hair, with hair colour having significant impact.(*228*)

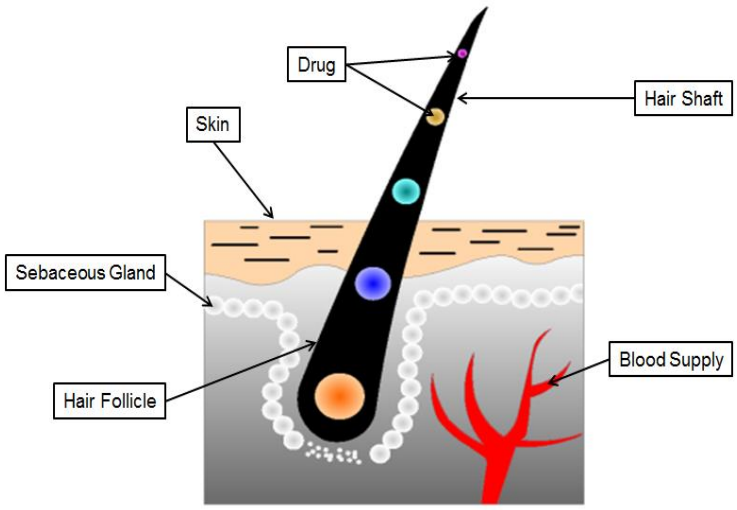


Figure 6-3: Schematic of a hair follicle showing the incorporation of drug into hair.

Drugs can also be present in hair samples due to contamination through the smoking or handling of drugs. In order to remove this external contamination,

hair samples must undergo thorough decontamination wash procedures.(*228, 229*) However, it has been shown that it may not be possible to remove all external contamination from hair.(*230*) It is also important to ensure that hair is collected from acceptable sites, that an adequate volume is collected and that appropriate cut-off values are set. The Society of Hair Testing (SoHT) have recommended procedures, guidelines and cut-off values to help aid with the interpretation of hair concentrations.(*231*)

Hair analysis is not a new technique having originally been applied to forensic analysis in the 1850's when the presence of arsenic was identified in a body exhumed 11 years after burial.(*232*) Despite this matrix having such a long history, its use is still limited due to the long sample preparation, incubation times and limited compatibility with automation. The low concentrations detected often require highly sensitive instrumentation which not all laboratories have access to such as LC-MS/MS, and interpretation can be difficult due to changes in drug concentrations with time due to cosmetic treatments.

Despite these difficulties, hair analysis in itself is relatively straight forward as no extensive metabolism or excretion occurs after the drug has been deposited. This makes is particular applicable to NPSs where the metabolites of these substances may not always be known, or commercially available. To date there has been no analysis of NBOMes in hair; as a result there are no recommended cut-off values from the SoHT.

6.2 Aims

The aim of this study was to establish a validated urine and hair method for the detection of 25B, 25C and 25I-NBOMe. The method should be fully validated in accordance with SWGTOX and SoHT guidelines.

6.3 Materials & Methods

6.3.1 Chemicals & Reagents

All NBOMe reagents were purchased from Sigma Aldrich (Poole, UK). $NaH_2PO_4.H_2O$, Na_2HPO_4 and NaOH were purchased from BDH (Poole, UK). HCOOH

and $NH_4CH_3CO_2$ were purchased from Sigma Aldrich (Poole, UK). All other solvents were of analytical grade and were purchased from VWR (Poole, UK).

Drug free urine and hair were provided from willing donors and screened prior to analysis. All analysis was carried out using pooled urine and hair samples from at least 10 donors. All solutions were prepared using Gilson PIPETMAN Classic™ accurate pipettes which had recently been calibrated in house. All urine was pipetted using an accurate Gilson MICROMAN® positive displacement pipette which had also recently been calibrated in house.

6.3.1.1 2M $NH_4CH_3CO_2$

$NH_4CH_3CO_2$ (3.85 g) was transferred to a 25 mL volumetric flask. This was then filled up to the mark with dH_2O and inverted several times to ensure thorough mixing. This solution was then stored for up to 6 months in a labelled amber glass bottle at 4°C.

6.3.1.2 0.1M pH7.4 Phosphate Buffer

$NaH_2PO_4.H_2O$ (3.1 g) and Na_2HPO_4 (10.9 g) were weighed by accurate balance and dissolved in approximately 500 mL dH_2O. This solution was then transferred to a 1 L volumetric flask, made up to the mark with additional dH_2O and inverted several times. This was stored at 4°C for up to 1 month in a clear glass bottle.

6.3.1.3 Mobile Phase A

HCOOH (1 mL) and 2 M $NH_4CH_3CO_2$ (1 mL) were transferred to a 1 L volumetric flask. This was then made up to the 1 L mark with dH_2O and mixed. This was stored at room temperature for up to 1 month in a clear glass bottle.

6.3.1.4 Mobile Phase B

HCOOH (1 mL) and 2 M $NH_4CH_3CO_2$ (1 mL) were transferred to a 1 L volumetric flask. This was then made up to the 1 L mark with MeOH and mixed by inverting several times. This was stored at room temperature for up to 1 month in a clear glass bottle.

6.3.2 Preparation of Stock & Working Standards

A stock solution (10 µg/mL) was prepared by transferring 1 mL of 25B-NBOMe, 25C-NBOMe and 25I-NBOMe (100 µg/mL) to a 10 mL volumetric flask. This flask was then made up to the mark with MeOH before being stoppered and inverted several times to ensure thorough mixing. This calibration working solution was then transferred to a clean amber bottle, and stored at -20°C for up to 1 year. A second stock solution was made following the same procedure, by a different analyst producing the same final 10 µg/mL concentration. This was used for all QC's.

Three different working solutions were prepared at concentrations of 0.01, 0.1 and 1 µg/mL from the calibration stock solution. The 1 µg/mL solution was prepared by transferring 1 mL of the 10 µg/mL solution to a 10 mL volumetric flask and making it up to the mark with MeOH. This 1 µg/mL solution was then used to make the 0.1 µg/mL solution by transferring 1 mL into a new 10 mL volumetric flask and making it up to the mark using MeOH. The same 1:10 dilution was then carried out using the 0.1 µg/mL solution, making a 0.01 µg/mL solution. All solutions were transferred to labelled amber bottles which were stored at -20°C for up to 1 year.

Another 3 working solutions were prepared from the QC stock solution to make QC working solutions (1, 0.1 and 0.01 µg/mL). Again these solutions were prepared by a different analyst, transferred to amber bottles and stored for up to 1 year at -20°C.

A 1 µg/mL 25B-NBOMe-D_3 I.S. solution was prepared. A 100 µg/mL 25B-NBOMe-D_3 solution was purchased, which was then transferred to a 10 mL volumetric flask using a Pasteur pipette. The flask was then made up to the mark using MeOH, inverted several times and stored at -20°C for up to 1 year. This was labelled as the stock solution. One mL of the stock solution was then transferred to another clean 10 mL volumetric flask and again made up to the mark using MeOH. This was inverted several times, and stored at -20°C for up to 1 year. The same procedure was followed by another student to make duplicate solutions for use when preparing QC's.

6.3.3 Calibration & QC Preparation

6.3.3.1 Urine Calibrators & QCs

During this study it was not possible to purchase NBOMes with different lot numbers within the same company as only 1 lot had been produced. It was also not possible to buy all of these drugs from alternative companies as LGC only began selling these substances in the summer of 2015. No NBOMe standards are currently produced in the UK and the importation of these substances from the USA takes approximately 3 months, falling out with the completion time of this research. All QC solutions were prepared by a different analyst on different days to ensure the methods were as robust as possible.

Urine calibrations and QCs were produced by spiking 1 mL of pooled drug-free urine with the relevant working solution as shown in Table 6-1 and Table 6-2.

Table 6-1: Urine calibrator concentrations and the volumes of each working solution needed to produce them.

Level	Calibration Concentration (ng/mL)	NBOMe Standard Working Solution (0.01 µg/mL) Volume (µl)	NBOMe Standard Working Solution (0.1µg/mL) Volume (µl)	NBOMe Standard Working Solution (1 µg/mL) Volume (µl)
1	0.1	10	0	0
2	0.25	25	0	0
3	1.0	100	0	0
4	5.0	0	50	0
5	25.0	0	0	25
6	50.0	0	0	50
7	100.0	0	0	100

Table 6-2: Urine QC concentrations and the volumes of each working solution needed to produce them.

Level	Calibration Concentration (ng/mL)	NBOMe Standard Working Solution (0.01 µg/mL) Volume (µl)	NBOMe Standard Working Solution (0.1µg/mL) Volume (µl)	NBOMe Standard Working Solution (1 µg/mL) Volume (µl)
QC 1	0.18	18	0	0
QC 2	4.20	0	42	0
QC 3	84.0	0	0	84

6.3.3.2 Hair Calibrators & QCs

Hair calibrations and QC's were produced by spiking 40 mg of human hair with the below amounts from the relevant working solution as shown in Table 6-3 and Table 6-4.

Table 6-3: Hair calibrator concentrations and the volumes of each working solution needed to produce them.

Level	Calibration Concentration (ng/mg)	NBOMe Standard Working Solution (0.1 µg/mL) Volume (µl)	NBOMe Standard Working Solution (1 µg/mL) Volume (µl)
1	0.025	10	0
2	0.05	20	0
3	0.125	50	0
4	0.250	100	0
5	0.625	0	25
6	1.25	0	50
7	2.5	0	100

Table 6-4: Hair QC concentrations and the volumes of each working solution needed to produce them.

QC LEVEL	Calibration Concentration (ng/mg)	NBOMe Standard Working Solution (0.1 µg/mL) Volume (µl)	NBOMe Standard Working Solution (1 µg/mL) Volume (µl)
QC 1	0.105	42	0
QC 2	1.05	0	42
QC 3	2.10	0	84

6.3.4 Instrumentation

Analysis of samples was carried out using an Agilent Technologies 6420 series triple quadrupole LC-MS/MS (Agilent, USA) coupled with an Agilent 1260 binary pump, autosampler, degasser and thermostated column compartment. An electrospray ionization (ESI) source was used in positive ionisation mode. The column used was a Phenomenex Gemini® 5 µm C18 110 Å (15 x 2 mm) with a Phenomenex Gemini C18 guard column (4 x 2.0mm).

Urine samples were mixed and rotated using a Denley Spiramix 10 (Billinghurst, UK). A pierce Reacti-Vap™ III nitrogren (Thermoscientific, Loughborough, UK) manifold coupled with a Technie Dri-Block DB3 (Thermoscientific, Loughborough, UK) heating block was used for sample evaporation. A Sigma 4-16 centrifuge (Sigma-Aldrich, Dorset, UK) was used and hair samples were weighed using a Sartorius TE64-0CE accurate balance (Surrey, UK). All pipetting was carried out using Gilson PIPETMAN classic pipettes (Luton, UK). Positive displacement pipettes used were Gilson Microman® (Luton, UK).

6.3.5 Operating conditions

Isocratic elution of mobile phase A & B (50:50) was used, with a flow rate of 0.3 mL/min. The total run time was 4 minutes with the column temperature kept at 40°C. The gas temperature was 350°C with a flow of 11 L/min. The nebulizer was operated at 30 psi and the capillary voltage was 4000 V. LC and MS parameters are summarized in Table 6-5.

Table 6-5: Summary of LC-MS/MS parameters

LC Parameters	
Column	Gemini® 5 µm C18 110 Å (15 x 2 mm)
Mobile phase	A & B (50:50)
Flow rate	0.3 mL/min isocratic
Injection volume	30µl
Column temperature	40°C
Run time	4 mins

Table6-5: Summary of LC-MS/MS parameters (cont.)

MS Parameters	
Operating mode	ESI
Gas temperature	350°C
Gas flow	11 L/min
Nebulizer pressure	30 psi
Capillary Voltage	4000 V
Scan mode	MRM

The MRM transitions, dwell time, fragmentor voltage and collision energies used to monitor each of the analytes and I.S. are shown below in Table 6-6. All drugs had previously been optimised by another postgraduate student using Mass Hunter Optimiser software.

Table 6-6: LCMS parameters used to monitor each NBOMe and I.S.

Analyte	Precursor Ion	Product Ion	Dwell	Frag (V)	CE (V)	RT (mins)
25B-NBOME-D_3	383	124	50	110	20	2.2
25B-NBOME	380	121	50	115	20	2.2
	380	91	50	115	50	1.9
25C-NBOME	336	121	50	105	18	1.9
	336	91	50	105	50	1.9
25D-NBOME	428	121	50	120	20	1.8
	428	91	50	120	60	1.8

An example chromatogram showing the elution time of all three analytes and the 25B-NBOMe-D3 I.S is shown below in Figure 6-4.

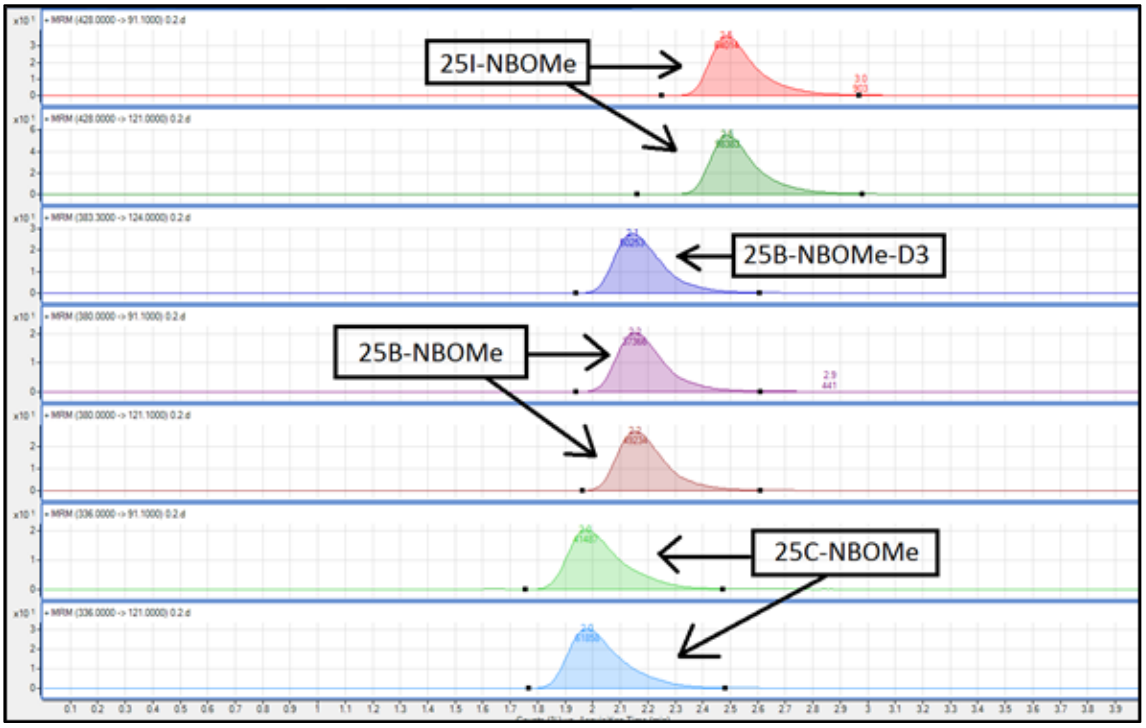


Figure 6-4: Chromatogram showing the retention times for each analyte and I.S.

6.3.6 LLE Urine Extraction Method

As determined in section 2.5.3. LLE is a suitable method for the extraction of NBOMes from urine. This LLE method has also been used for the detection of these compounds previously in the literature.

One mL aliquots of urine were transferred into glass culture tubes. Calibrators and QC's were then produced by adding the relevant solution volumes to each tube as shown in Table 6-1 and Table 6-2. Calibrations were run in duplicate and QC's were run in triplicate. To each tube 100 µl of 0.01 µg/mL 25B-NBOMe-D3 I.S. was added, as well as 500 µl of 0.1 M NaOH solution. Extraction solvent (3 mL) was then added to each test tube (50:50 hexane:EtOAc) and samples were rotated and mixed for 10 minutes using a Denley Spiramix 10. They were then centrifuged for 5 minute at 4000 rpm. These test tubes were then placed in the freezer (-20oC) for 5 minutes, freezing the bottom aqueous layer. The organic top layer was then decanted into another new glass culture tube. Samples were evaporated to dryness under nitrogen at room temperature. The samples were then reconstituted with 75 µl mobile phase mixture (50% mobile phase A: 50% mobile phase B). This solution was then transferred to autosampler vials for analysis by LC-MS/MS.

6.3.7 SPE Hair Extraction Method

Hair QCs, QC's and samples were washed with dH_2O (3 mL) and CH_2Cl_2 (3 mL). Each wash was sonicated for 30 minutes at room temperature and any excess solvent was decanted into fresh 7 mL vials by pasteur pipette for later analysis. In between each wash any remaining solvent was evaporated to dryness using a gentle stream of nitrogen at room temperature. Hair was then cut into 1-2 mm segments and 40 mg (±1 mg) was weighed and transferred to new 7 mL vials. These were then spiked with 50µl of 25B-NBOMe-D_3 (1 µg/mL). Calibrators and QC's were made by adding the relevant solution volume as stated in Table 6-3 and Table 6-4. To each vial, 3 mL of pH7.4 phosphate buffer were added before sonicating for 1 hour. Vials were then transferred to the oven where they were left to incubate overnight (12 hrs) at 40°C. Calibrators were run in duplicate and QC's were run in triplicate. A schematic of this process is shown below in Figure 6-5.

After incubation, the samples were then centrifuged at 4000 rpm for 10 minutes. UCT Cleanscreen cartridges were conditioned using 2 mL MeOH, and 1 mL 0.1 M pH7.4 phosphate buffer. The supernatant from the centrifuged samples was then transferred to the conditioned SPE cartridges. SPE cartridges were then washed with 3 mL of dH_2O and 1 mL of 1 M acetic acid before being dried for 10 minutes under vacuum. MeOH (3 mL) was then added to the cartridges before subsequent drying again under full vacuum for 1 minute. Elution was then carried out using a 3 mL CH_2Cl_2/IPA/NH_3 solution, before being evaporated to dryness under a slow stream of nitrogen, at room temperature. The samples were then reconstituted in 75 µl of mobile phase mixture (50% mobile phase A: 50% mobile phase B), vortex mixed for 30 seconds and transferred to vials for analysis by LC-MS/MS

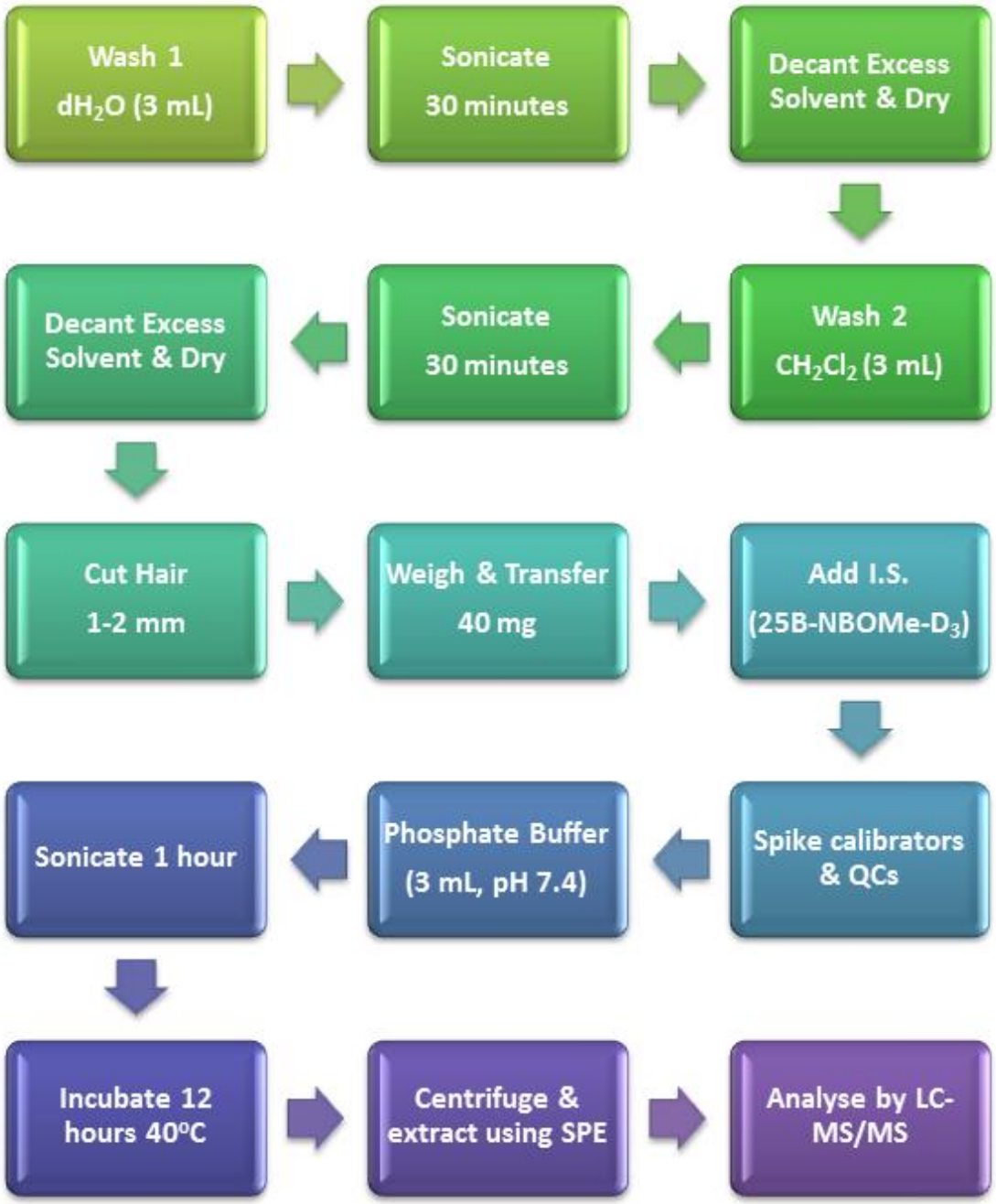


Figure 6-5: Schematic of hair preparation prior to SPE clean up steps and LC-MS/MS analysis.

6.3.8 Validation

LC-MS/MS validation was carried out in accordance with ISO17025 standards and SWGTOX recommendations where possible. All parameters covered were the same as that for the GC-MS validation in Chapter 3 with the additional parameter of matrix effects.

6.3.8.1 Linearity

Linearity was assessed by plotting the PAR of each NBOMe compound to I.S. against the corresponding concentration. The calibrators and QCs were prepared fresh each day using their respective working solutions. In order for the calibration to be deemed acceptable the calculated correlation coefficient (R^2) had to be greater than 0.99. The concentrations of each QC were then calculated using the linear equation from each calibration curve. QCs were deemed acceptable if their accuracy fell within ±10% of the true value and their %CV values were <15%. The calibration model best suited to the analysis was also assessed for each drug.

6.3.8.2 LOD and LOQ

Both the instrumental and the experimental limits of detection were assessed. In order to assess the instrumental LOD, volumes ranging from 10-100 µl of a 1 ng/mL mixed drug solution were evaporated and reconstituted in 75 µl of mobile phase and analysed. In order to determine the urine method LOD the same procedure was carried out, spiking 1 mL of urine with the same concentrations and extracting analytes as in section 6.3.6. A similar procedure was applied to hair samples, spiking 40 mg of hair with 10-100 µl of a 1 ng/mL mixed drug solution before extraction and analysis.

LOD was determined as per section 3.3.10 using Equation 3-1. Hair analysis LOD results were then divided by 40 to produce the LOD per single mg of hair.

6.3.8.3 Precision and Bias

The accuracy and precision of each NBOMe extracted from urine was calculated using Equation 3-6 to Equation 3-10 as shown in section 3.3.12. In order to calculate intra-day precision triplicate analysis of QC1 (0.18 ng/mL), QC2 (4.20 ng/mL) and QC3 (84.0 ng/mL) was carried out. The resulting values were averaged over 5 batches. Inter-day precision was calculated by analysing each QC in triplicate over a period of 5 batches and entering the data into Equation 3-8 and Equation 3-9.

The same procedure was carried out for each NBOMe extracted from hair. In order to calculate intra-day precision triplicate analysis of QC1 (0.105 ng/mg), QC2 (1.05 ng/mg) and QC3 (2.10 ng/mg) was carried out.

The percentage coefficient of variance (%CV) was then calculated, with values deemed to be acceptable when <20%.

6.3.8.4 Carryover

Carry over was assessed by triplicate injection of calibrator 7 followed by a mobile phase (50:50) flush. Carryover was deemed to have occurred if analytes were identified in the subsequent flush. This was carried out for both urine and hair validations. Calibrator 7 was deemed an acceptable level to assess as this is much higher than concentrations reported in literature and thus not likely to be encountered in samples.

6.3.8.5 Selectivity

In order to determine the selectivity of the method all analytes shown in Table 6-7 were tested. In order to determine exogenous interferences, 100 µl of each 10 µg/mL solution in Table 6-7 was transferred to a culture tube, evaporated to dryness and reconstituted in 75 µl of mobile phase. These were selected based on their high prevalence in everyday life such as paracetamol, caffeine and nicotine. GHB was chosen as it is endogenous to the human body, and the remaining drugs were chosen based on what was available at FMS. The same procedure was carried out using blank drug-free matrix from 10 different sources for both urine and hair to ensure that no components within the matrix itself interfered with the results.

Table 6-7: List of analytes used to check for interferences caused by exogenous compounds listed alphabetically.

Analytes			
2-DPMP	Cocaine	Ketamine	Norbuprenorphine
3-MeO-PCE	Codeine	Lamotrigine	Oxazepam
3-MeO-PCP	Cotinine	Lignocaine	Paracetamol
5-APB	Cyclizine	Lorazepam	Paroxetine
6-APB	Desipramine	MDA	Phenazepam
6-MAM	Diazepam	MDEA	Phenytoin
7-aminoflunitrazepam	Diltiazem	MDMA	Pregabalin
Amitriptyline	Diphenhydramine	MDPV	Procyclidine
Amphetamine	Dipipanone	Mephedrone	Procyliding
Atenolol	DMD	Methadone	Promethazine
Benzedrone	Dosulepin	Methamphetamine	Propoxyphene
Benzoylecgonine	Doxepin	Methandone-D9	Quetiapine
Buprenorphine	Ecgonine	Methedrone	Sertaline
Butylone	Ethylone	Methiopropamine	Sildenafil
Caffeine	Etizolam	Methyl Ester	Temazepam
Chlordiazepoxide	Flephedrone	Methylone	THC
Chlorpheniramine	Fluoxetine	Mirtazapine	THC-COOH
Chlorpromazine	Gabapentin	Morphine	Tramaol
Citalopram	GHB	MXE	Trazodone
Clomipramine	Haloperidol	Naphyrone	Venlafaxine
Clonazepam	Hydrocodine	Nicotine	Verapamil
Cocaethylene	Imipramine	Nitrazepam	Zolpidem

Due to the short run-time of this method chromatographic separation between 25B-NBOMe and its deuterated I.S was not always achieved, therefore it was necessary to ensure that neither were interfering with the others PA response. This was achieved by analysing 25B-NBOMe and 25B-NBOMe-D_3 separately and examining the resulting data file for peaks after monitoring the respective MRM transitions.

6.3.8.6 Matrix Effects & Extraction Efficiency

Analyte ionisation suppression or enhancement is commonly encountered in LC-MS. This may significantly affect concentrations detected, especially in the case

of suppression where concentrations may be reduced below that of quantification. In order to assess any suppression/ enhancement the Matuszewski method was used.(*233*) This method involved the analysis of 3 different batches of samples: pure standard, pre-extraction and post-extraction. Matrix effect was determined using QC3 calibrators for both hair and urine. Pooled matrix from at least 10 different individuals was used.

Pure standard – 84 µl of 25B, 25C and 25I-NBOMe working standard mixture (1 µg/mL) was added to 6 culture tubes. The methanolic solution was then evaporated to dryness using a steady stream of nitrogen at room temperature before reconstitution with 75 µl of mobile phase A and B mixture (50:50).

Pre-extraction – QC3 samples (n=6) were produced as normal adding both I.S and drug mixture to each matrix prior to extraction. Samples were then extracted as in sections 6.3.6 and 6.3.7.

Post-Extraction- Blank hair or urine calibrators (n=6) were extracted as normal without the addition of any I.S. or drug solution. Once extracted these blank matrix samples were spiked with 84 µl of drug mixture (1 µg/mL) and 50 µl of I.S. They were then evaporated to dryness using a steady stream of nitrogen and reconstituted in 75 µl mobile phase A and B mixture (50:50).

All matrix samples were then analysed by LC-MS/MS with mobile phase flushes between each sample, after the highest level calibrator and between each QC to prevent carryover. Separate "pure standards" were made for each matrix being examined. Recovery, process efficiency and the matrix factor were then calculated using Equation 6-1 to Equation 6-3.

Equation 6-1: Recovery % calculation

$$Recovery\ (\%) = \frac{Pre - extraction}{Post - extraction} x\ 100$$

Equation 6-2: Process efficiency calculation

$$Process\ Efficiency: (\%) = \frac{Pre - extraction}{Pure\ standard} x\ 100$$

Equation 6-3: Matrix factor calculation

$$Matrix\ Factor = \frac{Post - extraction}{Pure\ standard} x\ 100$$

No matrix effects are seen if the result of Equation 6-3 = 1. Suppression is determined if the result of Equation 6-3 <1 where as if it is > 1 then enhancement may be taking place. In order for the matrix factor to be deemed acceptable all results had to be within ±0.25.

In section 2.4.1.12, recoveries were assessed by the addition of I.S. after the extraction had taken place and the comparison of these PAR's with those of pure standards at the same concentration. Due to the possibility of matrix effects enhancing or suppressing the peak areas observed this approach is not appropriate for LC-MS. Therefore, extraction recovery was determined using Equation 6-1, eliminating the effect the matrix may have on the extraction procedure.

6.3.8.7 Dilution Integrity

The dilution of a sample may be necessary if the concentration detected in initial analyses is above the highest calibrator concentration, or the sample volume is below that required for the method. This method produces a wide concentration range, up to 100 ng/mL, and it is unlikely that urine samples will be received with concentrations higher than this, and thus low sample volume would be the most likely reason for sample dilution.

Dilution integrity of urine samples was assessed by preparing 12 replicates of QC3 (84 ng/mL) and diluting these with the addition of 1 to 4 mL of blank urine, producing triplicate dilutions of 1:2. 1:3, 1:4 and 1:5. Each QC was then vortex mixed before transferring 1 mL of each diluted QC to separate culture tubes and extracting as in section 6.3.6. These were then run alongside calibrators and QCs prepared as in section 6.3.3.1. The concentration of each diluted calibrator was then calculated using the linear equation of each calibration curve.

Hair samples would not undergo dilution but insufficient sample volume may be something which analysts encounter. In order to assess this, black rat hair samples (see Chapter 7) were analysed using 10, 20 and 40 mg of sample.

The dilution of each sample should not affect the calculated concentration. In order for integrity to be maintained, the mean concentration for each set of diluted QCs had to be within ± 15% of the true values, with %CV values <15%.

6.3.8.8 Stability

It is important that forensic toxicologists understand the stability of the samples they are interpreting as this may have an effect on the results obtained. In order to assess autosampler stability each QC was analysed in triplicate at 0, 24, 48 and 72 hours after initial preparation. This is important as samples may not be analysed straight away, either due to the large number of samples being analysed or due to instrumental failure overnight or over a weekend.

As NBOMe concentrations in biological matrices tend to be very low, the reconstitution volume for this method was reduced, as a result it is unlikely after 48 hours that any sample would remain in the vial to be analysed as it would have evaporated. In order to avoid sample evaporation, quadruple the amount of analyte and I.S. was used for this study. Samples were then reconstituted in quadruple the amount of mobile phase A and B mixture (50:50). This procedure was followed for both urine and hair calibrators.

Another factor of analyte stability is its ability to undergo freeze/thaw and warm/cool cycles, as samples may need to be reanalysed, or analysed for additional drugs after initial screening. This is only a factor for samples which would be stored in fridge/ freezer environments and thus only applies to urine samples as hair samples would always be kept at room temperature.

For the freeze/thaw and cool/warm stability, six of each urine QC were prepared as in section 6.3.3 with all volumes multiplied by 4, e.g. for QC1 4 mL of urine was spiked with 72 µl of NBOMe standard working solution (0.01 µg/mL). Each QC was then extracted and analysed as in sections 6.3.5, 6.3.6 and 6.3.7x. before storage. Three of each QC was stored at 4^{o}C in order to assess the effects of cool/warm cycles and the remainder stored at -20^{o}C to assess the effect of freeze/thaw cycles. These samples were then removed from storage, allowed to come to room temperature (22^{o}C) and analysed as before. This was carried out a

total of 3 times to assess the impact of freeze- thaw cycles on these samples, alongside fresh calibrators and QCs.

The PARs of each analyte were then plotted alongside the number of freeze thaws undergone to monitor for sample degradation. The analytes were considered stable if the PAR in comparison to those at time zero were within ±20%.

6.4 Validation Results & Discussion

6.4.1 Linearity

The three NBOMe compounds were linear over the extracted concentration range for both urine and hair analysis as shown in Figure 6-6 and Figure 6-7 respectively. R^2 values were >0.99. The peak area from the relevant MRM transition of each analyte to m/z 121 for the I.S. was used to calculate the PAR.

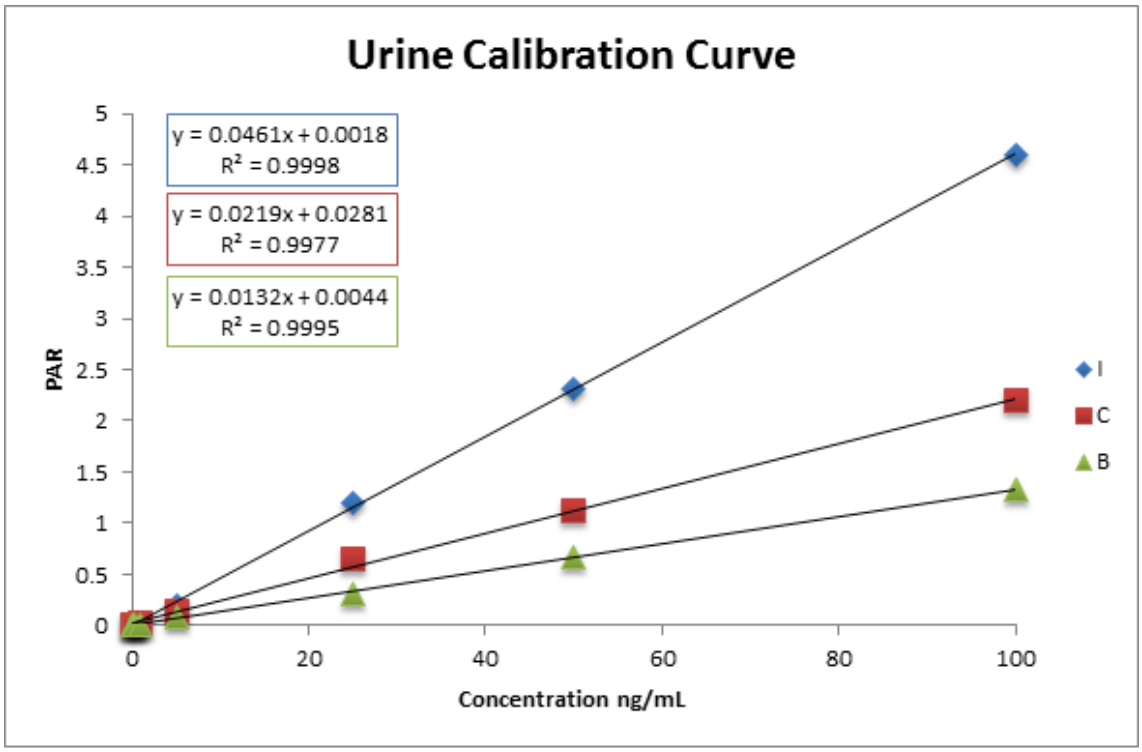


Figure 6-6: Example urine calibration graphs for 25B, 25C and 25I-NBOMe.

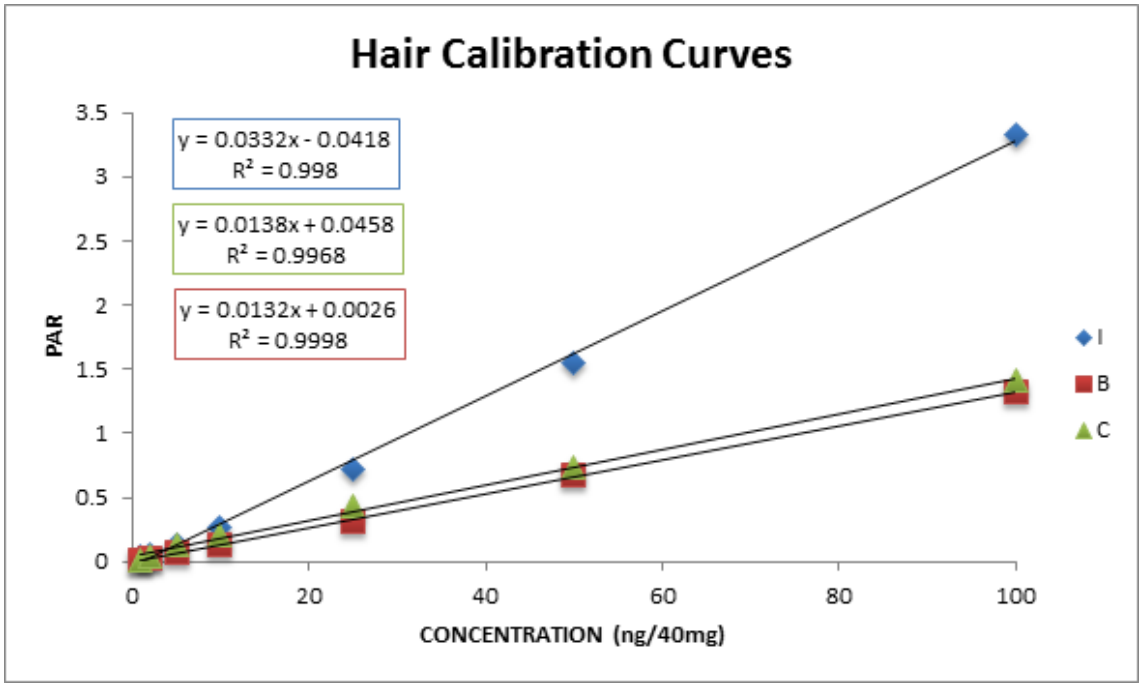


Figure 6-7: Example hair calibration graphs for 25B, 25C and 25I-NBOMe.

6.4.2 LOD and LOQ

The instrumental LOD and extraction LODs for urine and hair were identified and calculated as well as the LOQs for each matrix. The results of these are shown in Table 6-8. All LOD results produced signal to noise ratios > 3, all LOQ results produced signal to noise rations >10. Hair extractions were carried out using 40 mg of hair. The method LOD ranged from 250-500 pg/40 mg, which was detectable by the instrument, and higher than the urine LOD.

Table 6-8: Summary of LODs for each NBOMe in each matrix.

	Instrumental (pg/mL)	Urine (pg/mL)		Hair (pg/mg)	
	LOD	LOD	LOQ	LOD	LOQ
25B-NBOMe	4	5	50	3	6.25
25C-NBOMe	5	10	50	3	6.25
215I-NBOMe	10	25	50	5	12.5

The use of NBOMes have not been linked to DFSA cases as of yet and as there are no publications on the concentrations of NBOMes in hair it is unknown if these

limits would allow for the detection of single use exposure as required by the SOHT.(*231*)

6.4.3 Precision & Bias

The precision and bias for each analyte in urine and hair is shown below in Table 6-9 and Table 6-10 respectively. Both methods showed suitable acceptability with all results within the SWGTOX suggested ±20%. Hair analysis showed better accuracy than urine analysis with results falling within ±10% of the true values.

Intra-day precision results for urine QC's were all <15%, ranging from 1.3-10.2%. 25B-NBOMe showed the least intra-day precision, averaging at 2.3%, (1.3-3.4%). 25I-NBOMe showed the most intra-day precision averaging at 7.3% (ranging 2.4-14.9%).

Urine inter-day precision results were all <15%, with the exception of 25I-NBOMe QC2, ranging from 4.1%-15.7%. Again 25B-NBOMe showed the least variation, averaging at 7.8% (4.3-12.7%), whereas 25I-NBOMe showed the most inter-day variation averaging at 9.1% (ranging 4.1-15.7%).

25C-NBOMe showed the best accuracy results, all ranging within ±15%, where as 25B and 25I-NBOMe fell within ±20% off the expected values. This is unexpected as 25B-NBOMe showed the most inter and intra-day precision. These stronger precision results are most likely due to the used of 25B-NBOMe-D_3 as an I.S. As there is also a commercially available 25I-NBOMe-D_3 standard available, this may produce better intra and inter day precision results.

Table 6-9: 25-B, 25-C and 25-I NBOMe intra-day and inter-day precision and accuracy in urine.

Urine Results		25B-NBOMe	25C-NBOMe	25I-NBOMe
Intra-day Precision (%CV) (n=18)	QC 1 (0.18 ng/mL)	2.3	10.2	4.2
	QC 2 (4.2 ng/mL)	1.3	1.2	2.9
	QC 3 (84.0 ng/mL)	3.4	6.0	14.9
Inter-day Precision (%CV) (n=18)	QC 1 (0.18 ng/mL)	6.4	9.9	7.6
	QC 2 (4.2 ng/mL)	12.7	12.6	15.7
	QC 3 (84.0 ng/mL)	4.3	4.8	4.1
Accuracy (%) (n=18)	QC 1 (0.18 ng/mL)	80.3	105	118
	QC 2 (4.2 ng/mL)	117	98	99
	QC 3 (84.0 ng/mL)	104	114	113

All intra-day hair precision results fell below <15%. Unlike urine results however, it was 25C-NBOMe which showed the least variability averaging at 4.9%, (ranging 2.9-9.1%). 25B-NBOMe showed the largest intra-day variation averaging at 8.2% (ranging 2.6-12.1%).

Hair inter-day precision results also showed variation <15%. All NBOMes performed roughly the same averaging at 7.1, 8.0 and 6.2% for 25B, 25C and 25I-NBOMe respectively. Results ranged from 1.2-11.3%.

Hair accuracy results were better than urine, all falling within ±10% of the expected value. 25B-NBOMe however gave the most accurate results (103-109%), closely followed by 25C-NBOMe (96-109%). Again this is unsurprising as the I.S. used was 25B-NBOMe-D_3. Therefore, any differences observed in 25B-NBOMe analysis over the course of the validation should have been closely mimicked by 25B-NBOMe.

Table 6-10: 25B, 25C and 25I- NBOMe intra-day and inter-day precision and accuracy in hair.

Hair Results		25B-NBOMe	25C-NBOMe	25I-NBOMe
Intra-day (n=18)	QC 1 (0.105 ng/mg)	2.6	9.1	4.8
	QC 2 (1.05 ng/mg)	9.9	2.9	7.1
	QC 3 (2.10 ng/mg)	12.1	2.7	11.4
Inter-day Precision (%CV) (n=18)	QC 1 (0.105 ng/mg)	10.9	8.1	10.7
	QC 2 (1.05 ng/mg)	4.8	11.3	1.2
	QC 3 (2.10 ng/mg)	5.8	4.5	6.8
Accuracy (%) (n=18)	QC 1 (0.105 ng/mg)	109	96	102
	QC 2 (1.05 ng/mg)	104	105	110
	QC 3 (2.10 ng/mg)	103	109	94

6.4.4 Carryover

Carryover was observed after the highest urine standard as shown in Figure 6-8. The likelihood of having case samples with this concentration is extremely slim and it is therefore unlikely that this would result in carryover between case samples. However, if a high sample was observed the following sample would be re-injected after several flushes. The carryover observed was small in comparison to other standards accounting for <15% of the average area (n=6) for level 1 calibrators. It is therefore recommended that samples analysed after a high sample are re-injected and re-analysed.

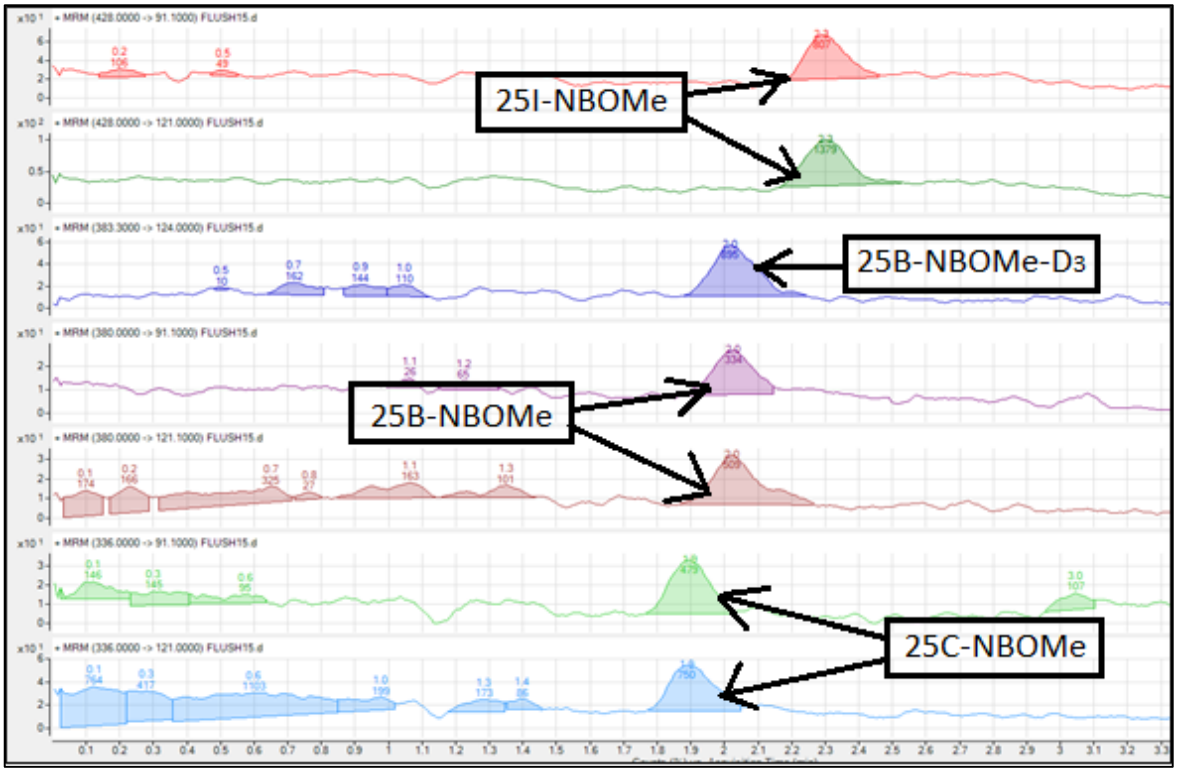


Figure 6-8: Observed carryover after running highest standard (100 ng/mL)

In order to determine an appropriate cut-off level for NBOMe concentrations identified in urine, a comparison was made between the flush after the highest calibration standard and the first calibration standard, the results of which are shown in Table 6-11. It is suggested from these results that to identify a sample as positive for the presence of 25B, 25C and 25I-NBOMe that the PA should be at least 3 times the peak found in the blank.

Carryover may not be seen for newer LC-MS/MS models which contain specific flush ports, which flush the system for set amounts of time after each injection and therefore reduce the presence of carryover.

Table 6-11: Carry over peak area versuss level 1 calibrator peak area.

	MRM Transition	Flush PA	Level 1 PA	% Difference
25B-NBOMe	428 →121	509	2687	18.9%
	428→ 91	334	2132	15.7%
25C-NBOMe	366 →121	750	5283	14.2%
	366→ 91	479	3953	12.1%
25I-NBOMe	380 →121	1379	7754	17.8%
	380 →911	807	4955	16.3%

6.4.5 Selectivity

None of the analytes tested in Table 6-7 interfered with the urine or hair method as shown in Figure 6-9. Figure 6-9 (a) shows the chromatogram from each analyte tested overlaid and although there are peaks present around the 2 minute mark these do not have the same retention times and therefore do not interfere with any of the analytes of interest as demonstrated by Figure 6-9 (b).

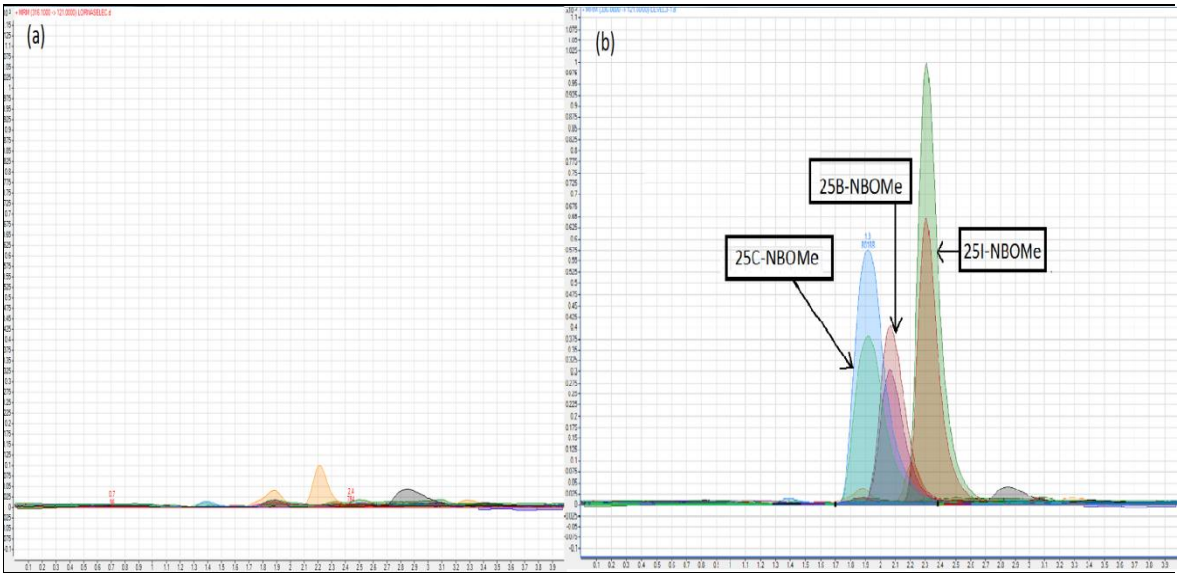


Figure 6-9: Overlaid chromatograms of analytes used to examine method selectivity. Figure (a) shows the interference analytes alone, and (b) shows the interference analytes with the target analytes overlaid (Level 3 calibrator).

I.S. and analyte interference results are shown in Figure 6-10. From these results it is clear that there is little interference caused between analyte and I.S. presence. Although there are peaks for the 25B-NBOMe transitions when I.S. was ran only, these are extremely low with peak areas of 50. Level 1 peak areas for 25B-NBOMe hair calibrators (n=6) averaged at 1712, thus the carryover peak represents 2.9% of this value. Urine level 1 calibrators (n=6) averaged at 3507, thus the carryover peak represents 1.4% of this value. It is therefore unlikely that the presence of either I.S. or analyte would significantly affect the other.

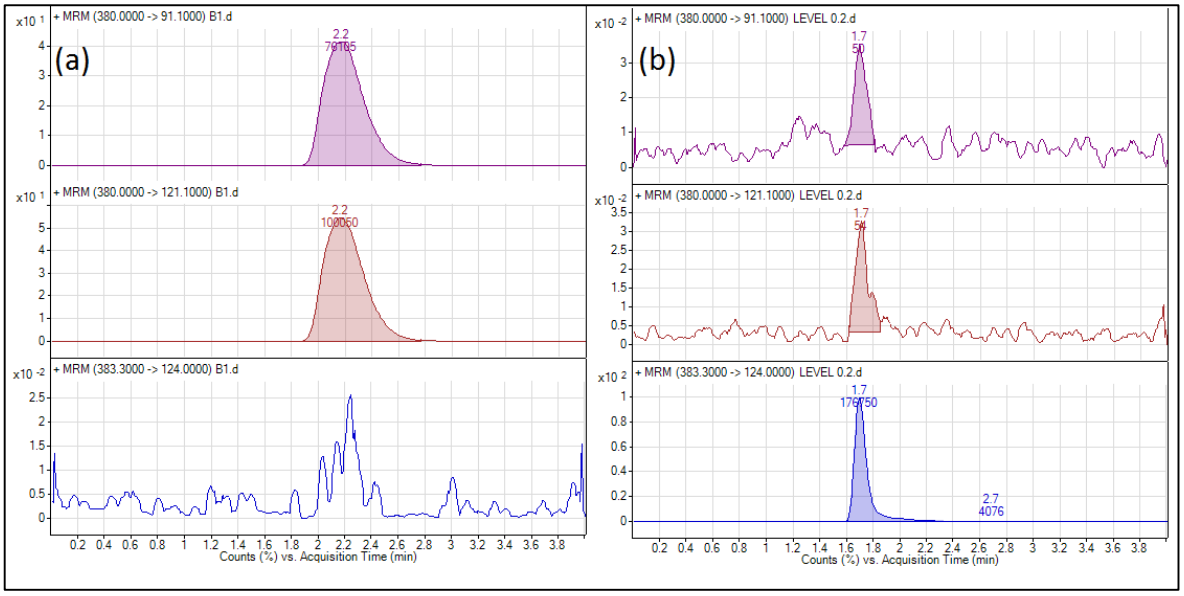


Figure 6-10: Analyte and I.S interference results.
Figure (a) shows the results of 25B-NBOMe analysed without the presence of 25B-NBOMe-D_3 and figure (b) shows 25B-NBOMe-D_3 analysed without the presence of 25B-NBOMe.

There were no interfering peaks from either matrix tested in this method as shown in Figure 6-11 and Figure 6-12.

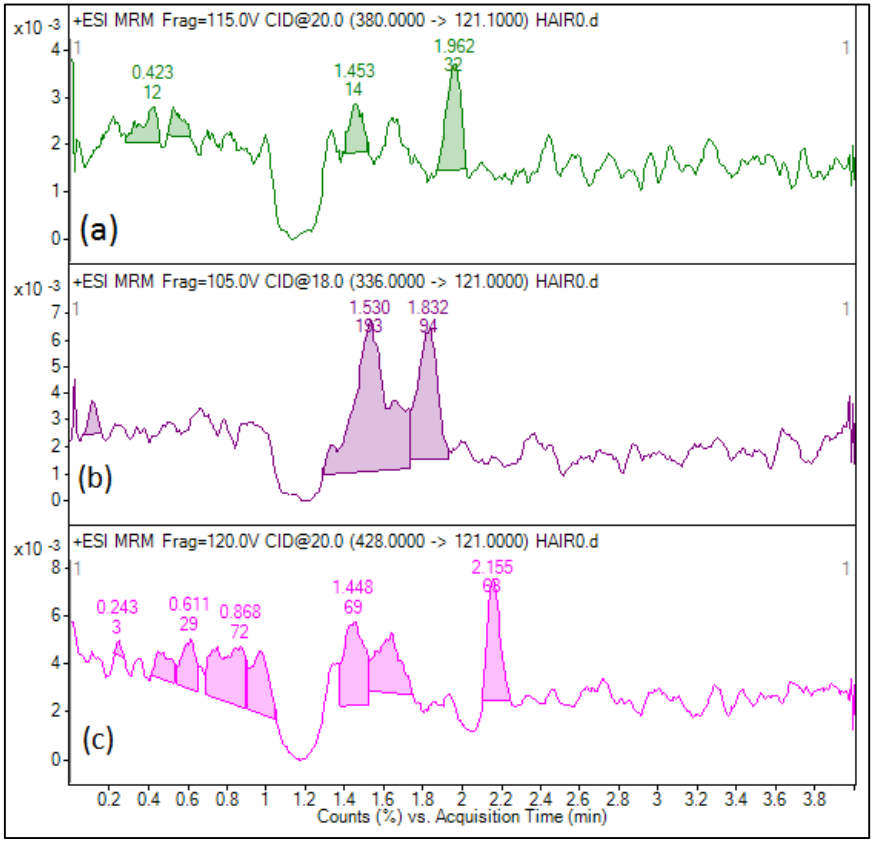


Figure 6-11: Blank pooled rat hair (n=55) showing no matrix interferences. (a) -25B-NBOMe, (b)- 25C-NBOMe, (c)-25I-NBOMe.

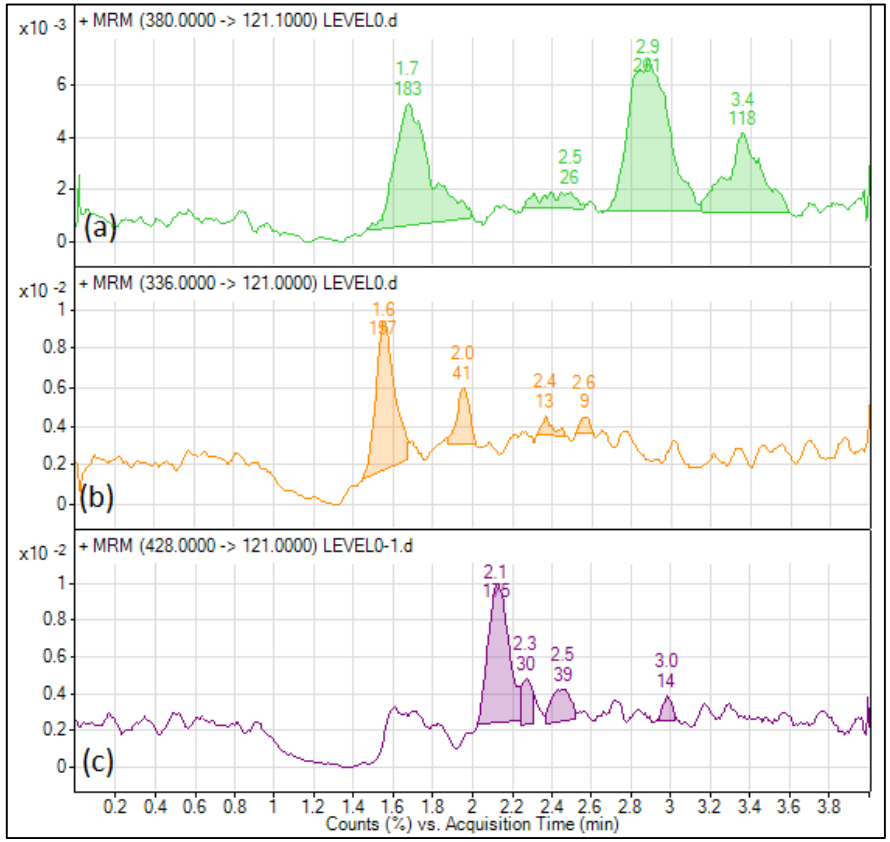


Figure 6-12: Blank pooled human urine (n=10) showing no matrix interferences.
(a) -25B-NBOMe, (b)- 25C-NBOMe, (c)-25I-NBOMe.

6.4.6 Stability

6.4.6.1 Freeze Thaw Stability (-20°C)

Repeated freeze thaws of urine samples stored at -20°C resulted in significant loss of sample over 3 freeze thaw cycles with an average loss of 32% across all analytes in QC1 as shown in Table 6-10.

For QC1, 25I-NBOMe was the most affected analyte seeing a decrease of 35%. From Figure 6-13 it can be seen that most analytes were stable up to the 3rd freeze thaw cycle when concentrations significantly decreased, in particular for 25I-NBOMe. The large increase in sample concentration was observed for the second freeze thaw cycle of 25I-NBOMe. This is most likely due to analysis error, as shown by the larger error bars associated with this point.

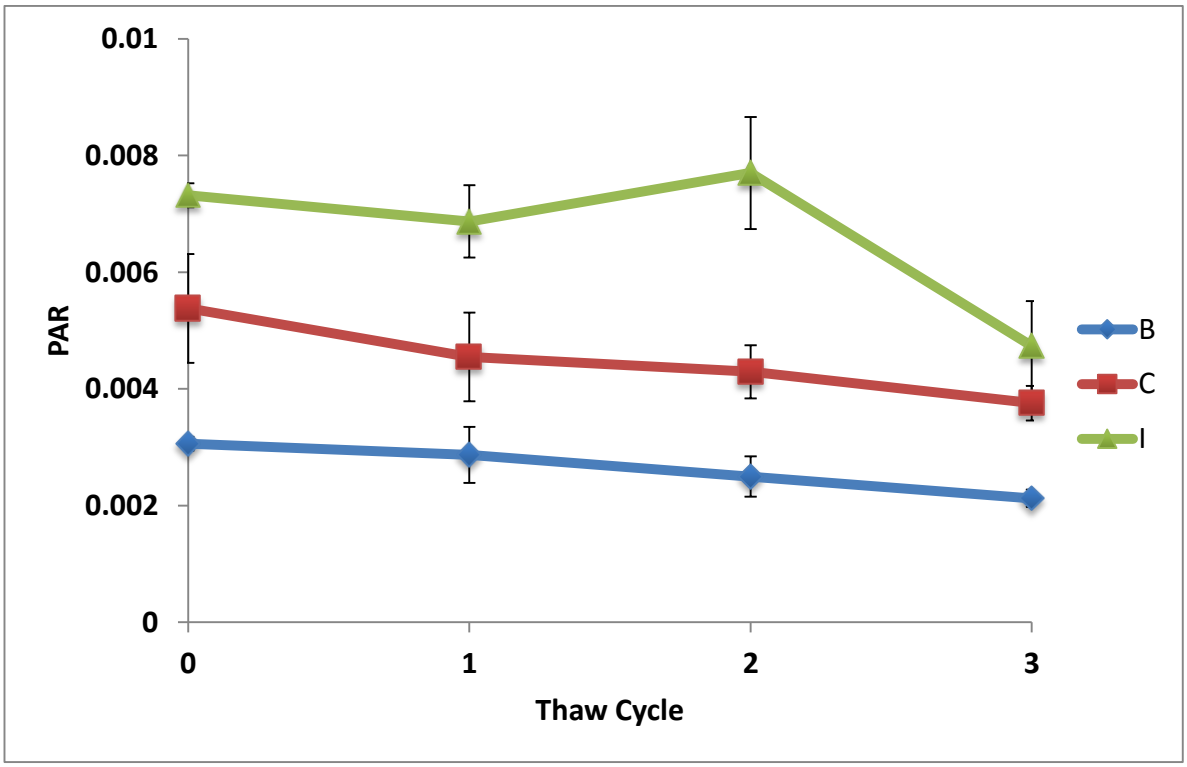


Figure 6-13: Freeze thaw results for urine QC1 containing 25B,25C and 25I-NBOMes stored at -20°C.

The same downward trend was also seen for QC2 and QC3 as shown in Figure 6-14 and Figure 6-15. QC2 lost an average of 37% of each analyte where as QC3 lost on average 58%. QC3 was the most affected losing on average 19% of

analytes after only 1 freeze thaw cycle. QC2 was within the ±20% range after 2 freeze thaw cycles.

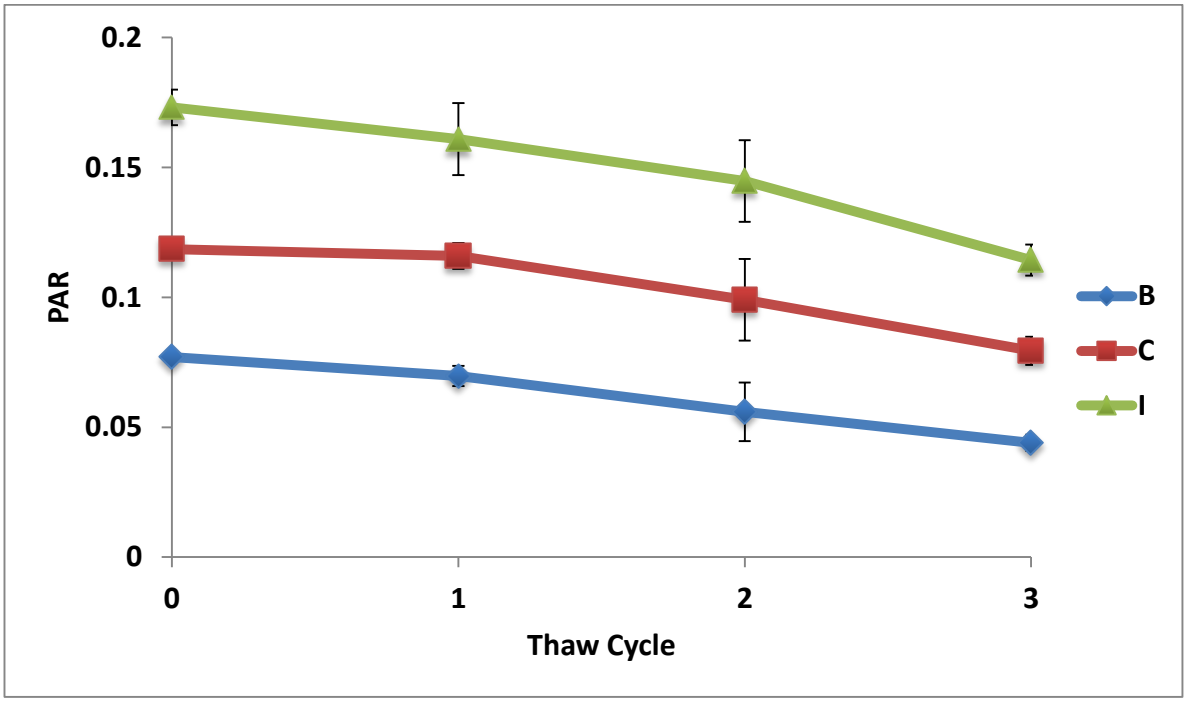


Figure 6-14: Freeze thaw results for urine QC2 containing 25B, 25C and 25I-NBOMes stored at -20 °C.

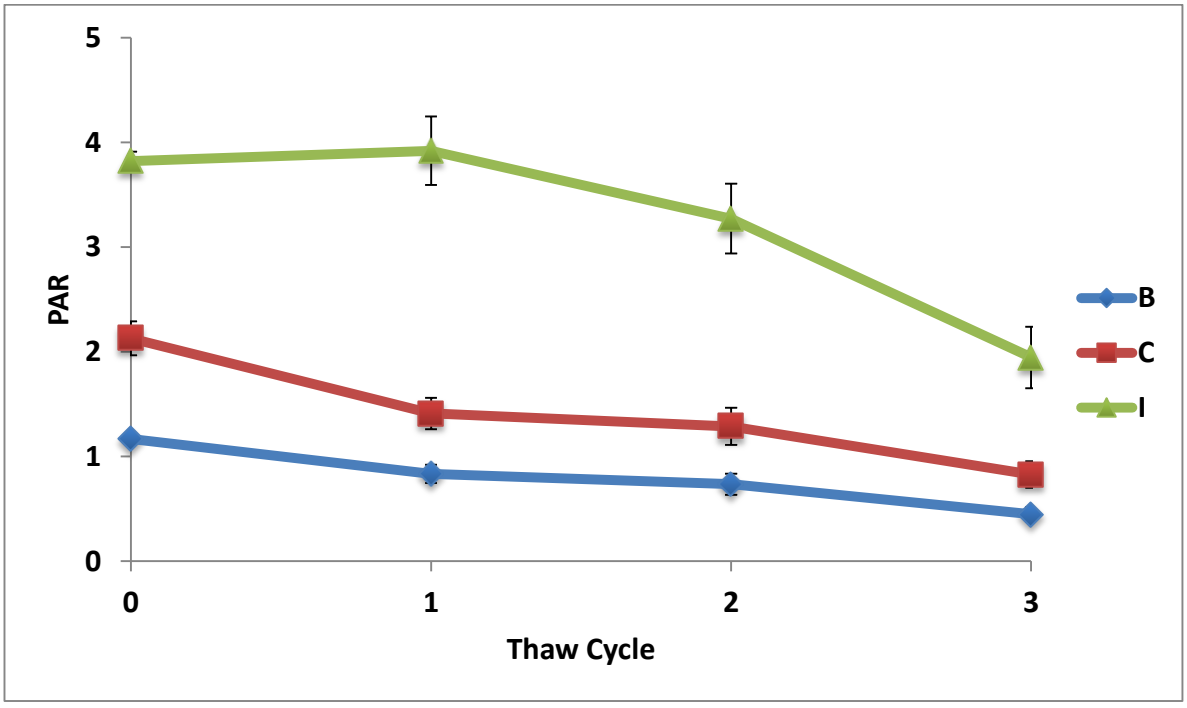


Figure 6-15: Freeze thaw results for urine QC3 containing 25B, 25C and 25I-NBOMes stored at -20°C.

6.4.6.2 Fridge Cool/ Warm Stability (+4ºC)

Samples may also be stored in the fridge and again a downward trend was shown for samples undergoing fridge thaw cycles. QC1 was stable up to 2 fridge thaw cycles, as shown in Figure 6-16. 25I-NBOMe showed an unsuspected increase after 2 fridge thaw cycles, however this was most likely due to analyst error as shown by the wider error bar associated with this point.

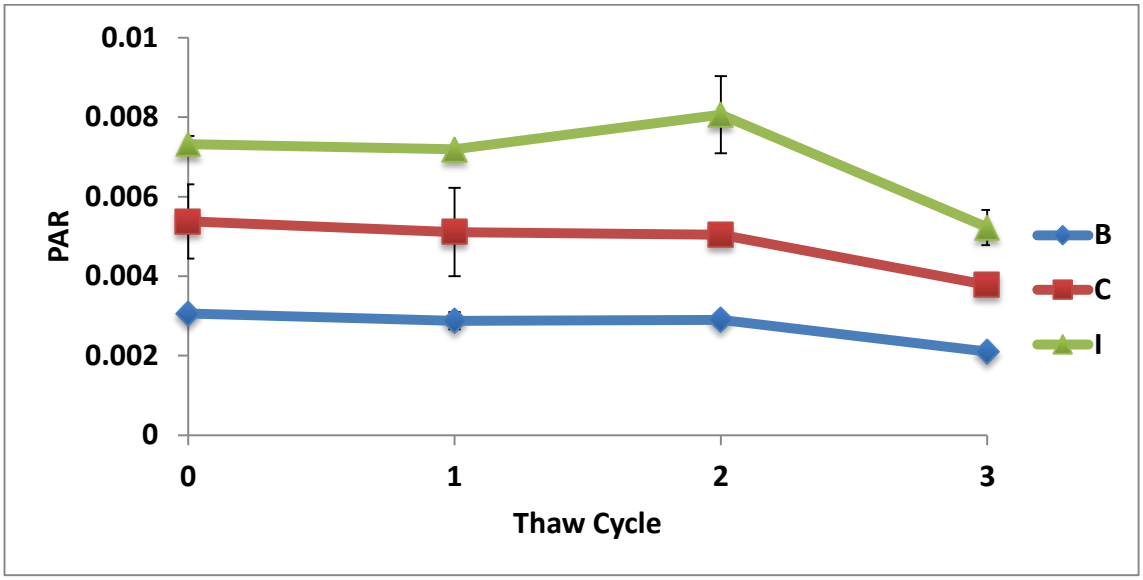


Figure 6-16: Fridge thaw results for urine QC1 containing 25B, 25C and 25I-NBOMes stored at -4ºC.

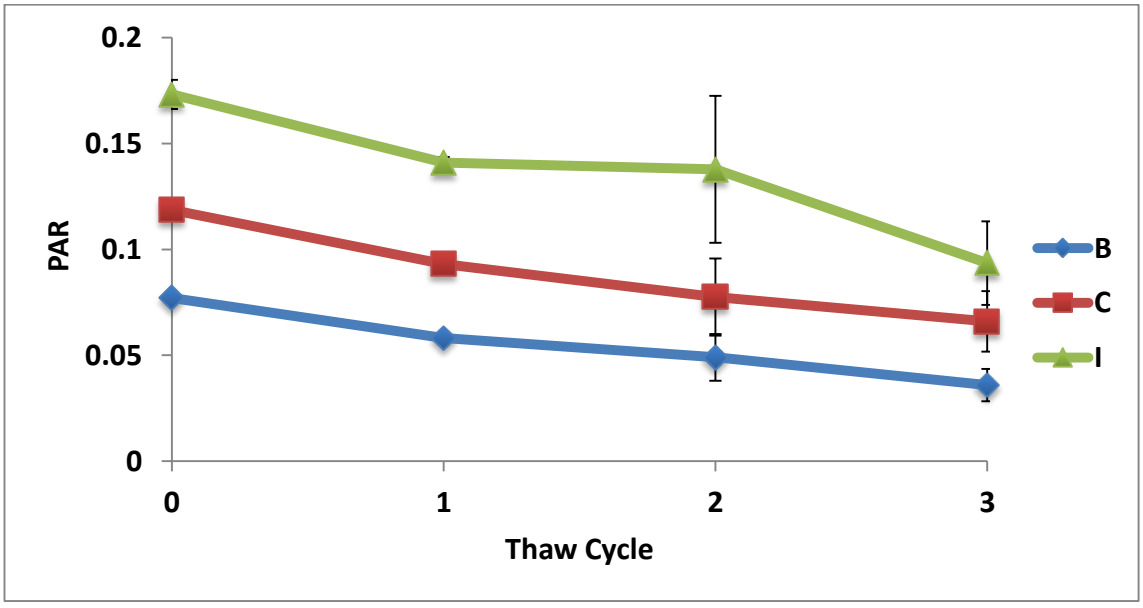


Figure 6-17: Fridge thaw results for urine QC2 containing 25B, 25C and 25I-NBOMes stored at -4ºC.

QC2 and QC3 were stable up to 1 fridge thaw cycle however after 3 cycles had lost 47% and 45% respectively as shown in Figure 6-17 and Figure 6-18. In both cased 25B-NBOMe was the most affected analyte losing 53% and 49% of analyte after the third cycle.

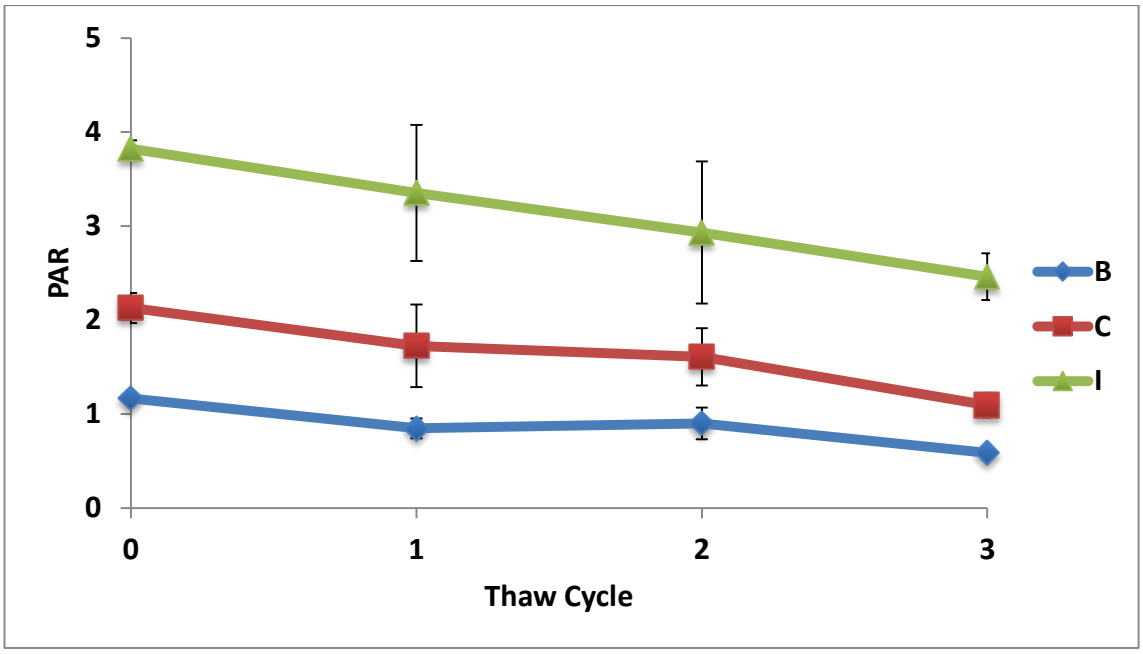


Figure 6-18: Fridge thaw results for urine QC3 containing 25B, 25C and 25I-NBOMes stored at -4°C.

These results show that any fridge/ freeze thaw cycles should be avoided when analysing for 25B, 25C and 25I-NBOMe. All QCs were stable for 1 thaw cycle however most analytes had lost more than 20% by the second cycle regardless of being stored in fridge (4°C) or freezer (-20°C).

6.4.6.3 Autosampler Stability

All analytes extracted from urine showed significant degradation whilst on the autosampler after 72 hours, with all QC1 and QC3 results failing to meet the <20% acceptability cut-off as shown in Table 6-12. Only the 25C and 25I-NBOMe QC2 met the ±20% requirements with recoveries of 84.6% and 89.5% respectively Table 6-12. 25B-NBOMe was the most affected analyte over the 72-hour time period, with an average recovery of 66.0% (50.9-75.0%). 25I-NBOMe was the least affected analysed over the 72-hour time period, with an average recovery of 82.4% (77.8-89.5%). Recovery for all analytes did not decrease by more than 20%

after 24 hours, with average % recovery values of 96.5, 9.3 and 92.8% respectively for all 3 QC's.

Table 6-12: Urine autosampler results.

		25B-NBOMe	25C-NBOMe	25I-NBOME
Q1 (PAR)	t=0	0.004	0.008	0.009
	t=24	0.004	0.007	0.008
	t=48	0.003	0.006	0.007
	t=72	0.003	0.005	0.007
	Recovery %	**75.0**	**62.5**	**77.8**
Q2 (PAR)	t=0	0.079	0.13	0.19
	t=24	0.079	0.13	0.18
	t=48	0.067	0.11	0.18
	t=72	0.057	0.11	0.17
	Recovery %	**72.2**	**84.6**	**89.5**
Q3 (PAR)	t=0	1.36	2.05	4.27
	t=24	1.22	1.83	4.05
	t=48	0.876	1.45	3.93
	t=72	0.692	1.325	3.41
	Recovery %	**50.9**	**64.6**	**79.9**

Hair autosampler stability results are shown in Table 6-13. The stability of hair samples is particularly encouraging however, as due to instrument failure no data could be collected at the 48 and 72-hour time period. Therefore these samples were analysed again at 120 hour. Both 25C and 25I-NBOMe showed acceptable stability results over the 120-hour time period with recovery values ±20% of the original. 25I-NBOMe however, fell slightly outside this for QC2 and 3 with recoveries of 123.3 and 123.7%. On average 25C-NBOMe remained the truest to its original recovery with an average result of 101.7% across all 3 QC's.

It is much more likely that hair samples would not be able to be re-extracted due to limited sample volume and thus this parameter is more important for this matrix should instrument failure occur and reinjection of samples is necessary.

Table 6-13: Hair autosampler results.

		25B-NBOMe	25C-NBOMe	25I-NBOME
Q1 (PAR)	t=0	0.12	0.15	0.15
	t=24	0.17	0.17	0.14
	t=120	0.14	0.13	0.17
	Recovery %	**116.67**	**86.67**	**113.33**
Q2 (PAR)	t=0	1.03	1.36	1.07
	t=24	1.46	1.82	1.00
	t=120	1.27	1.47	1.23
	Recovery %	**123.30**	**108.09**	**114.93**
Q3 (PAR)	t=0	2.19	2.79	2.25
	t=24	3.15	3.89	2.05
	t=120	2.71	3.08	2.61
	Recovery %	**123.74**	**110.39**	**116.00**

It is not known why there is such a marked difference between urine and hair results for autosampler stability (50.9-89.5% and 86.67-12.7% respectively for each matrix). Both use different extraction techniques and it is possible that matrix contamination is still present in urine samples extracted by LLE, which are negatively affecting the concentrations of these samples. It is also probable that as the PAR's for urine samples are so small due to the wider concentration range of this calibration curve that any differences observed have a greater impact on the % recovery results.

6.4.7 Matrix Effects and Extraction Efficiency

The matrix effect, process efficiency (%) and recovery (%) for both urine and hair are shown below in Table 6-14 and Table 6-15. From this information it can be shown that no significant ion suppression or enhancement from the matrix was observed.

LLE extraction of 25B, 25C and 25I-NBOMe from urine was extremely efficient with % recoveries ranging from 89-102%. Urine process efficiency was also good ranging from 89.4-102.6%.

Table 6-14: Matrix effects, process efficiency and recovery for urine QCs 1&3.

		25B-NBOMe	25C-NBOMe	25I-NBOMe
Matrix Effect	QC1	1.05	1.17	1.03
	QC3	0.96	1.09	0.98
Process Efficiency (%)	QC1	103.9	104.1	95.0
	QC3	98.5	101.5	89.4
Recovery (%)	QC1	99.1	89.1	92.4
	QC3	102.6	92.9	89.5

Hair extraction recovery (%) values were not as good as urine extraction, ranging from 80.5-107.5% over all compounds and QC levels. Matrix suppression was seen for 25I-NBOMe although this was within the ±0.25 limits of acceptability. 25I-NBOMe process efficiency was also low averaging at 61.63% across both QC's.

Table 6-15: Matrix effects, process efficiency and recovery for hair QCs 1&3.

		25B-NBOMe	25C-NBOMe	25I-NBOMe
Matrix Effect	QC1	1.05	1.12	0.82
	QC3	0.93	1.04	0.77
Process Efficiency (%)	QC1	98.21	93.30	68.28
	QC3	93.97	103.80	54.98
Recovery (%)	QC1	93.94	83.31	83.18
	QC3	86.99	107.46	80.48

6.4.8 Dilution Integrity

The dilution integrity for urine samples was assessed and the results are shown in Table 6-16. All dilution % accuracy results fall within the selected <20% criteria with the exception of the 1:4 dilution. For this particular dilution 25I-NBOMe fell slightly out with the accepted <±20% limit, at +21.7% and 25B-NBOMe fell further outside this limit at +29%. 25C-NBOMe was the only analyte to remain within ±20% for all dilutions. 25B and 25I-NBOMe samples should therefore only undergo a maximum of 1:3 dilutions in order to comply with original precision and bias requirements. 25C-NBOMe is able to undergo a 1:4 dilution if necessary, whilst retain acceptable accuracy results.

Table 6-16: Dilution integrity of urine samples.

	25B-NBOMe		25C-NBOMe		25I-NBOMe	
	Accuracy %	%CV	Accuracy %	%CV	Accuracy %	%CV
1:1	92.5	1.1	85.8	1.7	94.1	4.0
1:2	115.8	0.9	103.0	5.6	117.9	5.2
1:3	97.2	0.5	97.2	0.7	101.1	1.3
1:4	129.0	1.0	113.1	1.8	121.7	1.9

The brief hair study investigating the effects of limited sample weight showed that both 25B and 25C-NBOMe produced concentrations with accuracy results ±15% when only 10 or 20 mg of hair was used. 25I-NBOMe on the other hand fell slightly out with these limits with a % accuracy result of 81.8% (-19.2%) as shown in Table 6-17.

Due to limited sample volume it was not possible to analyse samples in triplicate and as a result the variation between the PARs of each sample may be due to analyst error. Ideally, once further hair samples become available this experiment should be repeated, with each weight analysed in triplicate.

Table 6-17: Hair sample results.

	PAR			Accuracy (%)
	10 mg	20 mg	40 mg	
25B-NBOMe	0.11	0.08	0.09	81.8
25C-NBOMe	0.15	0.15	0.15	100.0
25I-NBOMe	0.10	0.08	0.09	90.0

6.4.9 Comparison of LC-MS/MS method with GC-MS method

The LC-MS/MS method detailed in this chapter provided a much more robust and sensitive method when compared to the GC-MS method validated in Chapter 3. The MS/MS capabilities of the LC-MS/MS meant that the LOD and LOQ for urine samples were significantly lower. The LC-MS/MS method had an LOD ranging 5-25 pg/mL in contrast to the GC-MS method which only detected down to 200 pg/mL. The LC-MS/MS had a much better LOQ at 50 pg/mL in contrast to the GC-MS method which was only able to quantitate as low as 500 pg/mL. This is unsurprising as previously published methods all use LC-MS/MS for the detection

for NBOMes in biological matrices. Although this method was validated for urine and hair, it is likely that had it been validated for blood and applied to samples received from NMS (Chapter 3), the NBOMe concentrations present would have been quantified. This was not possible however, as GC-MS analysis took place in Philadelphia, whereas this method was validated in Glasgow. Samples were unable to be transferred between institutions due to on-going collaborative research with NMS and the FRFRF. The cost of sample transfer would also be significant for such a small number of samples as they would be sent via international courier on dry-ice.

The GC-MS analysis was carried out using a new column, with the injection port and liner changed after every 100 injections in order to keep the instrument as sensitive as possible. This additional maintenance was not required for the LC-MS/MS method, making this method for the analysis of these 3 NBOMes less time consuming, more sensitive and more robust than that of the GC-MS method.

6.5 Conclusion

A simple and quick quantitative LC-MS/MS method for the analysis of 3 common NBOMes was developed and validated for hair and urine samples according to SWGTOX guidelines. The speed of this method allows for high sample throughput, with time limiting steps being the sample preparation itself. The use of LLE for urine provided a rapid and yet efficient sample extraction technique with recoveries ranging from 89.5-102%. Both methods cover a wide concentration range; 0.1-100 ng/mL for urine and 0.025-2.5 ng/mg for hair.

To date there has been no previously published method for the detection of NBOMes in hair. Although the hair method is slightly more time consuming than that of the urine method, this could be improved by the use of an automated SPE system.

6.6 Future Work

Further work should be completed on the LC-MS/MS method to fully validate this to ISO17025 standards. Furthermore, the addition of newer NBOMes to the panel would be beneficial as and when more become available, in keeping with other

published methods and also keeping up with what drugs are available/ being abused.

This method only examined hair and urine as these were the matrices collected during the rat studies (Chapter 7). It would therefore seem logical to expand this to include whole blood, plasma and serum, especially as concentrations would typically be low and thus MS/MS would be required for their detection.

Autosampler stability should be re-examined. As loss of analytes has been shown, it may be useful to repeat this experiment analysing for the presence of metabolites alongside that of the parent compound.

Chapter 7: Detection of NBOMes in Rat Hair and Urine Samples

7.1 Introduction

In order for drugs to be excreted by the body they undergo metabolism, typically into more water soluble forms. Metabolism typically occurs in the liver, subject to route of administration, and it is here that these processes are catalysed by microsomal membrane bound enzymes in the hepatocytes such as the cyctochrome P450 system.(*234*) Many drugs undergo a combination of Phase I (oxidation, hydroxylation, de-alkylation and sulfoxide formation) and Phase II (conjugation) metabolism.(*170*) Phase I metabolism occurs via the CYP450 family of enzymes, specifically CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, the latter of which is involved in the metabolism of around 55% of drugs.(*235*) As a result, metabolism via CYP3A4 is typically the first to be assessed during metabolism studies.

Many drugs undergo rapid metabolism so that by the time of analysis it is not the parent drug which is analysed, but the metabolites. For this reason, it is important to know how particular drugs are processed by the body in order to determine which compounds will be present by the time of sample collection. For example, when analysing heroin it is not the parent drug diamorphine which is looked for, but instead 6-monoacetylmorphine and morphine.(*236*) By targeting metabolites, a much more sensitive and specific analysis can be achieved, thus enabling the toxicologist to provide a more accurate interpretation of results.

In vivo studies are extremely rare and difficult to carry out due to various ethical issues and although there are alternative *in-vitro* methods available, these can be expensive, and are much more complex to carry out.(*237*) An alternative to this is the use of animal models. Rodents are typically used as the ideal animal model as they are small, easily housed, and cheaper than other animals. The results of these tests can then be compared to “real life” human case samples to establish if the same metabolites are seen and to the same degree. This method has been routinely used for other compounds such as synthetic cathinones.(*101, 178-180, 238*) When using rodents they are typically

placed into metabolism cages which contain a raised wire mesh floor across the bottom of the cage, allowing for the separation of urine and faeces from the rodent. (*239*)

Another benefit of animal models is that these allow for the behavioural effects of compounds to be studied. As more NPSs are launched into the recreational drug market it becomes much more difficult for A&E staff to know the effects and behaviours exhibited by individuals on these drugs. As a result it is useful to use animal models to try and predict any adverse reactions which may be observed by users.

The use of animal models allows for testing to be carried out on both traditional and untraditional matrices. Therefore allowing toxicologists the chance to study matrices which they may not routinely come into contact with but which may be of scientific interest, especially for compounds which are not routinely seen in post mortem samples.

Many drugs cause the user to have reduced or heightened reactions to pain. Whether a drug has an analgesic effect can be determined by the use of tail flick experiments. These can be conducted using water baths or by placing the animal on a hot plate and monitoring the animal's reaction to heat.(*240, 241*) An increase in the animal's response time shows that the drug has an analgesic affect, whereas a reduction shows the drug enhancing pain. After time the effects may be reduced which signifies the animal has grown tolerant to the drug.

This research involves the analysis of NBOMes which are hallucinatory stimulant drugs and although analgesia is commonly linked to central nervous system depressants, it has been observed in other stimulants such as amphetamines and synthetic cathinones.(*242-244*) Administering drugs to animal models allows for more in-depth analysis of their effects and more importantly their toxicity which ethically cannot be completed in human patients.

There are however limitations to the use of animal models. Some drugs will not undergo metabolism the same way as humans do. As a result animals may or may not experience effects which are or aren't shown in humans.(*245*)

7.2 Aims

This work aims of this research was to extract 25B, 25C and 25I-NBOMe from rat hair by using a phosphate buffer incubation and SPE clean up method. Additional objectives including assessing any dose response relationship and determining whether the colour of hair affects concentration as seen with other phenethylamines. This work also aims to extract and quantitate 25B, 25C and 25I-NBOMe from rat urine and identify any metabolites which may also be present.

7.3 Materials & Methods

7.3.1 Subjects

Male Long Evans rats (Charles River, MA, USA) were used for this investigation. Animals were housed singly as shown in Figure 7-1, in a humidity and temperature controlled laboratory with 12:12 hour light:dark cycles. Animals entered the laboratory at age 3 weeks old and weighed 28-50g at the start of the study. Animals were given 7 days to acclimatise to their surroundings before any experimental work commenced and had *ad libitum* access to food and water. All procedures were conducted during the dark cycle, under protocols approved by the Institutional Animal Care and Use Committee (IACUC). The IACUC proposal and approval letter can be found in Appendix 12 and Appendix 13 respectively.

All individuals assisting with this research had completed the Collaborative Institutional Training Initiative (CITI) and received hands-on training prior to any experimental work. Citi training certificates can be found in Appendix 14.

Figure 7-1: Diagram of rat housing

7.3.2 Chemicals

Reference standards of 25B-NBOMe, 25C-NBOMe and 25I-NBOMe (10 mg powders) were purchased from Cayman Chemical Company. DMSO (Sigma Aldrich, MO, USA) was used as the injection vehicle in saline. Syringes and needles were purchased from Fisher scientific. Needles were replaced after each injection and syringes were changed daily for each drug used.

HCOOH was purchased from Sigma Aldrich (MO, USA). MeOH, acetic acid and all other solvents and chemicals were all of analytical grade and all purchased from VWR (TX, USA).

7.3.3 Instrumentation

The instrumentation used for this work was the same of that listed in Chapter 6. The Agilent Technologies 6420 series triple quadrupole LC-MS/MS (Agilent, USA) was operated using the same parameters as those listed in Table 6-5.

7.3.4 Injection Solutions

Each NBOMe drug (5 mg) was dissolved initially in 100 µL of DMSO. This was then further diluted with 4.9 mL of saline solution in a 5 mL volumetric flask, giving a 1 mg/mL solution. Saline was produced as per section 2.4.1.2. From this solution, 1.66 mL was transferred and further diluted with 3.34 mL saline solution to yield a concentration of 0.33 mg/mL. A further 1 mL of the 1 mg/mL solution was transferred to a volumetric flask and again made to the 5 mL mark by adding 4 mL of saline solution to produce the least concentrated solution of 0.1 mg/mL. The 1 mg/mL solution was used for rats receiving the highest dose (0.1 mg/kg), the 0.33 mg/mL solution was used for rats receiving the medium dose (0.03 mg/kg) and the 0.1 mg/mL solution was used for rats receiving the lowest dose (0.01 mg/kg). Due to the little information available about NBOMe doses LSD was used as a guideline when determining suitable doses.(*246*)

7.3.5 Tail Flicks and Injections

Rats were weighed upon arrival and daily at 9 am (±1 hour) throughout the experiment. Rats were visually inspected during weighing for signs of distress

such as porphyrin staining. Rats, like all vertebrate, have Harderian glands, producing lipids to lubricate the eye. In the case of rats these glands also produce porphyrin, and when stressed, ill or poorly fed over produce porphyrin. This dries around the eye colouring the surrounding area red, known as porphyrin staining. Any rats which did not show significant weight gain over the 1-week grace period or which showed signs of distress were excluded from the experiment. This amounted to a total of 4 of the 59 rats received leaving 55 rats fit for experiments. All rats (n=55) were initially shaved after the 1-week grace period along their backs, collecting both black and white hair separately in clean 7 mL vials.

Each rat was injected intraperitoneally (IP) with either the low (30 µg/kg), medium (100 µg/kg) or high dose (300 µg/kg) of a single NBOMe drug as shown below in Figure 7-2.

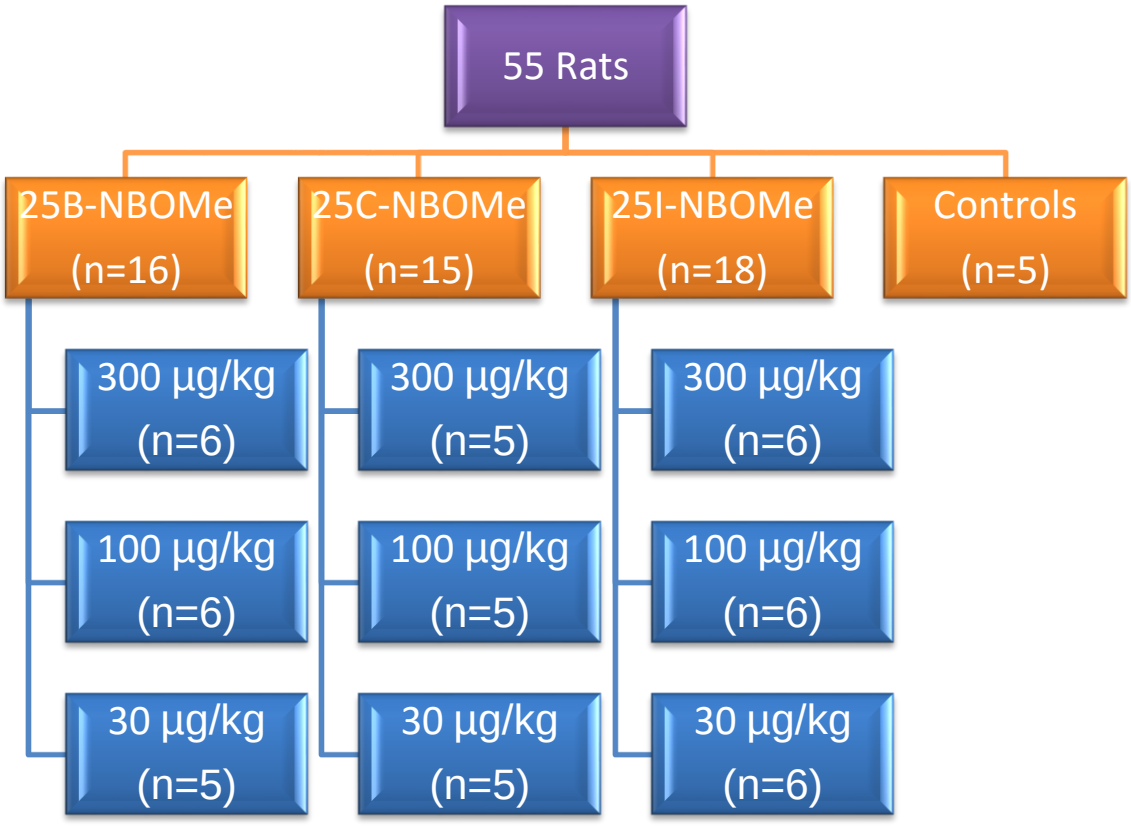


Figure 7-2: Schematic of rat dosing.

The amount of solution injected into each rat was calculated as per Equation 7-1 based on the rats weight. Control rats were injected with the equivalent volume of DMSO:saline solution.

Equation 7-1: Rat injection volume calculation

$$Injection\ Volume\ (mL) = dose\ (\mu g/kg)\ x\ rat\ weight\ (kg)$$

Rats were then left in their cages for 15 minutes before tail flick experiments were commenced. Tails were marked 8 cm from the tip prior to the beginning of the experiment with a permanent marker to ensure consistent tail immersion throughout the experiment. Tail flicks were conducted on 3 occasions prior to injections to produce a baseline for each rat. Each rat was wrapped in a small towel, leaving only their tails exposed. The tails of each rat were then immersed up to the 8 cm pen mark in a water bath (52^{o}C). The length of time taken from immersion to the rat "flicking" its tail out of the water was recorded by a stopwatch. The same individual recorded all time measurements to limit variability in this step.

A rat from the highest dose group for each drug was left in a metabolism cage after its tail flick test until all experiments had been completed for that day. After returning the rat to its normal cage urine was collected from the bottom of the metabolism cage, transferred to vials by pasteur pipette and stored at -20^{o}C for later analysis (see section 7.3.6). Only rats injected with the highest dose were kept in the metabolism cages as their urine was expected to provide the highest concentration of any detectable metabolites. This also reduced the number of animals which entered the metabolism cages as these can be stressful for the animals involved.(*247*) Having already lost 4 rats to stress it was deemed appropriate to keep the number of rats entering the metabolism cage as small as possible, should any show adverse signs. A different rat from each drug group was chosen each day to enter the metabolism cages, again to reduce any additional stress on the animals. Urine was pooled, and approximately 10 mL was collected over the 8-day period. Rats were injected over a period of 9 days at 10 am each day (±30 minutes). After 8 days of injections the rats were re-shaved in the same place as the initial shaving, with shavings separated by dose and colour. Hair from animals receiving the same drug at the same dose was pooled and stored in vials for later analysis (see section 7.3.7). At the end of the study all rats were sacrificed using CO_2 or frozen for further research out-with the remit of this thesis.

Tail flick data was analysed by SSPS software (version 19). This was used to test for any significant differences between the length of time of the tail flicks between the control group and those receiving doses of NBOMe's.

7.3.6 Urine Analysis

Rat urine samples were analysed using the validated LC-MS/MS method discussed in Chapter 6. The three urine samples were analysed in triplicate. Samples were extracted as per the LLE urine extraction method described in 6.3.6. Calibrators and QCs were run alongside the samples, again prepared in accordance with chapter 6.

In order to determine if any metabolites were present, the MRM method was altered to analyse for de-methylated metabolites using the MRM transitions shown in Table 7-1. These transitions relate to the parent compound minus 1, 2 or 3 methyl groups.

Table 7-1: MRM transitions monitored for demethylated metabolites.

Drug	-1 Methyl Group	-2 Methyl Groups	-3 Methyl Groups
25B-NBOMe	366	352	338
25C-NBOMe	322	308	294
25I-NBOMe	414	400	386

In order to identify if there were any additional metabolites present, the urine samples were also analysed using a Bruker QToF. This screening technique allows for the detection of compounds using their exact mass and can be used in a targeted way, where the exact mass of a substance is already known, or untargeted, where the QToF collects all the mass data relating to every molecule within a single sample.

7.3.7 Hair Analysis

Long-Evans rats were chosen specifically for this study as they grow both black and white hair as illustrated in Figure 7-3. This allows for the detection of drug in different coloured hair without the additional variables of drug dose, and individual rat metabolism.

Figure 7-3: Image of Long-Evans rat

Rat hair samples were analysed in triplicate as per the validated LC-MS/MS method discussed in Chapter 6. Samples were washed and extracted as per the SPE hair extraction method described in 6.3.7. Calibrators and QCs were run alongside samples; again these were prepared in accordance with 6.3.3.2. Hair wash solutions were stored for analysis in order to determine whether any NBOMe concentrations could be a result of external contamination, i.e. by injection solution dripping onto the hair of the animal prior to injection, sweat, urine or faeces.

7.4 Results & Discussion

7.4.1 Tail Flicks

Using the SSPS software package it was found that there was no statistically significant difference between the tail flick times of each drug group, or between drug groups and controls as illustrated by Figure 7-4 to Figure 7-6. There was also no statistically significant difference between differing doses or between the weight gains of each animal over the 8-day period. As a result these drugs were shown to not produce analgesia at the doses provided.

The stability of these compounds in DMSO:Saline is also not known, and as a result it cannot be guaranteed that the rats received the same amount of drug each day, or whether the solution degraded over the course of the 8 days.

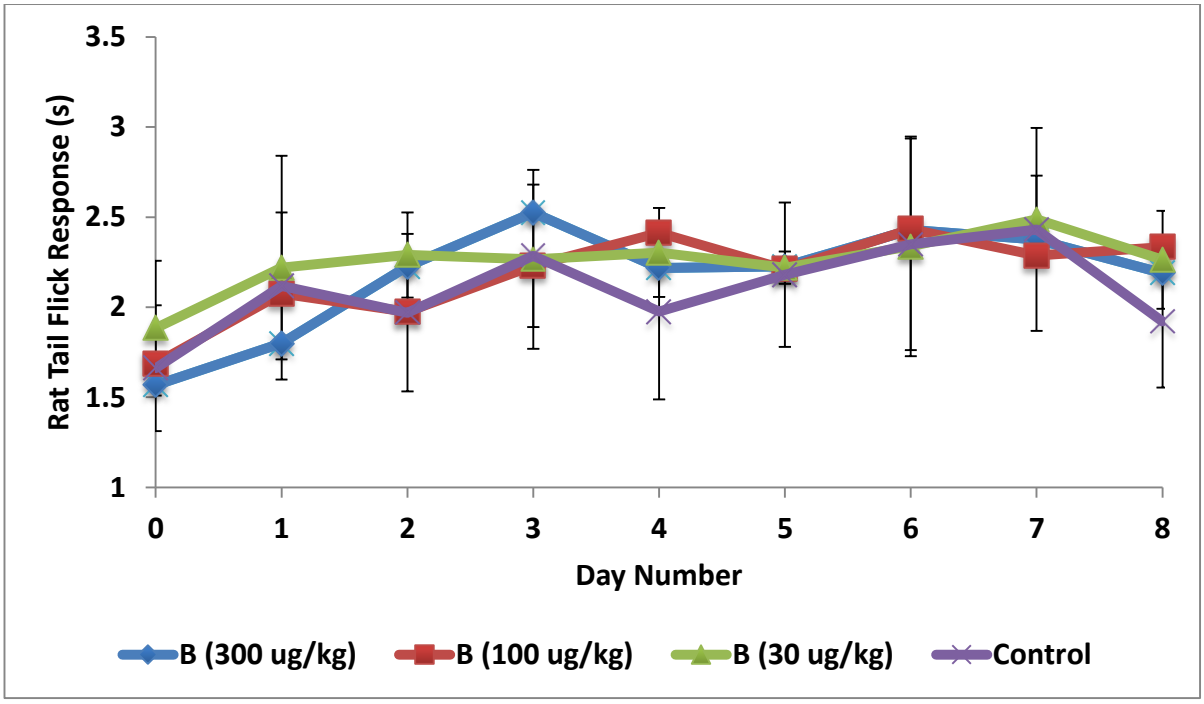


Figure 7-4: Average tail flick response times (seconds) from rats receiving 30, 100 and 300 µg/kg doses of 25B-NBOMe.

*B=25B-NBOMe

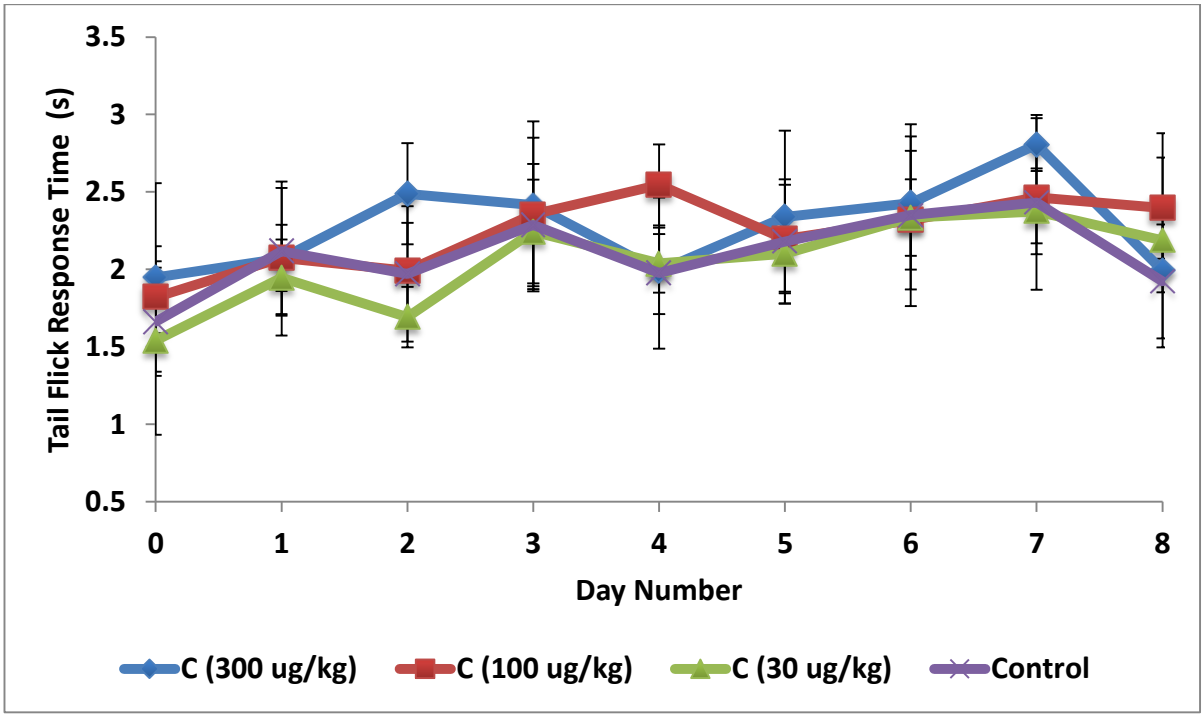


Figure 7-5: Average tail flick response times (seconds) from rats receiving 30, 100 and 300 µg/kg doses of 25C-NBOMe.

*C=25C-NBOMe

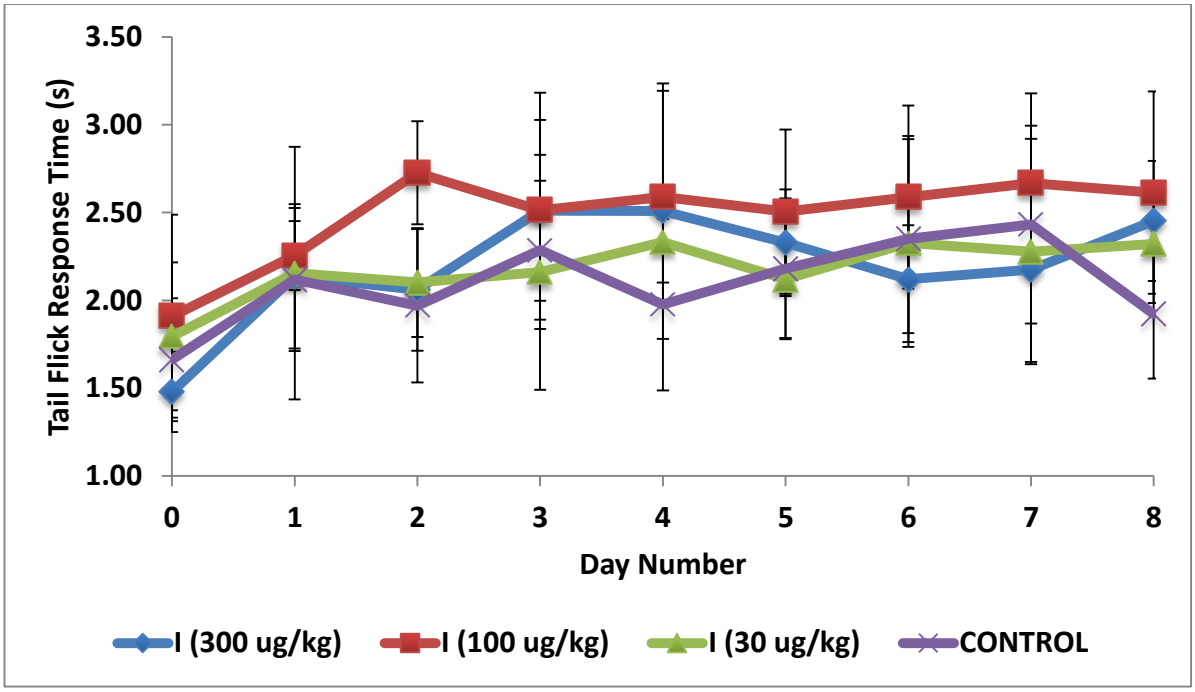


Figure 7-6: Average tail flick response times (seconds) from rats receiving 30, 100 and 300 µg/kg doses of 25I-NBOMe.

***I-25I=NBOMe**

7.4.2 Behaviour

As part of IACUC approval, all rats were monitored for 1-hour post injection. Rats injected with NBOMes were much more active than the control rats, displaying significant rearing, correlating with Poklis' findings.(*248*) Head twitching was observed which is typical of 5-HT_{2A} receptor activation, which has been identified by others. This was not quantified though as typically this involves mounting magnetics to the head of the rat so that the head twitches can be digitally recorded.(*249*) The mounting of magnets to the head of a rat involves surgery which fell out with the expertise of Arcadia University and the IACUC protocol.

Rearing is another common behaviour shown in rats undergoing 5-HT_{2A} receptor activity. While hallucinating, rats commonly rear and stretch in their cages. This can be monitored using an activity monitor such as a video path analyser.(*250*) Video path analysers were not available at Arcadia University and fell out with the purchasing limit of this project. Therefore, although rearing was observed in all drug dosed animals this was not quantified. These observations coupled with that of the head twitches shows strong indications that there was activity at the

5-HT_{2A} receptors. This correlated with previously published research, where head twitch responses and rearing were also witnessed.(*248*)

This study was conducted summer 2014 and at this time point very little information regarding doses was available. It was therefore not known what doses the rats would be able to handle without having adverse reactions such as seizures and possibly death. Due to the nature of the data collected from these animals they had to survive injections over multiple days. Since this research was carried out more rodent studies have been published using higher doses. The majority of these publications induced seizures and even death, which was not part of this IACUC proposal.(*251, 252*)

7.4.3 Urine Samples

All urine samples tested positive for their expected NBOMes as shown below in Figure 7-7. As seen with hair samples 25C-NBOMe provided the highest detected concentrations, however this was then followed by 25I-NBOMe, and finally 2B-NBOMe.

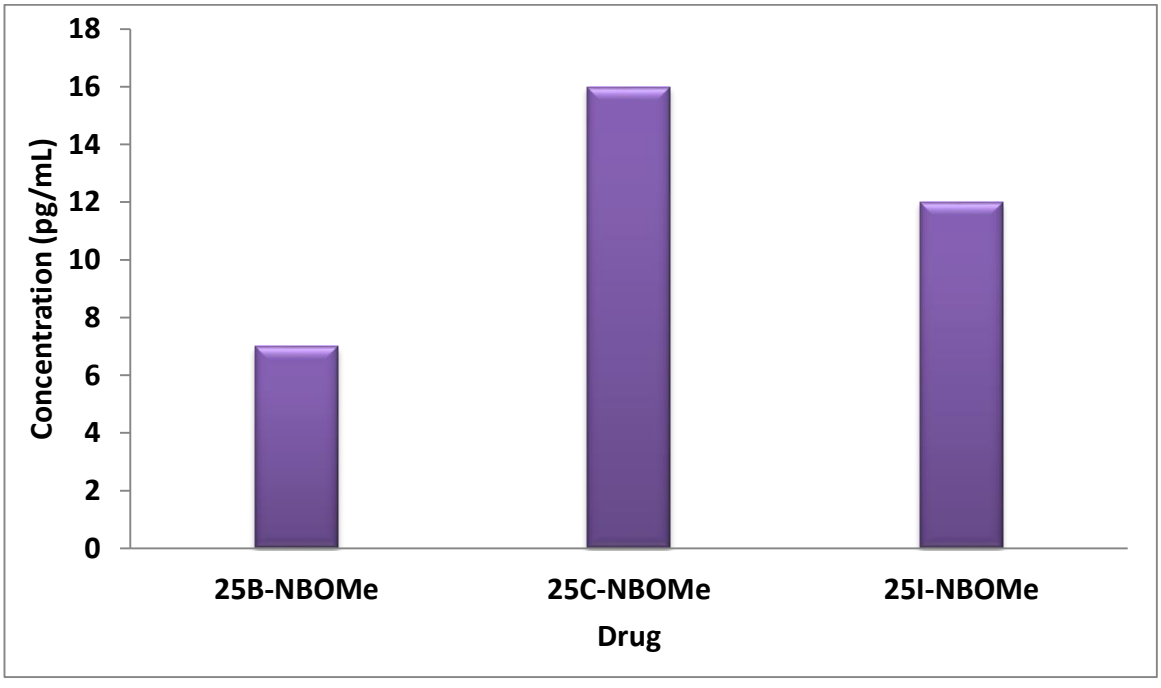


Figure 7-7: NBOMe concentrations detected in rat urine after dosing (300 µg/kg).

The urine samples were then re-injected, with peaks being observed from MRM transitions relating to demethylation at one site as shown in Table 7-1. In order

to confirm whether these “metabolite peaks” were due to metabolites themselves or to de-methylation taking place in the LC-MS/MS, 100 µl of 25B, 25C and 25I-NBOMe mixture (1 ng/mL) was evaporated and reconstituted in 75 µl of mobile phase. This was then analysed using the same method monitoring the demethylation ions as well as those of the parent. From this it is clear that demethylation of the parent compound is not occurring in the LC-MS/MS, as the reference standard compounds only produced peaks for the parent MRM transitions and not those associated with demethylation. This is shown in Figure 7-8 to Figure 7-10.

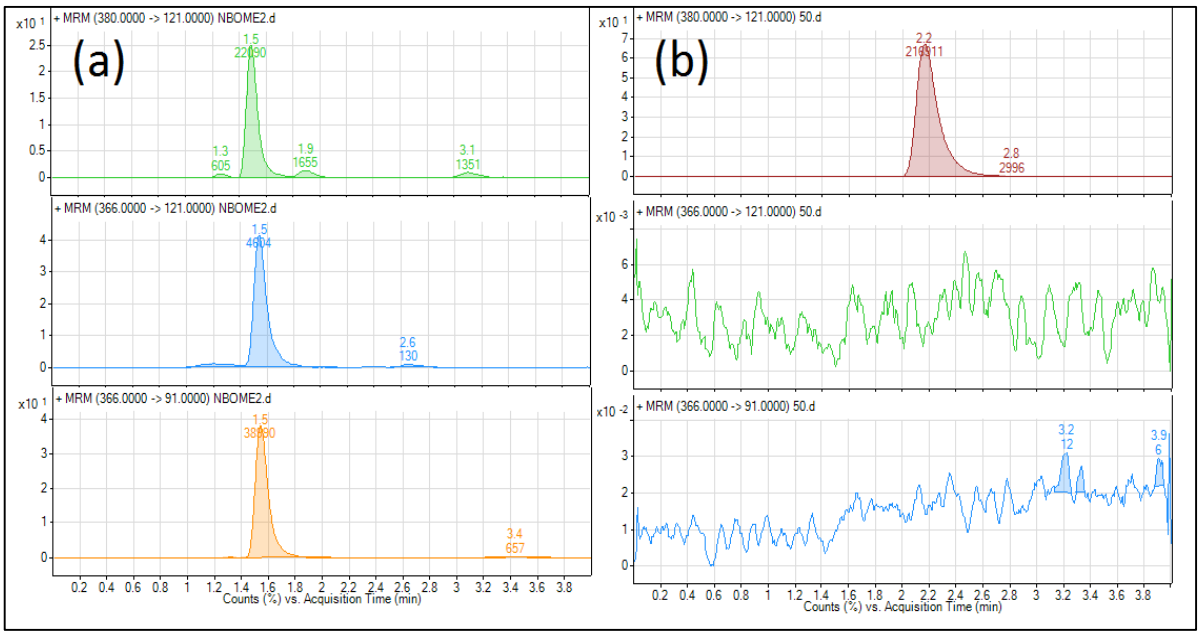


Figure 7-8: Chromatogram showing comparison of 25B-NBOMe rat urine sample (a), with 25B-NBOMe reference drug (b). The parent drug is shown in the top chromatogram with the metabolite peaks in the other 2.

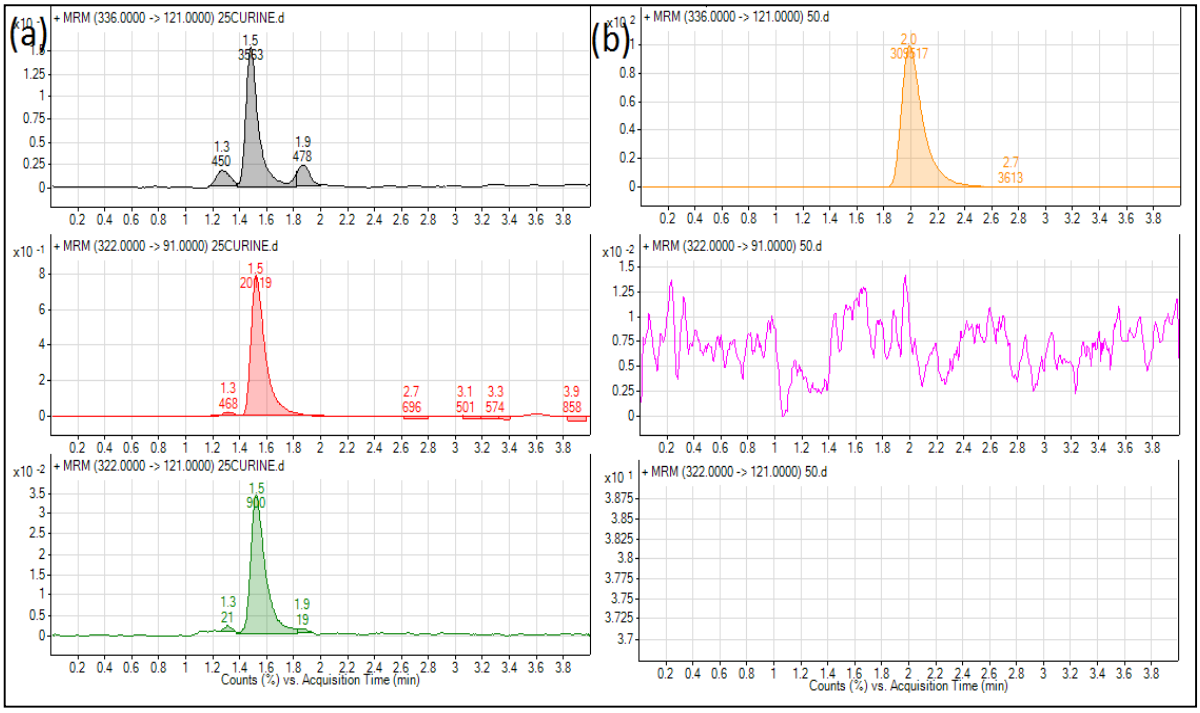


Figure 7-9: Chromatogram showing comparison of 25C-Urine rat sample (a), with 25C-NBOMe reference drug (b).
The parent drug is shown in the top chromatogram with the metabolite peaks in the other 2.

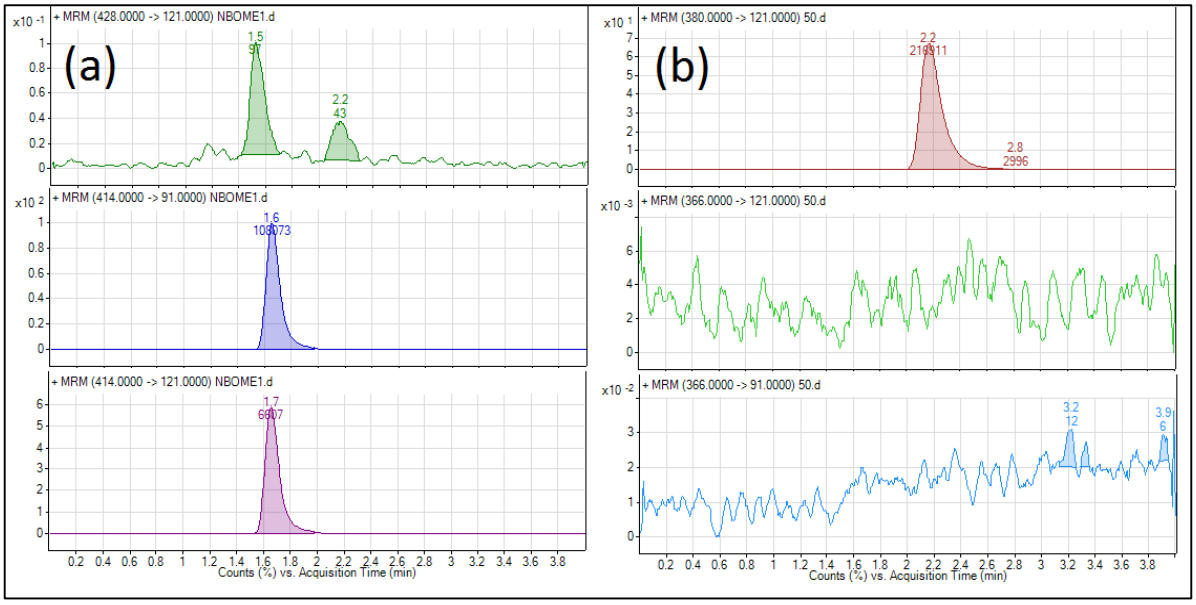


Figure 7-10: Chromatogram showing comparison of 25I-Urine rat sample (a), with 25I-NBOMe reference drug (b).
The parent drug is shown in the top chromatogram with the metabolite peaks in the other 2.

The urine samples were analysed by LC-QToF to determine if there were any additional metabolites which could be identified, however this was unsuccessful. The method was able to detect the parent compound in unextracted calibrations, but was not able to detect any analytes in the rat urine samples,

parent or metabolite. This could in part be due to a sensitivity issue, as previous analysis was completed using MS/MS which is a more sensitive analytical technique. The QToF instrument used in this project was 1st generation, and therefore not as sensitive as those currently on the market. It may be that a more up to date instrument would have been able to detect metabolites in these samples as instrument sensitivity has improved.

Due to the electronegative effects of the Br, Cl, or I atoms, it is predicted that *o*-demethylation would be occurring at the position meta on these atoms (a) however this cannot be confirmed without the use of additional techniques such as nuclear magnetic resonance. The o-demethylation is likely to occur at this position as the halogen group (B, C or I) would be able to stabilize the negative charge on the oxygen molecule.

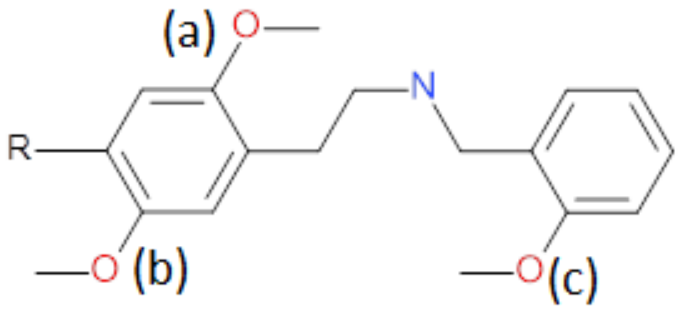


Figure 7-11: Chemical structure of 25X-NBOMe showing 3 sites for possible o-demethylation to take place (a-c).

7.4.4 Hair Samples

As predicted, the black hair incorporated all drugs to a higher degree than the white hair. A dose-dependent concentration increase was observed in the black hair as illustrated by Figure 7-12 to Figure 7-14.

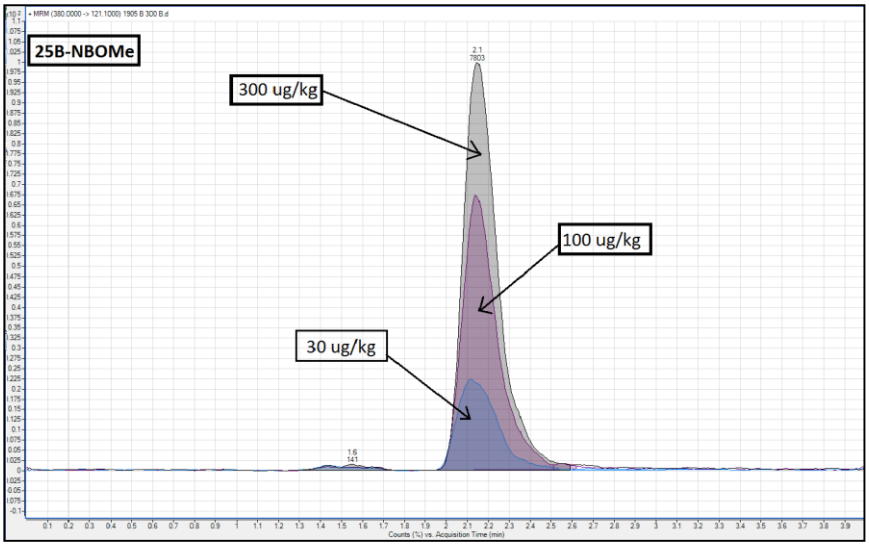


Figure 7-12: Overlaid chromatograms of black rat hair from each dose of 25B-NBOMe

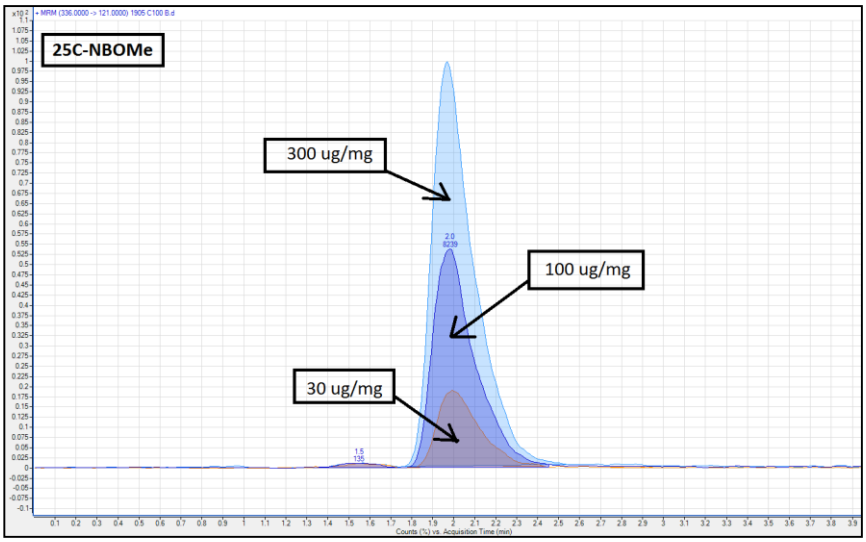


Figure 7-13: Overlaid chromatograms of black rat hair from each dose of 25C-NBOMe

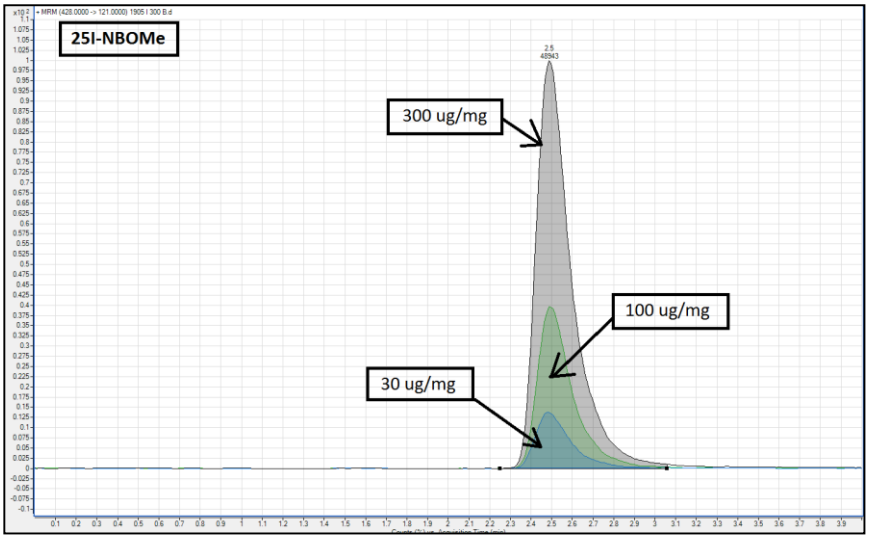


Figure 7-14: Overlaid chromatograms of black rat hair from each dose of 25I-NBOMe

25C-NBOMe incorporated into the black hair to the greatest extent for 300 ug/kg doses in comparison to 25B-NBOMe and 25I-NBOMe, +92%. 25C-NBOMe also incorporated into the hair of rats receiving the medium dose to the greatest degree, +10% in comparison to 25B-NBOMe and +37% in comparison to 25I-NBOMe. Of rats receiving the lowest dose of drug, hair from those administered 25B-NBOMe yielded the highest concentration of 37 pg/mg; 43% more than 25C-NBOMe rats and 62% more than 25I-NBOMe rats. Drugs were only detected and quantified in white hair from rats receiving the highest concentration of drug as illustrated in Table 7-2.

Concentrations marked with * fell below that of the level 1 calibrator (25 pg/mg) and thus were calculated using the LOQ calibration curve which was run on the same day.

Table 7-2: Concentration of each NBOMe detected in each hair sample.

Drug	Dose (ug/kg)	Black Hair		White Hair	
		Concentration (pg/mg)	STDEV (pg/mg)	Concentration (pg/mg)	STDEV (pg/mg)
25B-NBOMe	30	37	±3.3	Not Detected	
	100	51	±0.7	Not detected	
	300	92	±1.5	22*	±0.3
25C-NBOMe	30	21*	±2.8	Not detected	
	100	57	±3.3	11*	±1.0
	300	143	±1.0	30	±0.4
25I-NBOMe	30	14*	±1.6	Detected	
	100	36	±0.8	Detected	
	300	92	±4.3	Detected	

Overall, 25C-NBOMe> 25B-NBOMe > 25I-NBOMe when it came to the incorporation of each drug into black hair. This may be in part due to the electronegativity or size of the halogen ion on each NBOMe, with chlorine > bromine > iodine. Chlorine has already been shown to positively affect incorporation rates, although further work would need to be carried out in order to better assess this.(*253*)

The basicity of a drug is an important factor affecting the incorporation of drugs into hair. As the size of atoms increase their basicity decreases, and thus it follows that 25C-NBOMe incorporates greater than 25B-NBOMe and 25I-NBOMe.(*254*) These observations were seen regardless of hair colour with 25C-NBOMe being the only drug to be detected in white hair with a concentration above that of the level 1 calibrator.

The recovery of the method could also be responsible for this pattern as 25C-NBOMe extracted better from hair (averaging 95%) followed by 25B-NBOMe and then 25I-NBOMe. It is not known whether the buffer incubation step allowed for the extraction of each drug equally or that if it favoured the extraction of 25C-NBOMe. In order to determine whether each drug incorporated or extracted to a different degree further work would need to be carried out. A more powerful incubation solvent such as NaOH should be used to identify if this provides a

higher recovery rate and whether this impacts on the concentration of each drug detected.

The water and CH_2Cl_2 washes for each hair concentration were analysed as well as those from a blank rat hair sample. Both blanks (white and black) resulted in no detectable traces of NBOMes. DH_2O sample washes resulted in little to no NBOMe drugs detected (all distinguishable peaks had an area <500). This was not the case with the CH_2Cl_2 washes however, which did result in identification of NBOMes, although the PARs of these results were significantly lower than that of the level 1 calibrator, as shown in Figure 7-15.

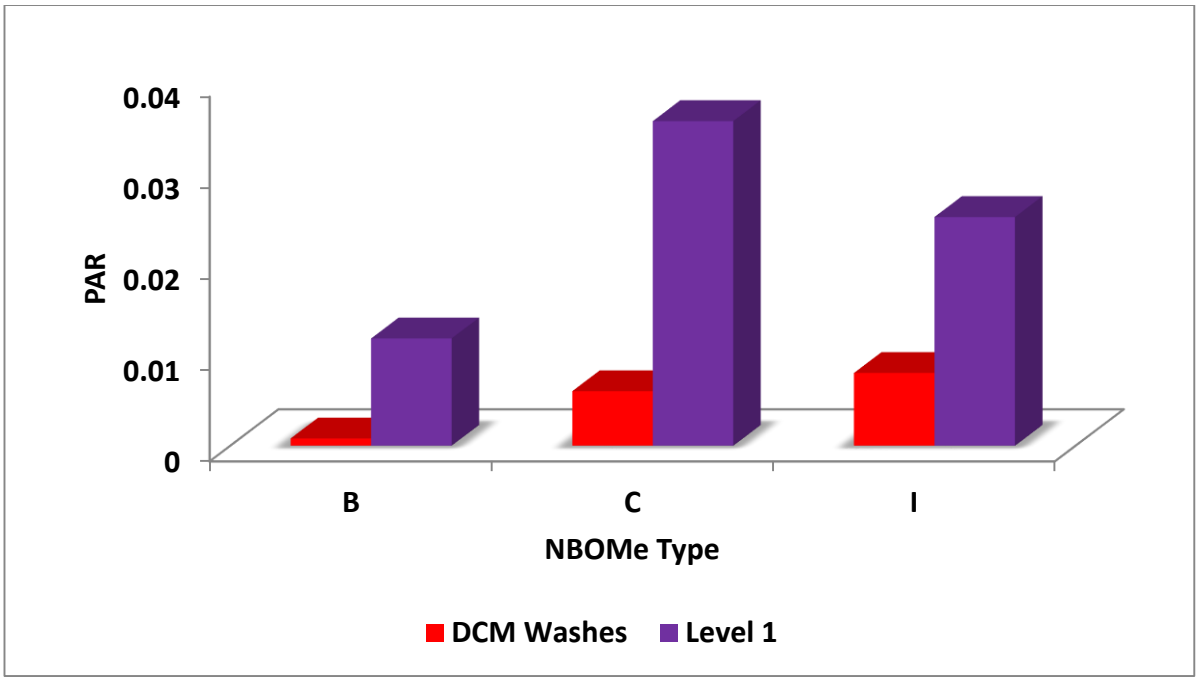


Figure 7-15: PARs of hair samples from rats dosed with 25B, 25C or 25I-NBOMe (300 µg/kg) and the PARs of a level 1 calibrator.

In order to assess whether the amount of NBOMe detected in the CH_2Cl_2 washes could be altered by the CH_2Cl_2 incubation time, 40 mg of hair from rats dosed with 25B-NBOMe (300 µg/kg) was initially washed with dH_2O before washing with CH_2Cl_2 for 15, 30 and 45 minutes under sonication. This was done in duplicate and the PAR of each was calculated. The results are shown below in Figure 7-16, alongside the PAR of the 25B-NBOMe rat sample following extraction. From this we can see that although 25B-NBOMe was detected in the washes, its PAR was approximately 20 times higher in the sample. We can also see that sonication of samples for different time periods did not affect the amount of 25B-NBOMe

detected in the washes, indicating that only surface contamination was removed.

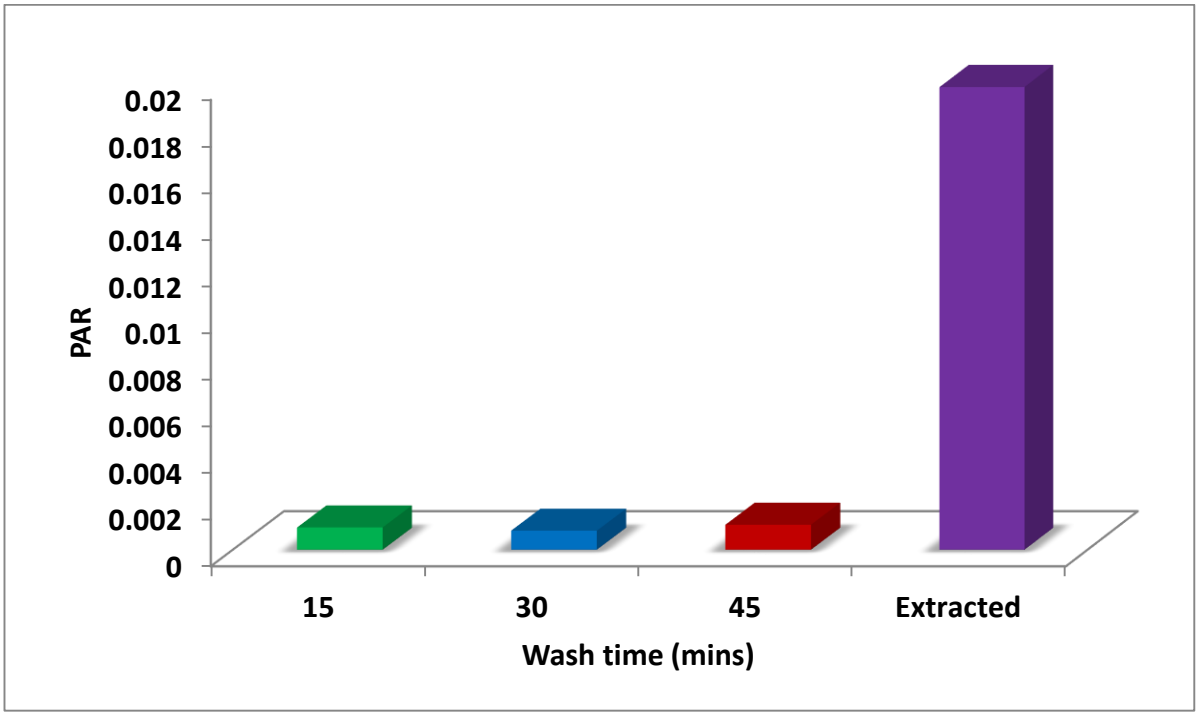


Figure 7-16: PAR of 25B-NBOMe rat hair samples washed for 15, 30 and 45 minutes and the amount found in the hair sample following extraction.

7.5 Conclusion

There was no analgesia observed at the doses given to the rats and as a result tolerance and dependence could not be assessed using the tail flick method. In order to better assess whether these drugs cause analgesia, tolerance and dependence higher concentrations would have to be used applying additional techniques.

Black hair concentrations ranged from 37-92 pg/mg for rats dosed with 25B-NBOMe, 31-143 pg/mg for rats dosed with 25C-NBOMe and 14-92 pg/mg for rats dosed with 25I-NBOMe. White hair from rats administered 25B-NBOMe was only positive from those receiving the highest dose (300 µg/kg), with 22 pg/mg detected. White hair from rats dosed with 25C-NBOMe was positive from animals receiving the medium and high doses (100 µg/kg and 300 µg/kg) with 11 pg/mg and 30 pg/mg detected respectively. All white hair from rats receiving 25I-NBOMe tested positive, but all were below the LOQ for this method. Although this indicates that each NBOMe incorporated into the black hair much more than

white, this cannot be confirmed without further testing on the extraction method itself.

The incorporation of each NBOMe improved with the basicity of each drug, the size, and electronegativity of the halogen group.

Urine NBOMe concentrations ranged from 7-16 pg/mL from rats administered doses of 300 µg/kg. A possible *o*-demethylated metabolite was identified in each sample, although this was not confirmed due to low concentrations detected.

7.6 Future Work

This research should be repeated using similar doses to those now published in the literature after stability studies have been conducted with these substances in DMSO and saline. Additional NBOMes should also be investigated, in particular mescaline-NBOMe as recreational doses for this substance are reported to be much higher than other NBOMes.(*133*) To date there have been no published mescaline-NBOMe case reports to support this claim and thus the use of animal models, and tail flick testing could help determining the accuracy of these remarks. Repeating this study at higher doses would also allow further investigations into potential dependency of the drug, establishing the length of time it took for tail-flick times to return to their baseline values.

The identification of a possible metabolite in each of the urine samples should be further investigated, with the development and enhancement of a QToF method or if standards are developed. It may be that as the rats received such small amounts of drug that human urine from case samples may facilitate the identification of metabolites better as concentrations would be higher. Alternatively, the use of human liver microsomes could be used to identify metabolites.

All rats used in this experiment were male, which again is another possible area of further work as previous publications have shown gender to be an important factor affecting the incorporation of drugs into hair.(*253, 255-257*)

The study of additional NBOMe compounds and their incorporation rates into hair would not only further the information available on NBOMe compounds but would allow further investigation into the effects different functional groups have on this process.

Chapter 8: Conclusion & Future Work

8.1 Conclusion

NPS pose a significant challenge to forensic toxicologists and clinical practitioners alike. It is difficult to keep up to date with what is currently being used within the recreational market, and by the time analysts are aware of the current substances these will have no doubt been replaced. The variation of substances between countries and regions makes the sharing of information much more difficult, as what is contained within a branded product in one geographical area may be completely different in another area. As a result, getting accurate information to law enforcement, toxicologists, health professionals and users is much more difficult.

A GC-MS method for the simultaneous quantification of 25 analytes in urine and 23 analytes in blood was successfully developed and validated. This method utilised SPE for sample clean-up and PFPA:EtOAc derivatisation. This method was validated to SWGTOX 2013 guidelines. The GC-MS was operated in SIM mode to enhance sensitivity, with a lowered injection port temperature of 225^{o}C to prevent analyte degradation at the injection port.

The extraction of various NPS substances from urine, blood, plasma and serum using a variety of cartridges was assessed. Cleanscreen CSDAU133 cartridges provided the best recovery rates for blood and urine whereas Cleanscreen ZSDAU020 cartridges proved to be the optimum for plasma and serum samples. The extraction efficiency of two LLE methods was also evaluated; however neither method recovered all analytes from urine. As the GC-MS method looked for 10 and 8 NBOMe compounds in urine and blood respectively, instrument sensitivity was paramount. As LLE is a relatively crude extraction method, and as this instrument was used by more than one individual, it was only applied to urine samples. SLE was also investigated for the extraction of NPS from blood, urine, serum and plasma. This method extracted 68% of analytes to a greater extent than the CSDAU0133 SPE method. The CSDAU133 SPE cartridge performed better for the extraction of NPS from urine with 64% of analytes achieving higher recovery rates using this method.

The developed method was applied to 12 blood samples from 8 different cases obtained from NMS labs, Willow Grove, PA. These samples were found to contain 25C-NBOMe, 25I-NBOMe, methoxetamine and methylone. Previous analysis, including the NMS labs method, for NBOMes has utilised LC-MS/MS due to the high potency of these substances and therefore the low concentrations identified within clinical and forensic laboratories. Although this GC-MS method was not able to identify NBOMes in all 8 samples confirmed positive by NMS laboratories, it was possible to identify and quantitate these substances in 5 samples. It is not known if this was due to a lack of sensitivity of the method or due to issues with the long term stability of the NBOMes in question.

The stability of mephedrone samples in bovine blood was assessed over a period of ten weeks. Bovine blood was spiked at 1 mg/L and stored at room temperature (20^{o}C), in the fridge (4^{o}C) or in the freezer (-20^{o}C). Samples were individually aliquoted to prevent any effects from freeze thaw cycles. The effect of preservatives was also investigated with samples stored unpreserved, with citrate preservative or with fluoride/oxalate preservative. Samples stored at room temperature were stored in the dark to mimic the conditions of the fridge and freezer and thus avoiding the additional variable of light. Samples were analysed daily for 1 week and then weekly for the remaining time period. This work identified that mephedrone samples should be stored at -20^{o}C with the addition of a citrate preservative. Mephedrone could not be detected in unpreserved samples stored at room temperature after 21 days.

The cross reactivity of NPS analytes with amphetamine, methamphetamine and ketamine ELISA kits was assessed. Cross reactivity was identified for 5-APB, 6-APB, butylone, ethylone, flephedrone, mephedrone, methedrone and methylone using the methamphetamine kit. Cross-reactivity was also seen when using the amphetamine kit for 5-APB, 6-APB, ethylone, mephedrone, methedrone and methylone, however this was to a lesser extent. Methoxetamine, 3-MeO-PCE and 3-MeO-PCP all cross reacted with the ketamine ELISA kit.

Another screening technique used in clinical settings is the POCT. POCT cross-reactivity is important as many NPS users will come into contact with A&E services and thus correct identification of the substances people have ingested can be crucial for their treatment. Three different POCT were examined,

WorldCassette™, DRUGCHECK® and Nal von Minden. Cross reactivity using each of the POCT was seen when using 1 mg/mL concentrations, however these are far higher than have been reported in the literature and so cross reactivity in a "real-life" setting would be unlikely.

A LC-MS/MS method for the simultaneous detection and quantification of 25B-, 25C- and 25I-NBOMe in urine and hair was established and validated using SWGTOX 2013 guidelines. This method covered a wide concentration range for forensic and clinical application. Urine samples were extracted using a simple NaOH and hexane:EtOAc LLE using 1 mL of sample. Hair samples were extracted using SPE after a 12-hour incubation in pH 7.4 phosphate buffer. Total instrument run-time was 4 minutes. As NBOMe compounds are so potent, hair concentrations are expected to be extremely low. As a result 40 mg of hair was used, which allowed the instrument to detect concentrations as low as 3-5 pg/mg.

No analgesic effects were seen after Long Evans rats were administers 25B-, 25C- or 25I-NBOMe at doses of 30, 100 or 300 µg/kg over an 8-day period. Urine collected from rats receiving 300 µg/kg doses of each NBOMe tested positive using the previously mentioned LC-MS/MS method, with concentrations ranging from 7-12 pg/mL. The *o*-demethylated metabolites of each NBOMe were also identified in these urine samples. White and black hair from the rats was also collected and analysed by LC-MS/MS. All black hair samples tested positive for the presence of NBOMes regardless of the dose with concentrations identified ranging from 21-143 pg/mg. 25I-NBOMe was identified in white hair for all doses although concentrations fell below the method LOQ. 25B-NBOMe was only identified in rats receiving the 300 µg/kg dose (22 pg/mg) and 25C-NBOMe was only identified in rats dosed at 100 µg/kg (11 pg/mg) and 300 µg/kg (30 pg/mg). A dose response relationship was seen with all black hair samples.

Although it is likely that laboratories will move towards new instrumentation for the detection of NPS such as UPLC-QToF, this thesis highlights that they can be detected using current equipment. In particular, the identification of NBOMes using GC-MS is important as although it was not able to detect the presence of these substances in all samples tested, it was able to identify them in 62.5% of samples. This is important in the case of developing nations and small

laboratories which may not see NPS frequently enough to warrant large investments into new instrumentation.

8.2 Future Work

The constant influx of new substances onto the recreational market means that the GC-MS and LC-MS/MS methods discussed here will need to be updated and re-validated to SWGTOX guidelines to include these. It is likely in the future that screening instruments such as UPLC-QToF will replace GC-MS and LC-MS/MS for the identification of NPS and that these instruments will be used for more targeted analysis only.

The optimisation of the SLE extraction procedure should occur to ensure that a fair comparison between SPE and SLE takes place. This involves looking at cartridges from different manufactures, and using different elution solvents which did not occur here due to the limited number of cartridges supplied by Biotage®. Examining other SPE cartridges as and when new versions are launched should also take place to ensure that CSDAU cartridges remain the optimum cartridge for the extraction of the analytes examined in this thesis.

The LC-MS/MS urine and hair method should be expanded to include more NBOMes which are now commercially available. These methods should also be applied to case samples to ensure their applicability to “real-life” scenarios. This is particularly important for the LC-MS/MS hair method as the concentrations in hair remain unknown. It may be that the calibration levels in the hair method need adjusted as and when this information becomes available.

Further work on the analgesic properties of NBOMes should also take place. In particular comparing the doses of mescaline-NBOMe to identify if the trip reports are correct in stating higher doses of this substance are required to obtain recreational effects.

Finally, both the GC-MS and LC-MS/MS method should be used to assess the current prevalence of these substances within the living population, ensuring that the analytes included in these methods are still seen in forensic and clinical work. There is a financial implication in looking for many analytes at once as

these need to be included in calibrators and QCs. It may be that although this method is able to detect and quantify these substances, they appear too infrequently to all be included in routine analysis.

Chapter 9: Reference List

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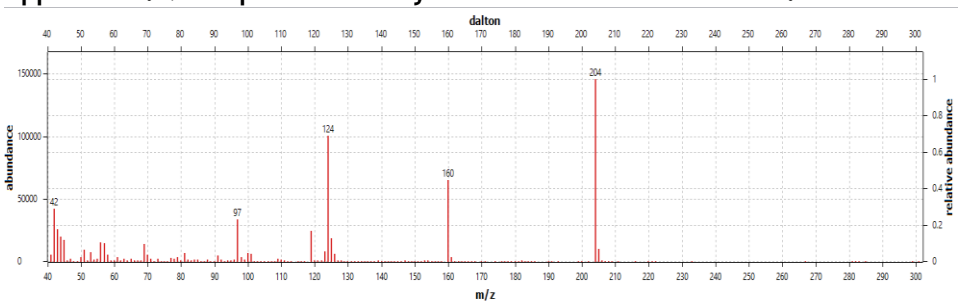
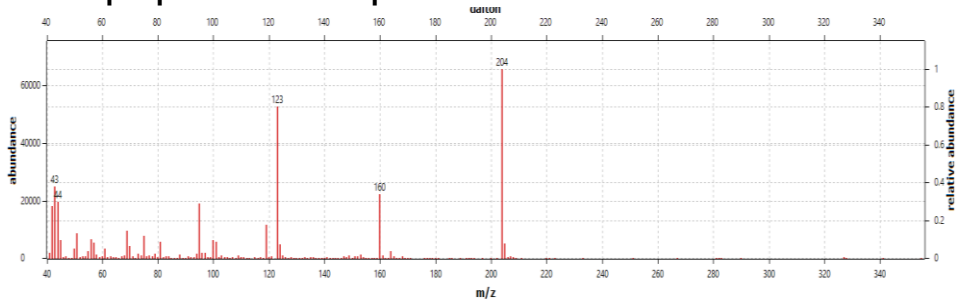
Appendices

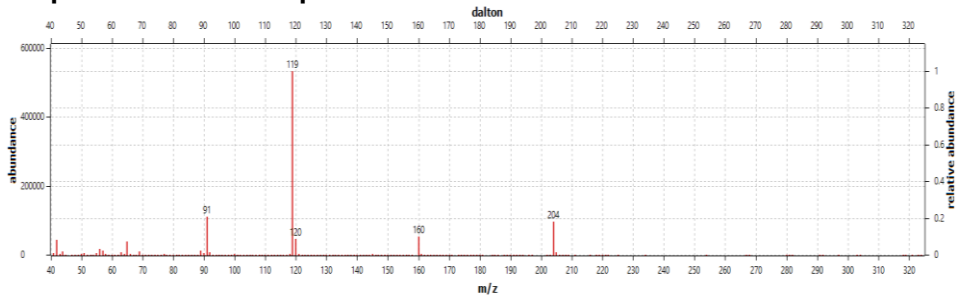
Appendix 1: Mass spectra of analytes after PFPA derivatisation.



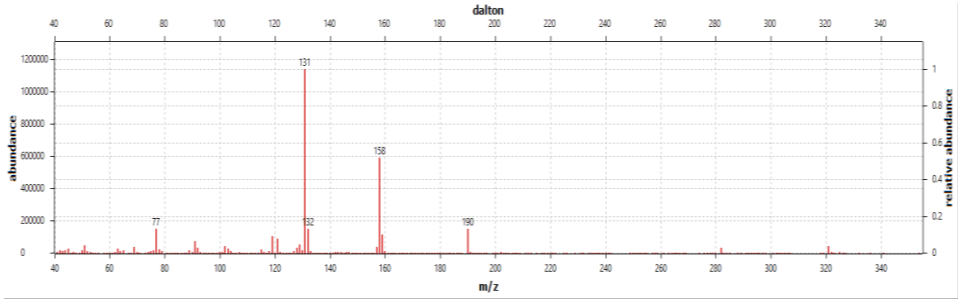
Methiopropamine mass spectrum after PFPA derivatisation



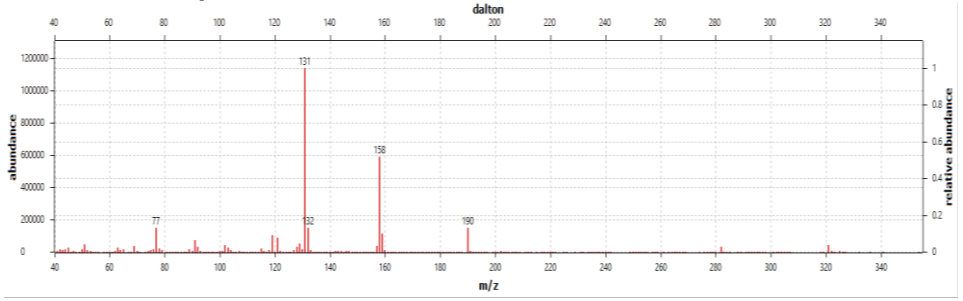
Flephedrone mass spectrum after PFPA derivatisation



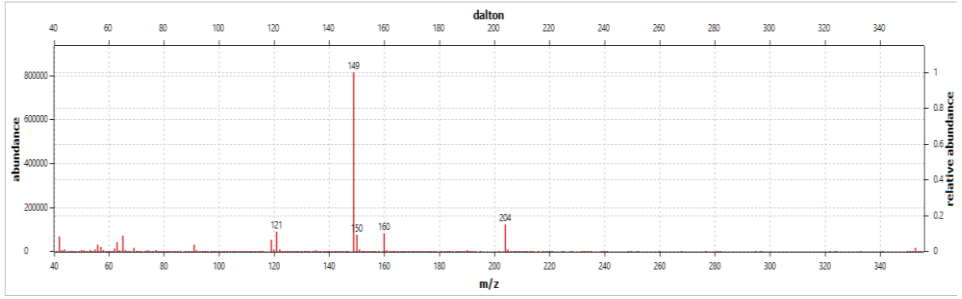
Mephedrone mass spectrum after PFPA derivatisation



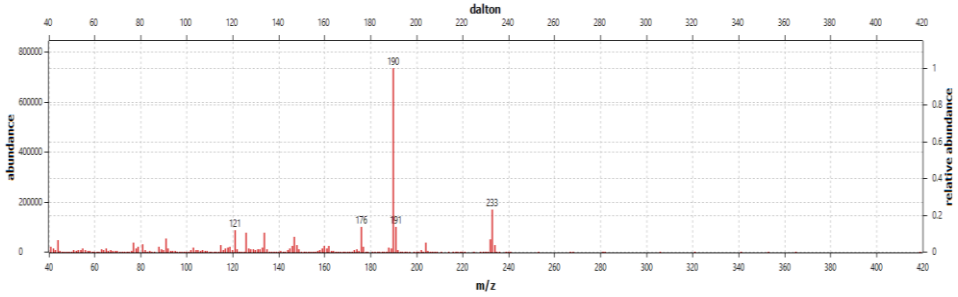
5-APB mass spectrum after PFPA derivatisation



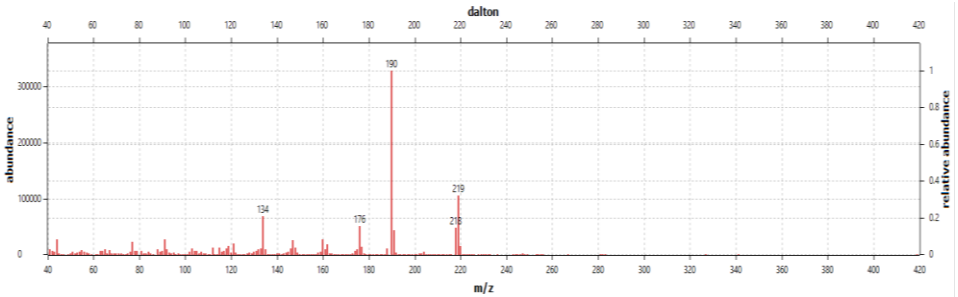
6-APB mass spectrum after PFPA derivatisation



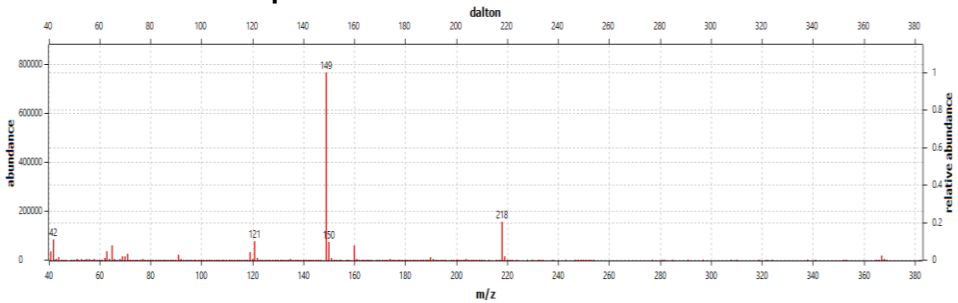
Methylone mass spectrum after PFPA derivatisation



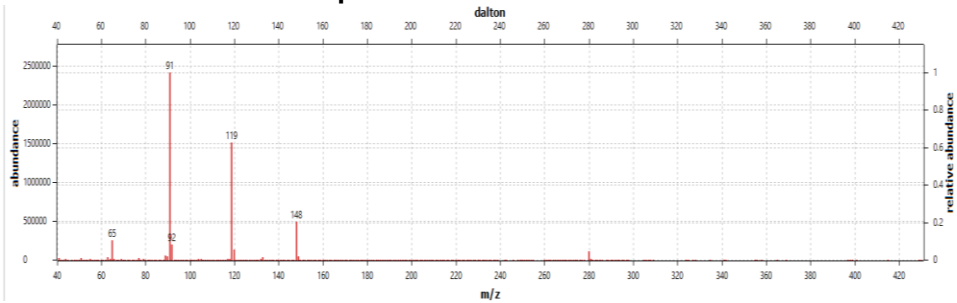
3-MeO-PCE mass spectrum after PFPA derivatisation



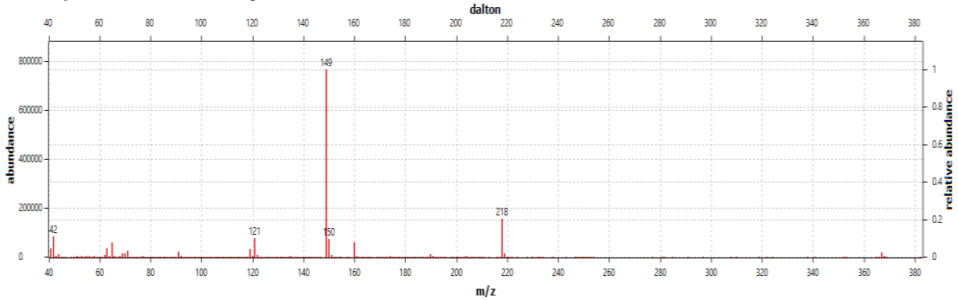
Methoxetamine mass spectrum after PFPA derivatisation



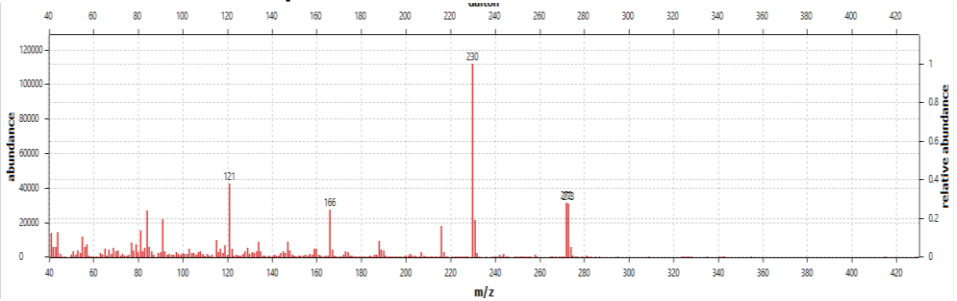
Butylone mass spectrum after PFPA derivatisation



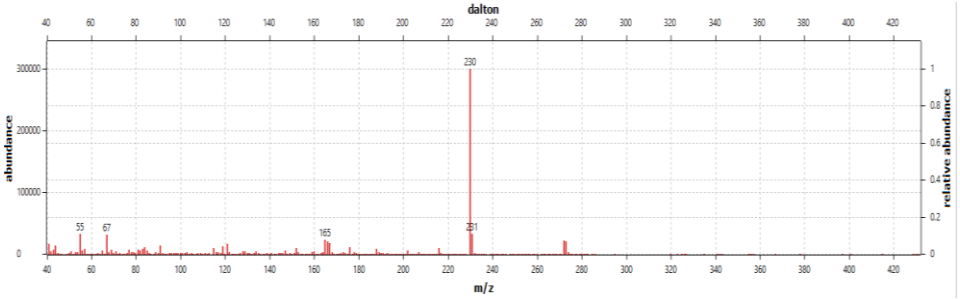
Benzedrone mass spectrum after PFPA derivatisation



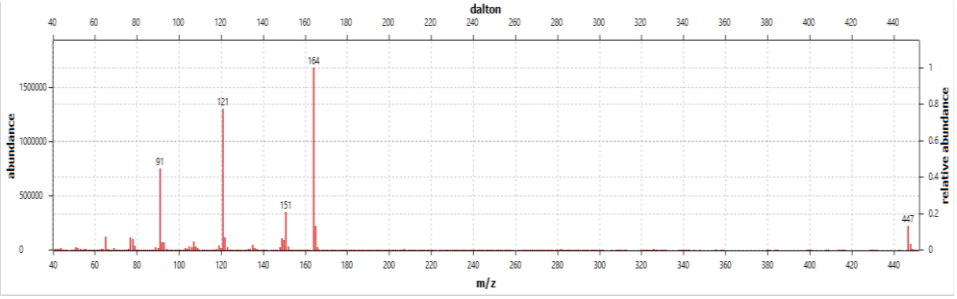
Ethylone mass spectrum after PFPA derivatisation



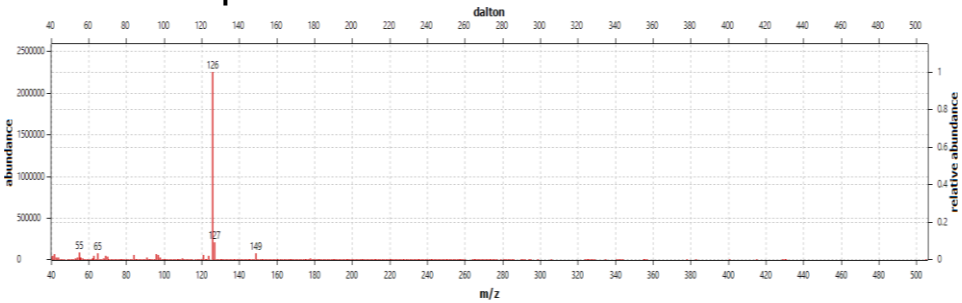
3-MeO-PCP mass spectrum after PFPA derivatisation



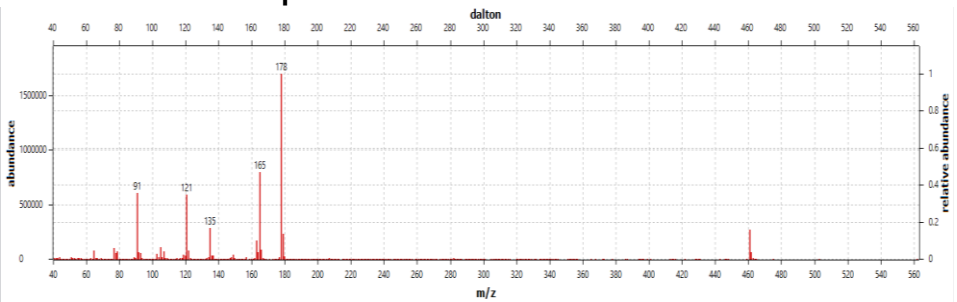
2-DPMP mass spectrum after PFPA derivatisation



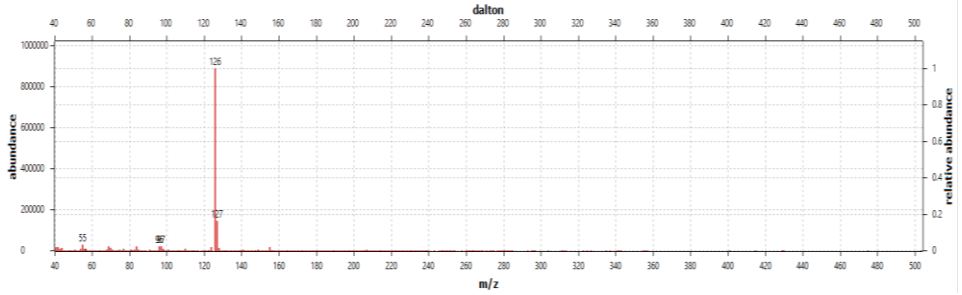
25H-NBOMe mass spectrum after PFPA derivatisation



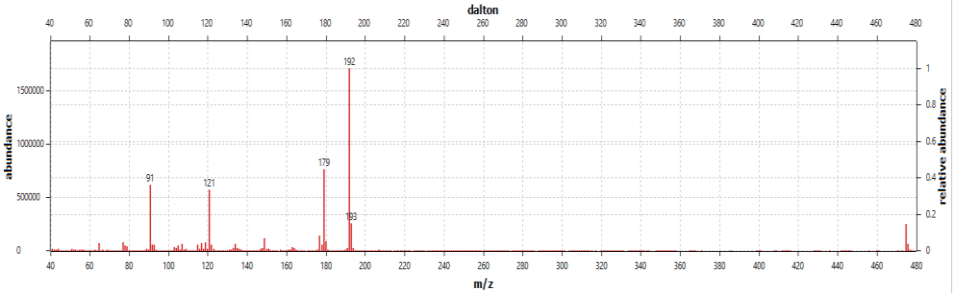
MDPV mass spectrum after PFPA derivatisation



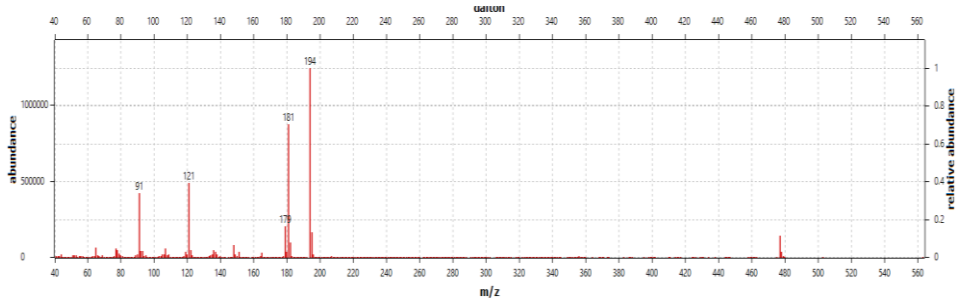
25D-NBOMe mass spectrum after PFPA derivatisation



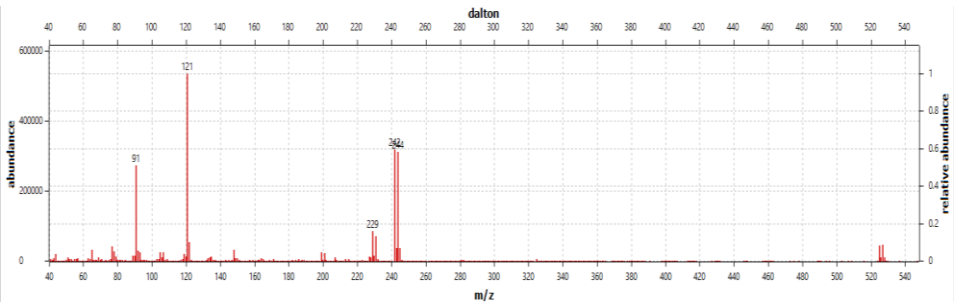
Naphyrone mass spectrum after PFPA derivatisation



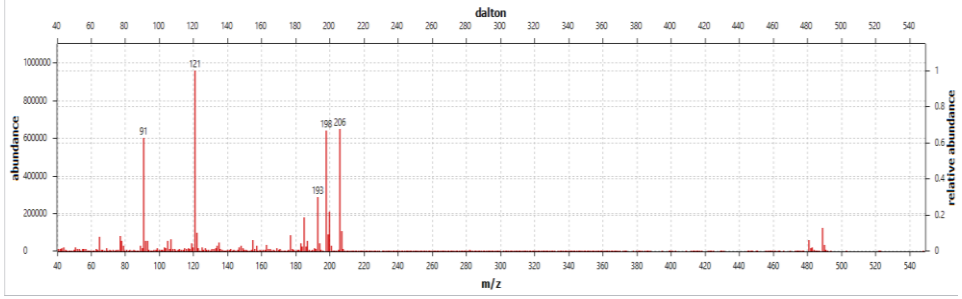
25E-NBOMe mass spectrum after PFPA derivatisation



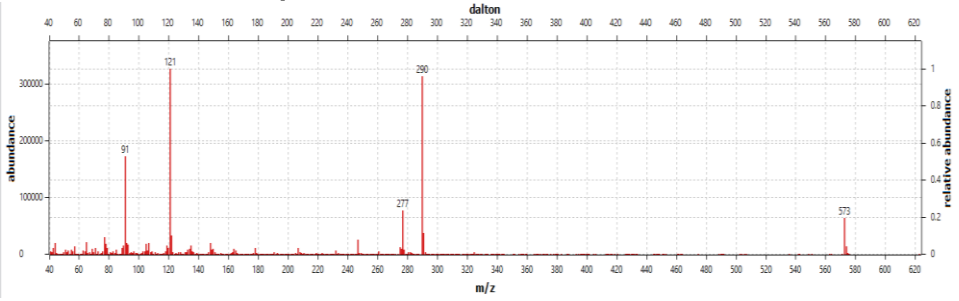
Mescaline-NBOMe mass spectrum after PFPA derivatisation



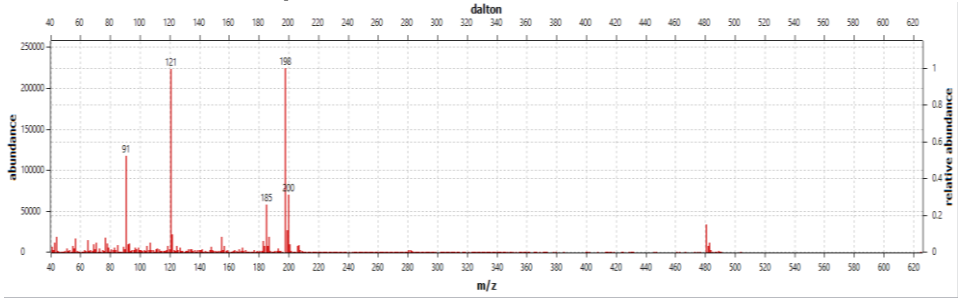
25B-NBOMe mass spectrum after PFPA derivatisation



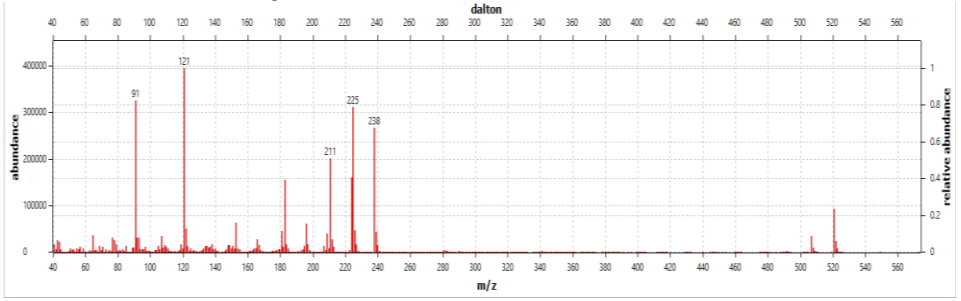
25P-NBOMe mass spectrum after PFPA derivatisation



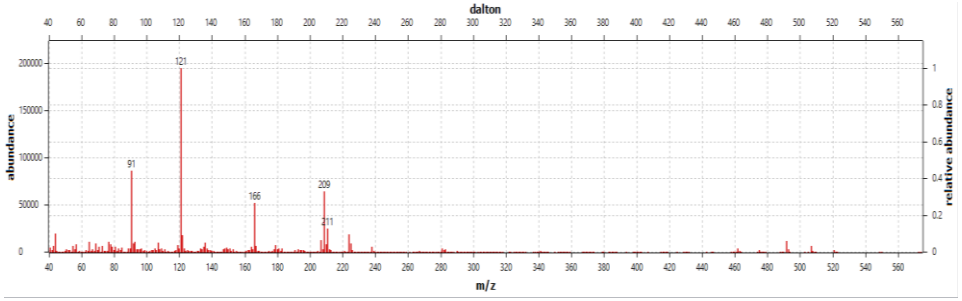
25I-NBOMe mass spectrum after PFPA derivatisation



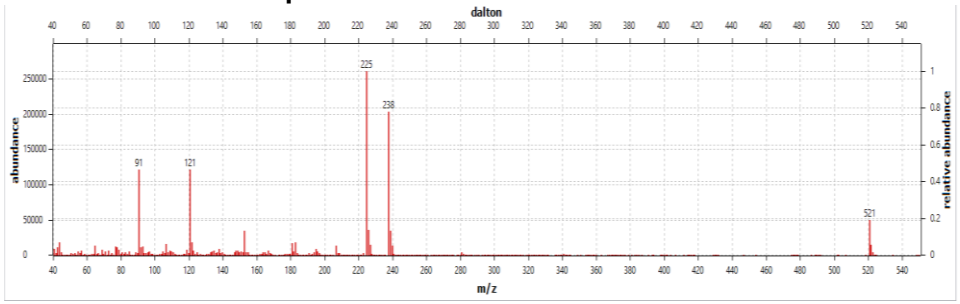
25C-NBOMe mass spectrum after PFPA derivatisation



25T2-NBOMe mass spectrum after PFPA derivatisation

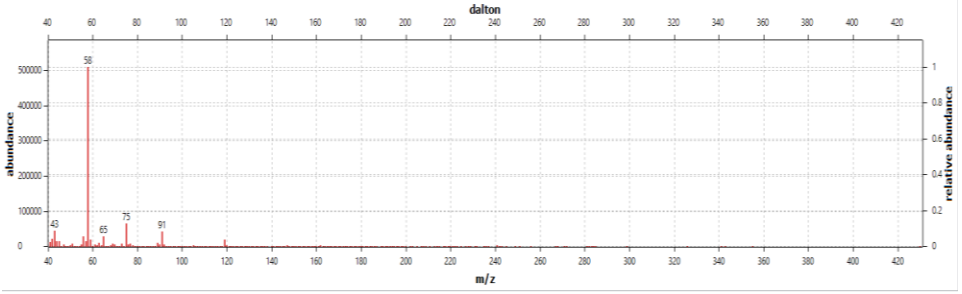


25N-NBOMe mass spectrum after PFPA derivatisation

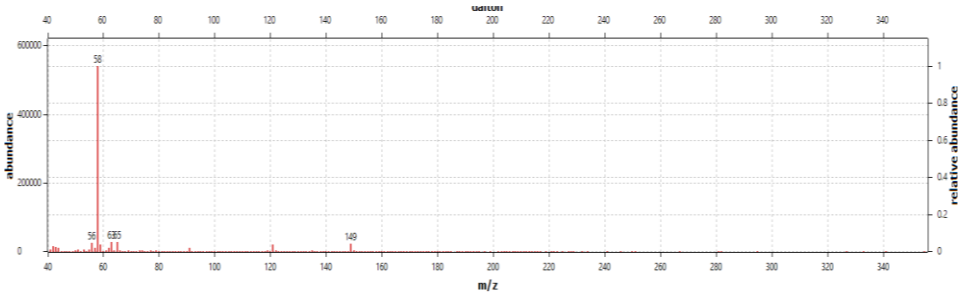


25T7-NBOMe mass spectrum after PFPA derivatisation

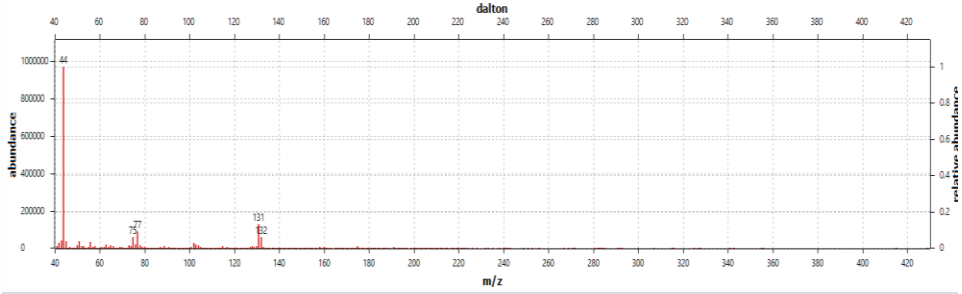
Appendix 2: Mass spectra of analytes after MSTFA derivatisation.



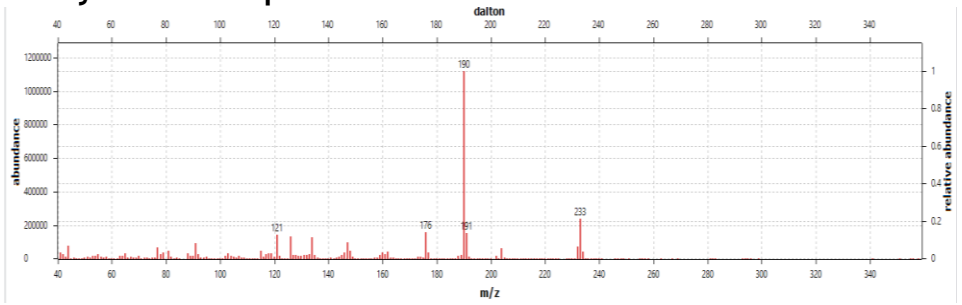
Mephedrone mass spectrum after MSTFA derivatisation



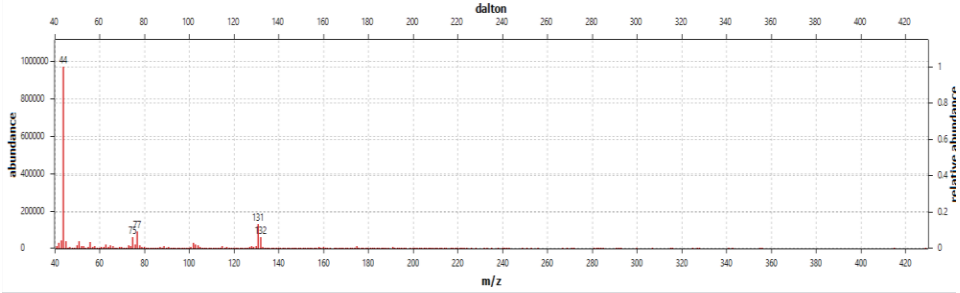
Methylone mass spectrum after MSTFA derivatisation



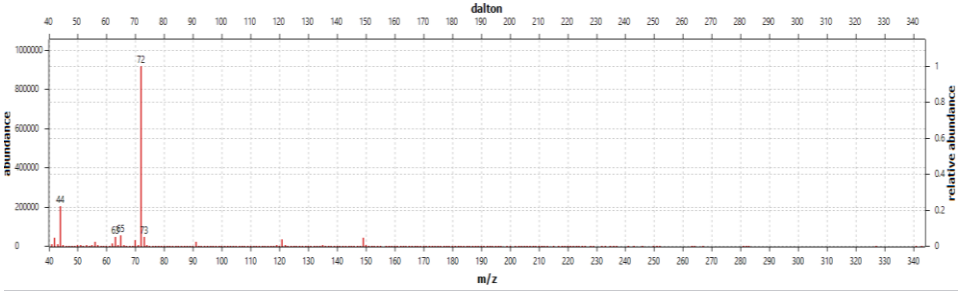
5-APB mass spectrum after MSTFA derivatisation



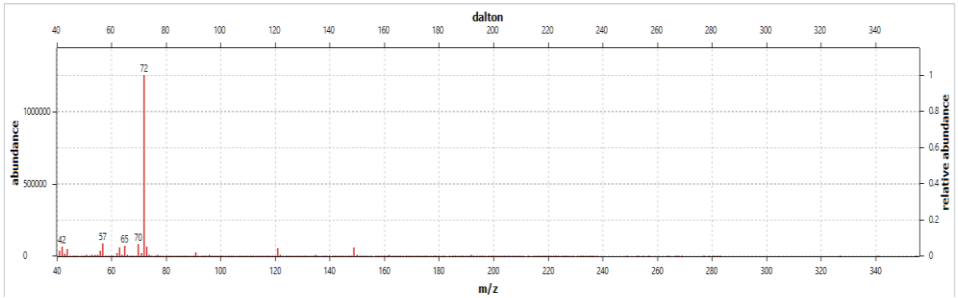
3-MeO-PCE mass spectrum after MSTFA derivatisation



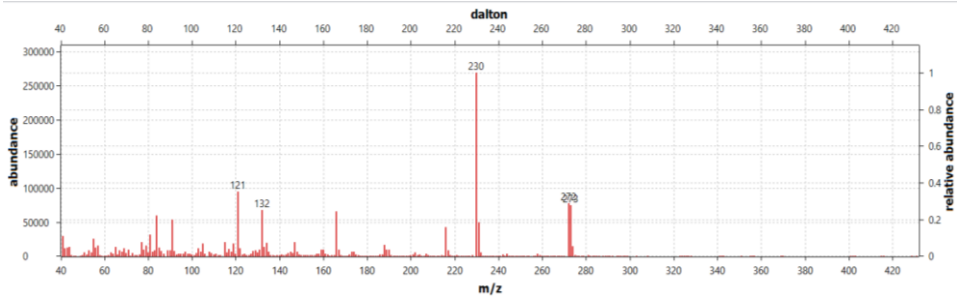
6-APB mass spectrum after MSTFA derivatisation



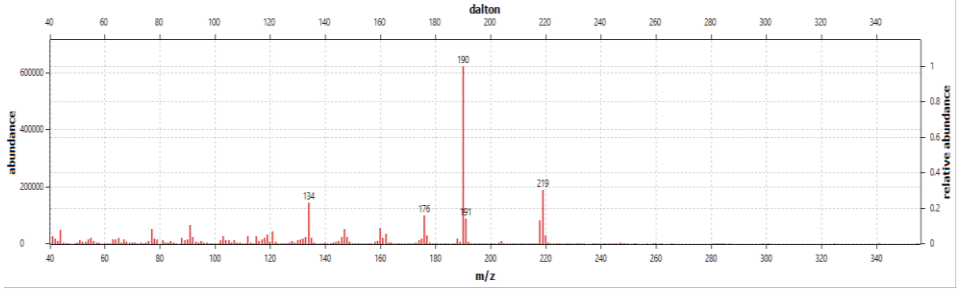
Butylone mass spectrum after MSTFA derivatisation



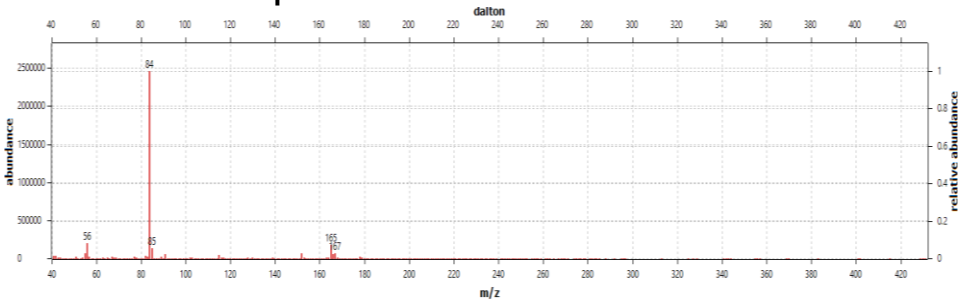
Ethylone mass spectrum after MSTFA derivatisation



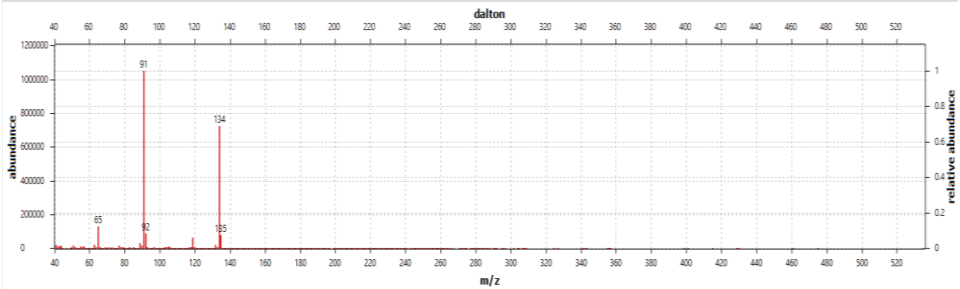
3-MeO-PCP mass spectrum after MSTFA derivatisation



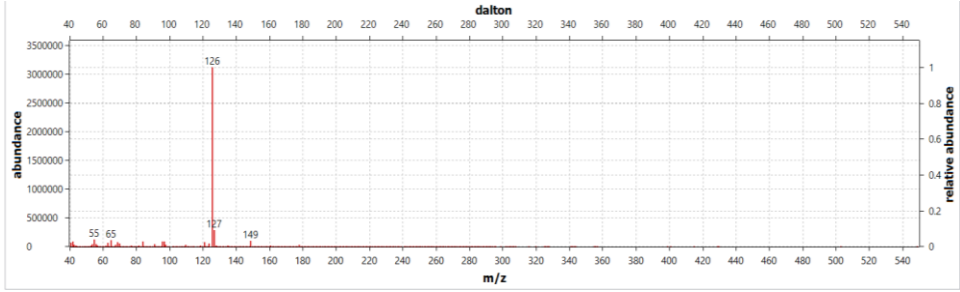
Methoxetamine mass spectrum after MSTFA derivatisation



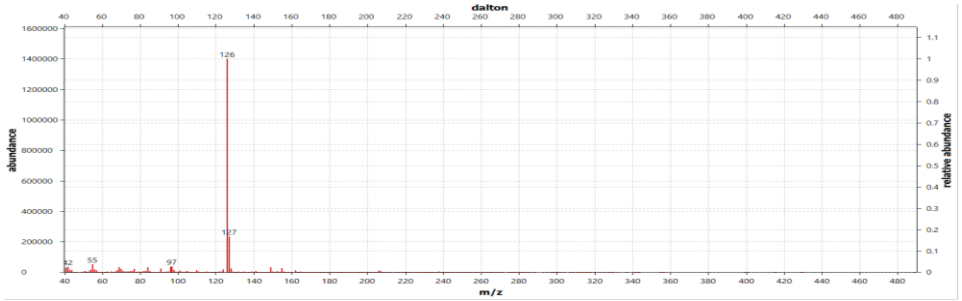
2-DPMP mass spectrum after MSTFA derivatisation



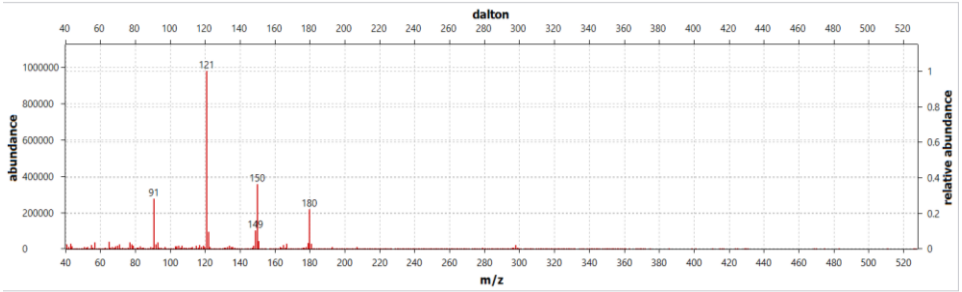
Benzedrone mass spectrum after MSTFA derivatisation



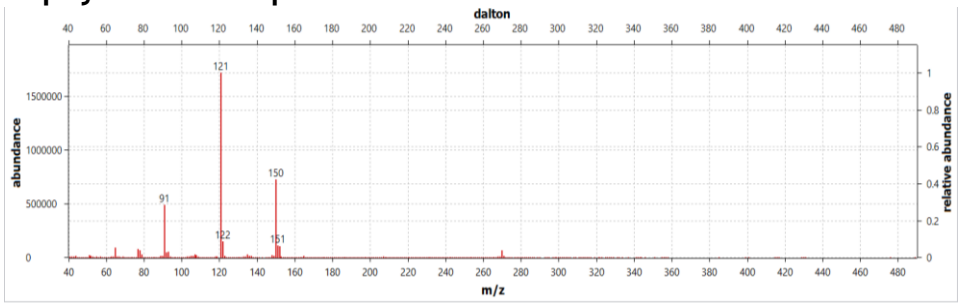
MDPV mass spectrum after MSTFA derivatisation



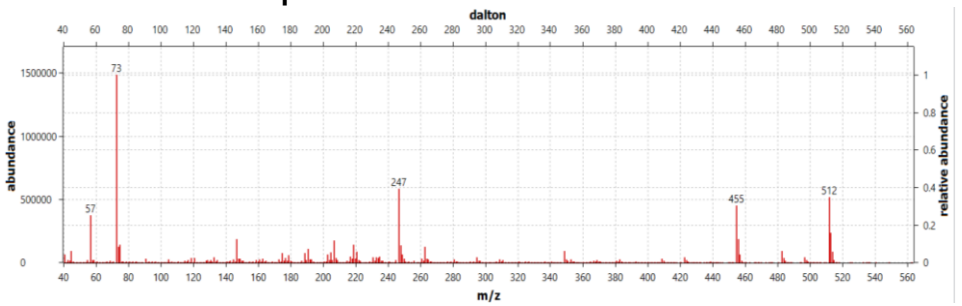
Naphyrone mass spectrum after MSTFA derivatisation



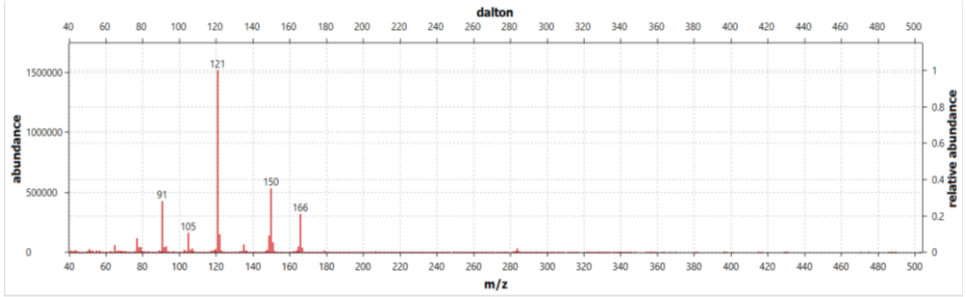
25E-NBOMe mass spectrum after MSTFA derivatisation



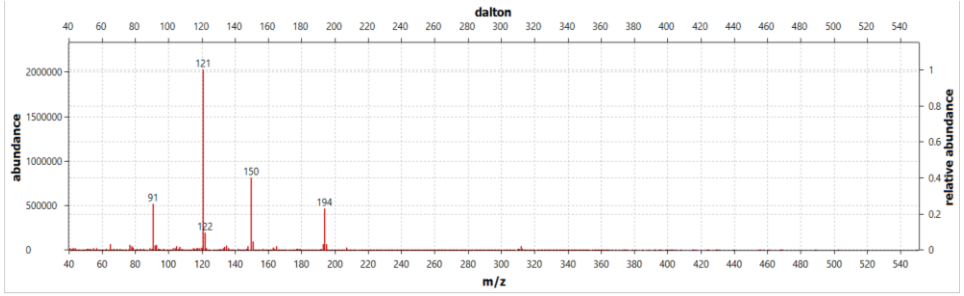
25H-NBOMe mass spectrum after MSTFA derivatisation



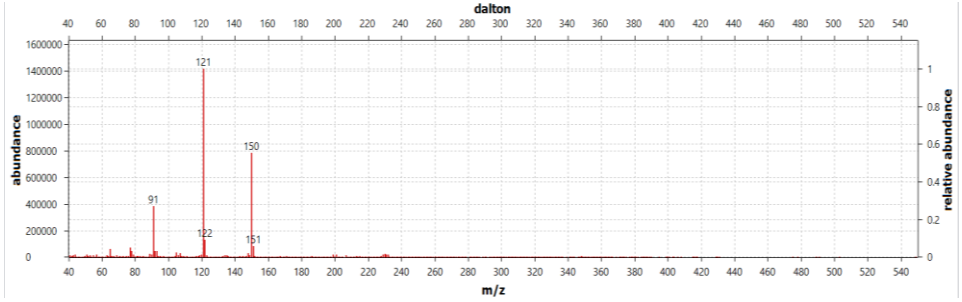
Mescaline-NBOMe mass spectrum after MSTFA derivatisation



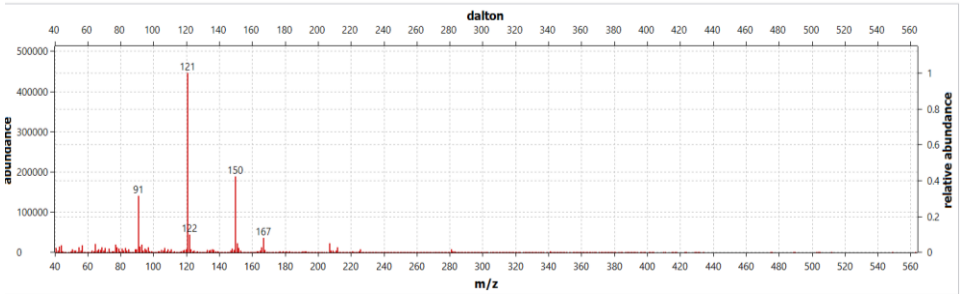
25D-NBOMe mass spectrum after MSTFA derivatisation



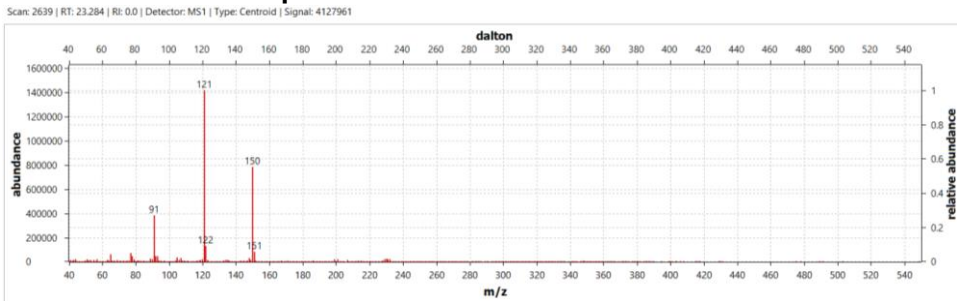
25P-NBOMe mass spectrum after MSTFA derivatisation



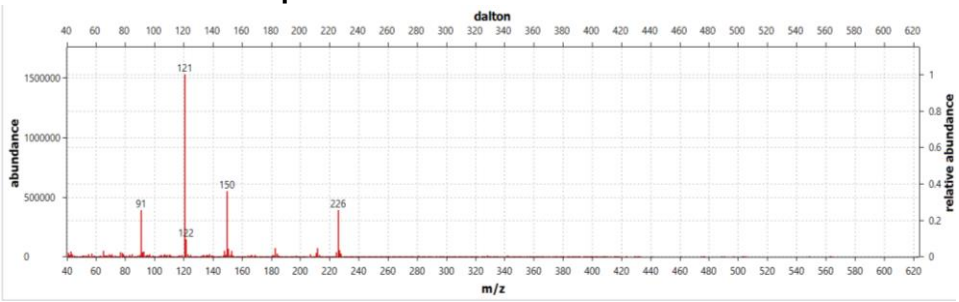
25C-NBOMe mass spectrum after MSTFA derivatisation



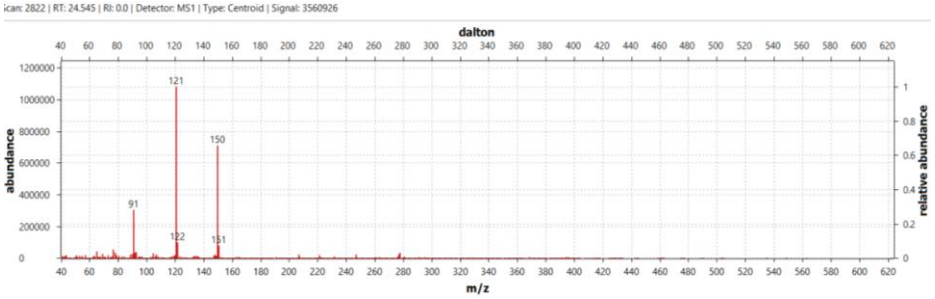
25N-NBOMe mass spectrum after MSTFA derivatisation



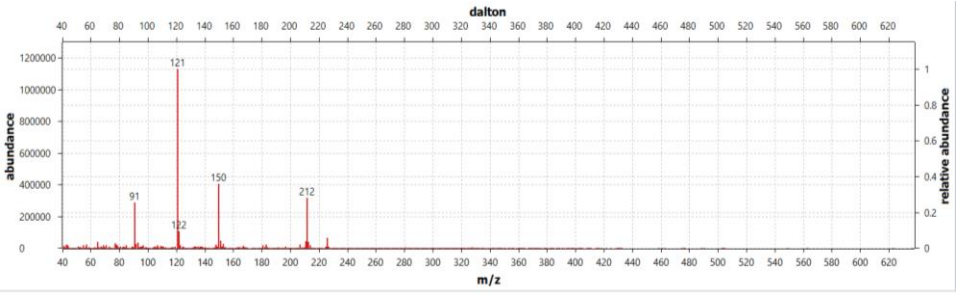
25B-NBOMe mass spectrum after MSTFA derivatisation



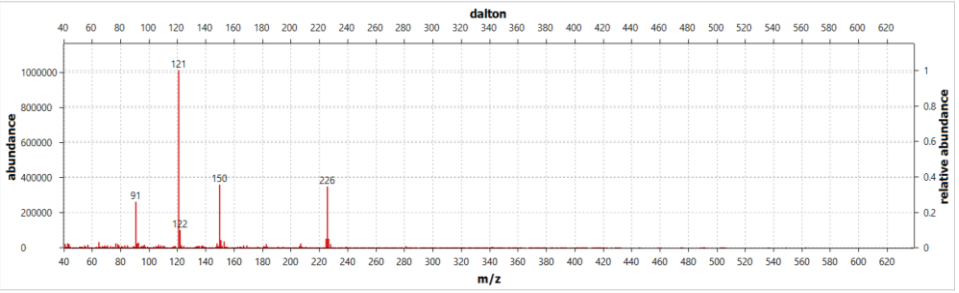
25T4-NBOMe mass spectrum after MSTFA derivatisation



25I-NBOMe mass spectrum after MSTFA derivatisation

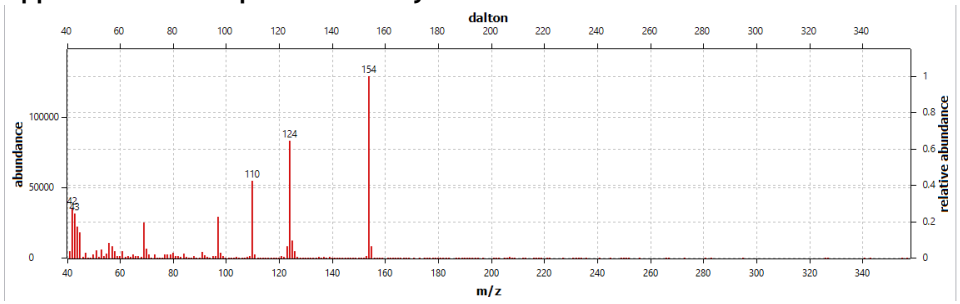


25T2-NBOMe mass spectrum after MSTFA derivatisation

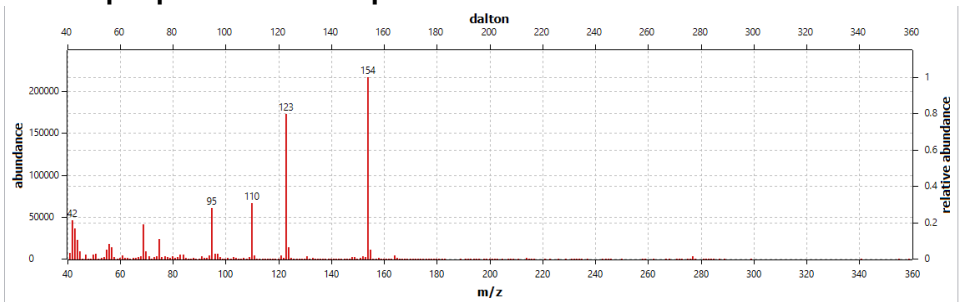


25T7-NBOMe mass spectrum after MSTFA derivatisation

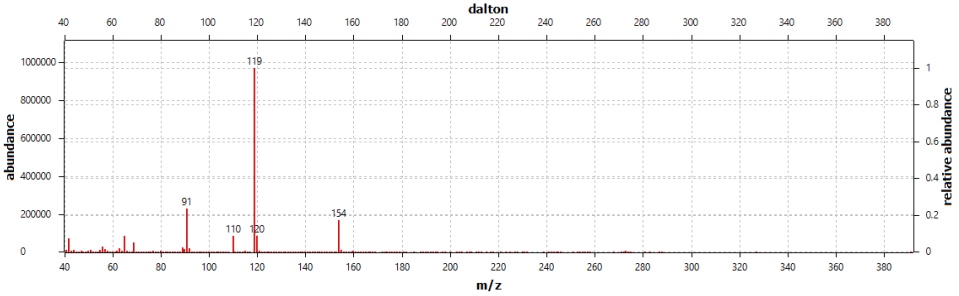
Appendix 3: Mass spectra of analytes after TFAA derivatisation.



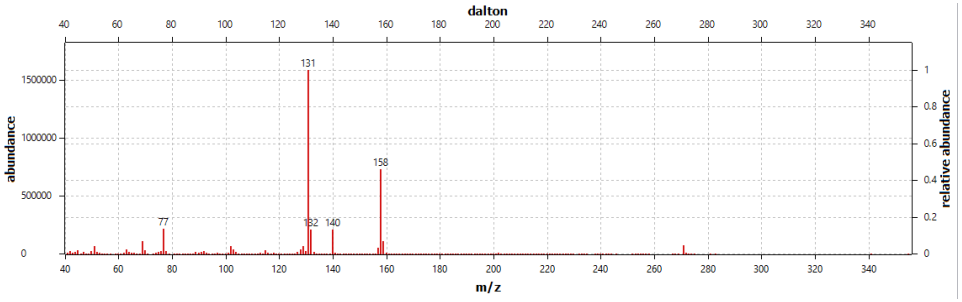
Methiopropamine mass spectrum after TFAA derivatisation



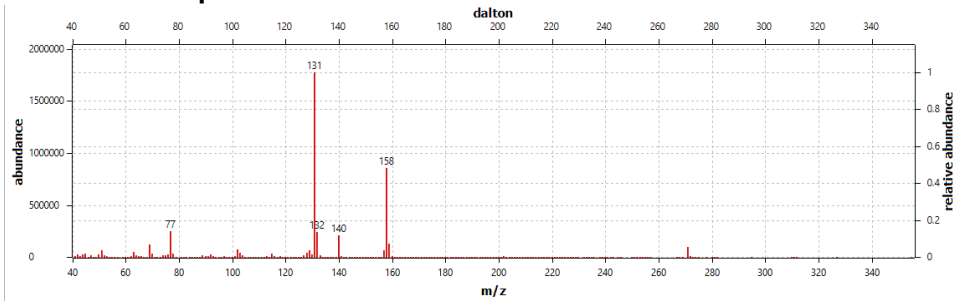
Flephedrone mass spectrum after TFAA derivatisation



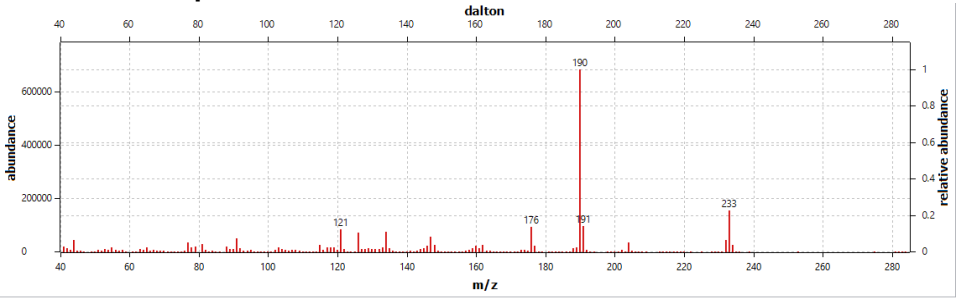
Mephedrone mass spectrum after TFAA derivatisation



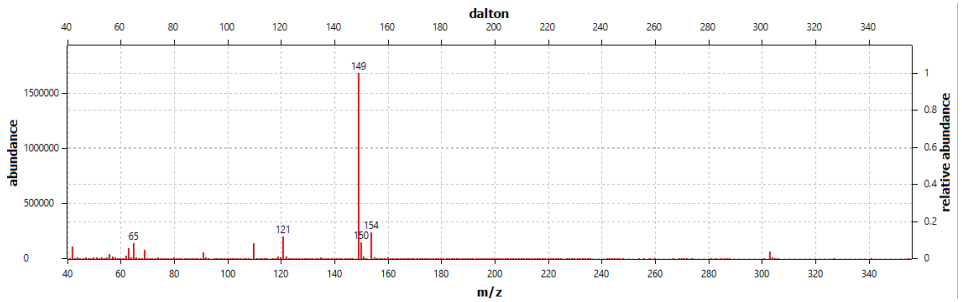
5-APB mass spectrum after TFAA derivatisation



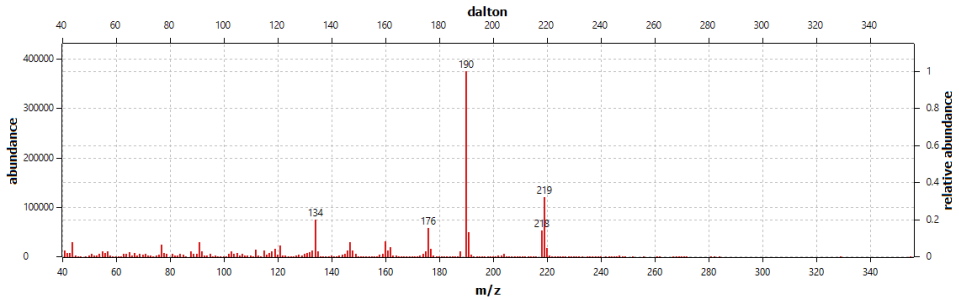
6-APB mass spectrum after TFAA derivatisation



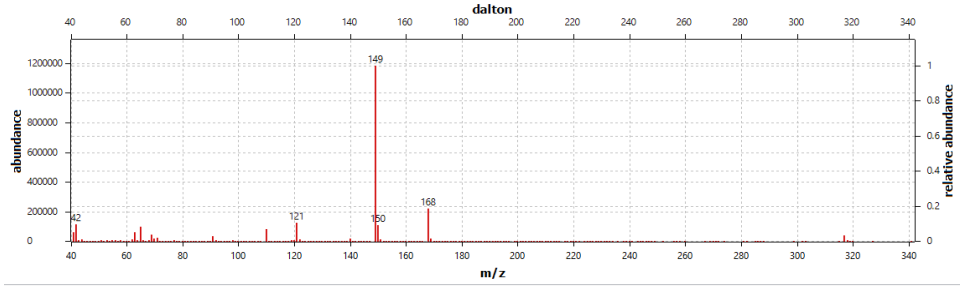
3-MeO-PCE mass spectrum after TFAA derivatisation



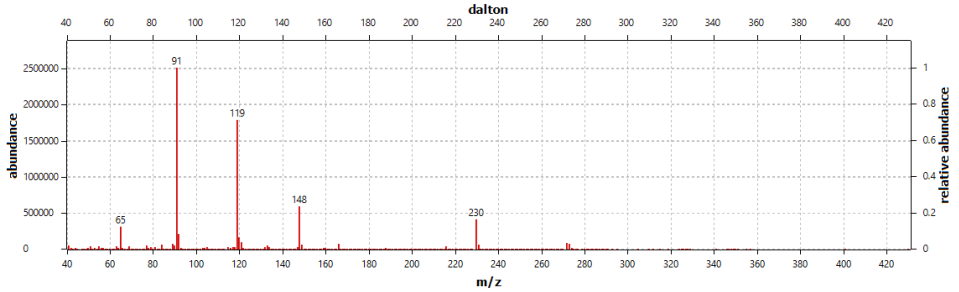
Methylone mass spectrum after TFAA derivatisation



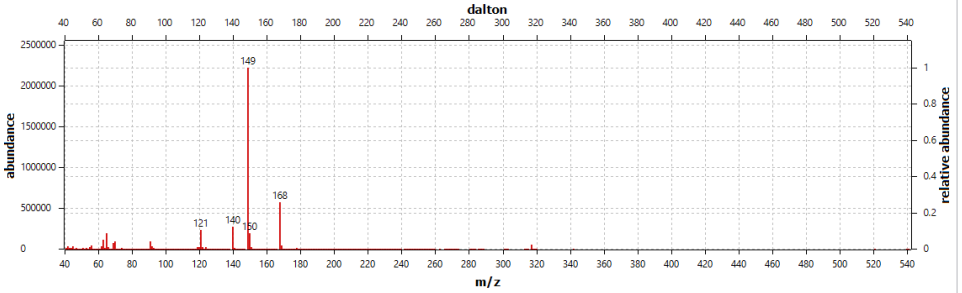
Methoxetamine mass spectrum after TFAA derivatisation



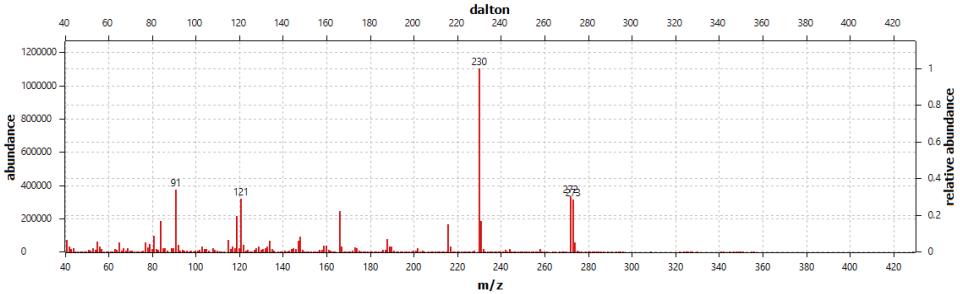
Butylone mass spectrum after TFAA derivatisation



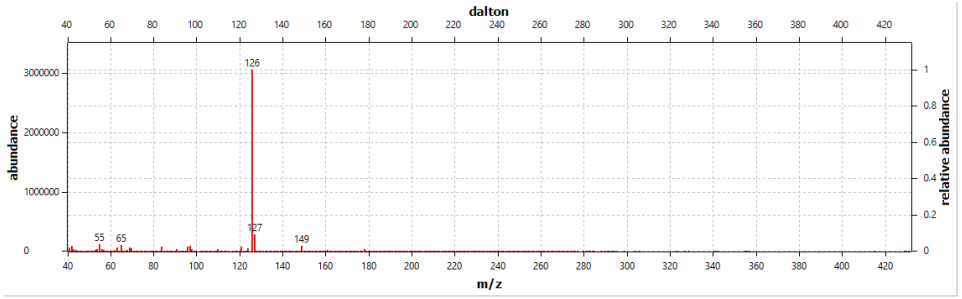
Benzedrone mass spectrum after TFAA derivatisation



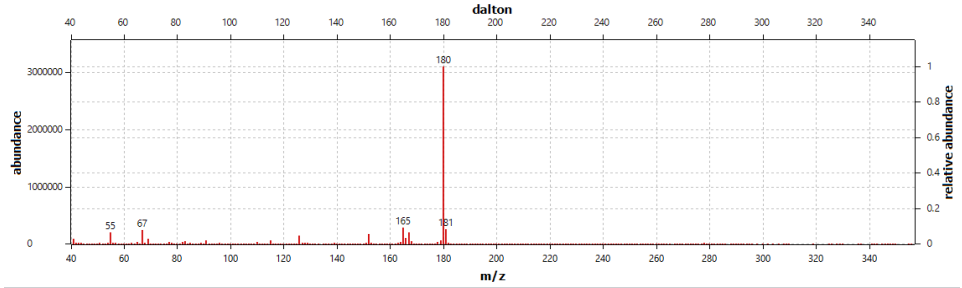
Ethylone mass spectrum after TFAA derivatisation



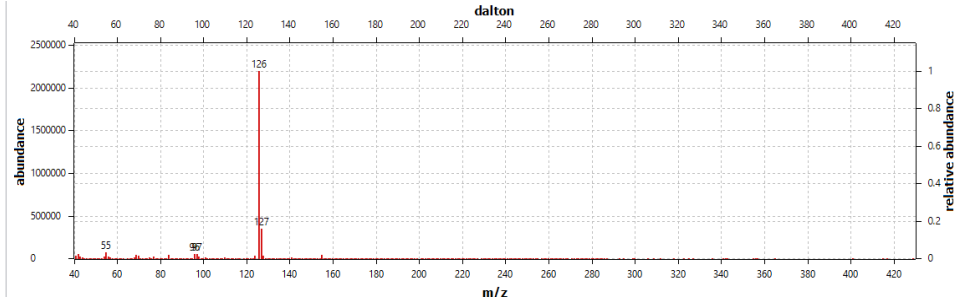
3-MeO-PCP mass spectrum after TFAA derivatisation



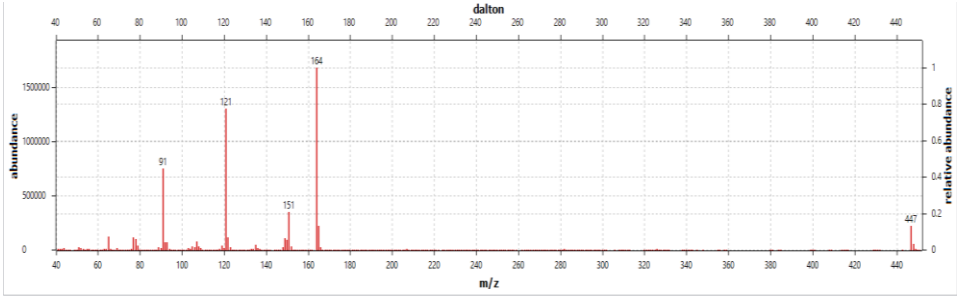
MDPV mass spectrum after TFAA derivatisation



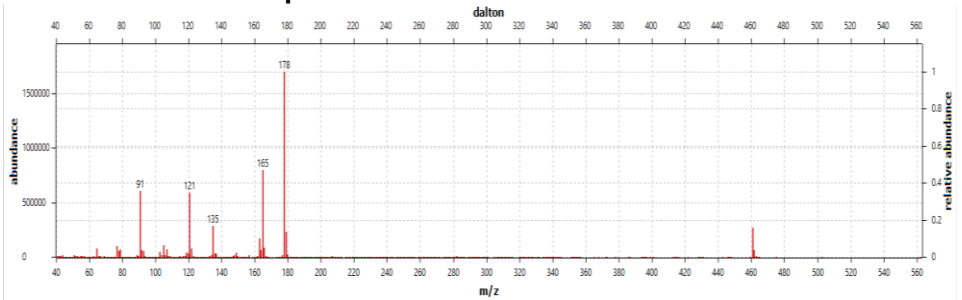
2-DPMP mass spectrum after TFAA derivatisation



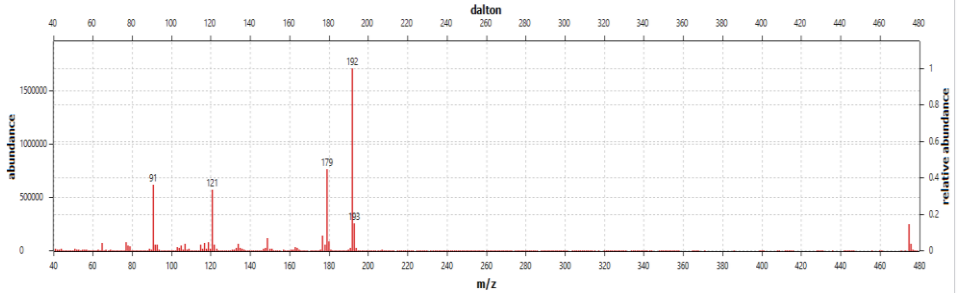
Naphyrone mass spectrum after TFAA derivatisation



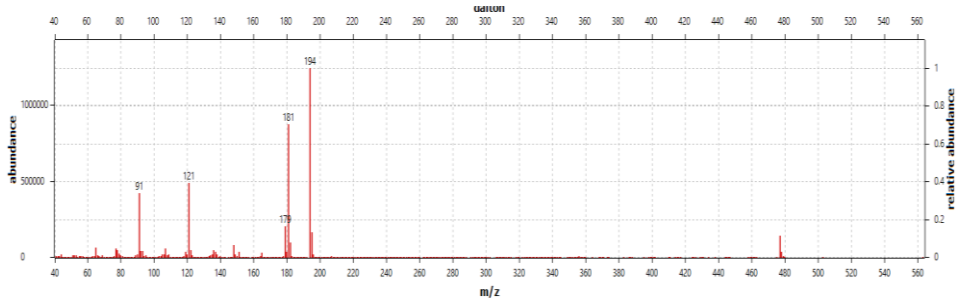
25H-NBOMe mass spectrum after TFAA derivatisation



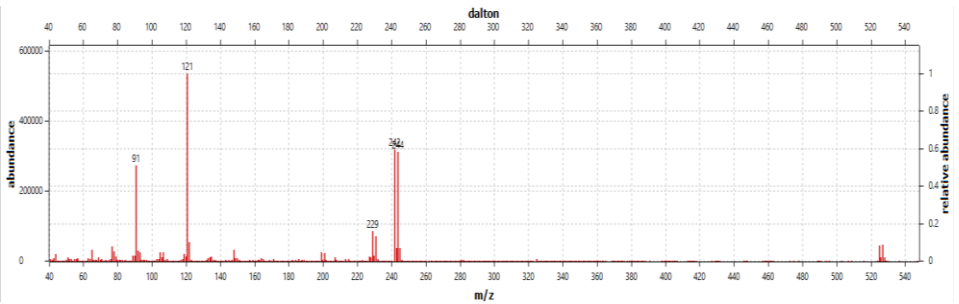
25D-NBOMe mass spectrum after TFAA derivatisation



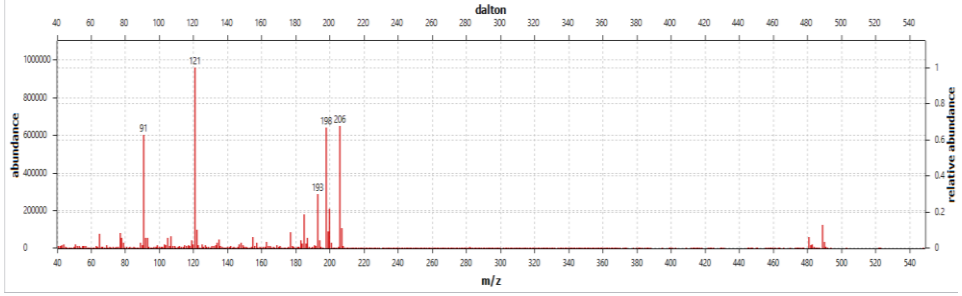
25E-NBOMe mass spectrum after TFAA derivatisation



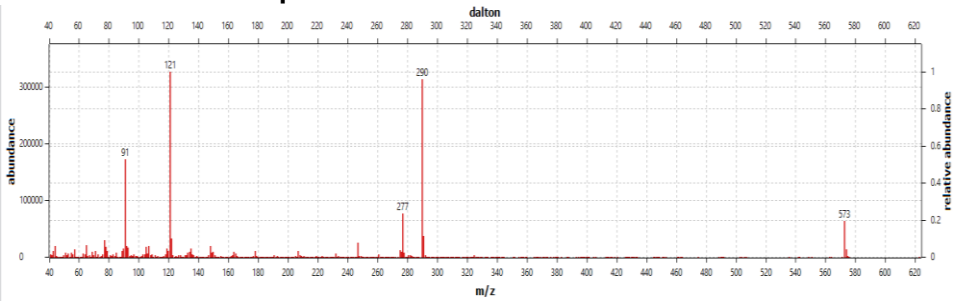
Mescaline-NBOMe mass spectrum after TFAA derivatisation



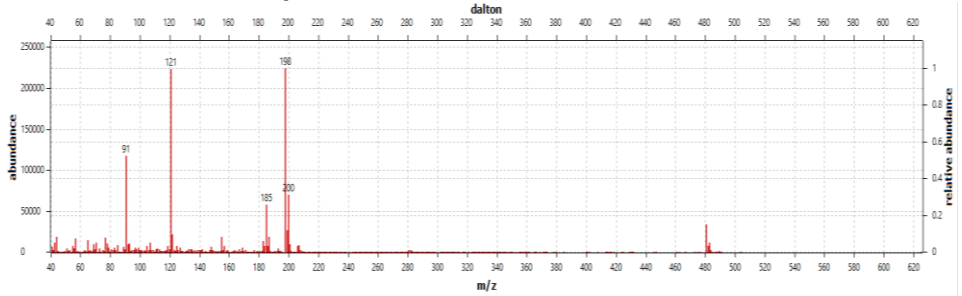
25B-NBOMe mass spectrum after TFAA derivatisation



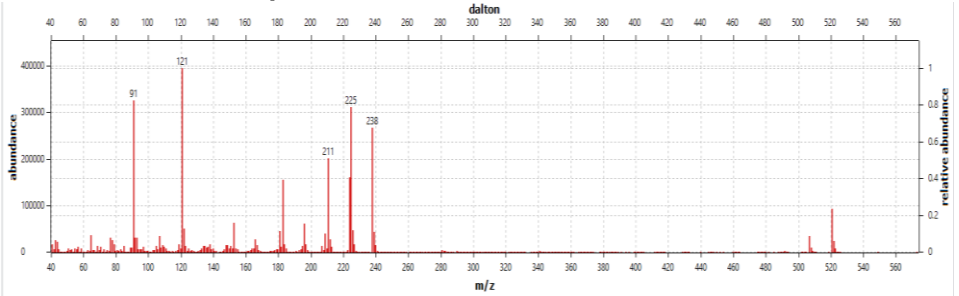
25P-NBOMe mass spectrum after TFAA derivatisation



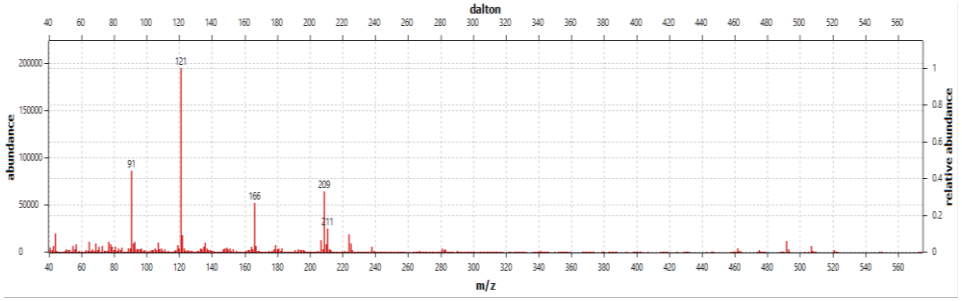
25I-NBOMe mass spectrum after TFAA derivatisation



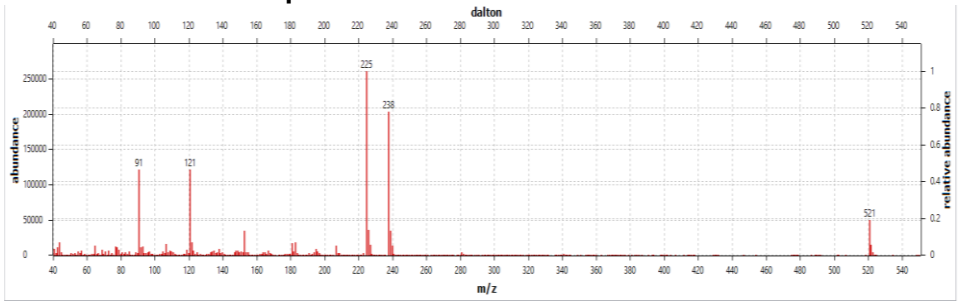
25C-NBOMe mass spectrum after TFAA derivatisation



25T2-NBOMe mass spectrum after TFAA derivatisation

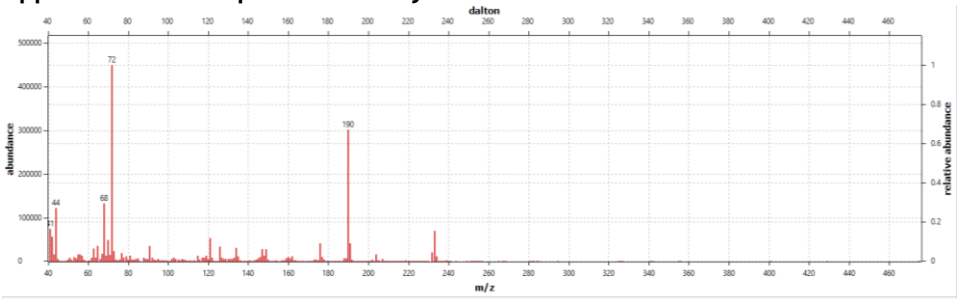


25N-NBOMe mass spectrum after TFAA derivatisation

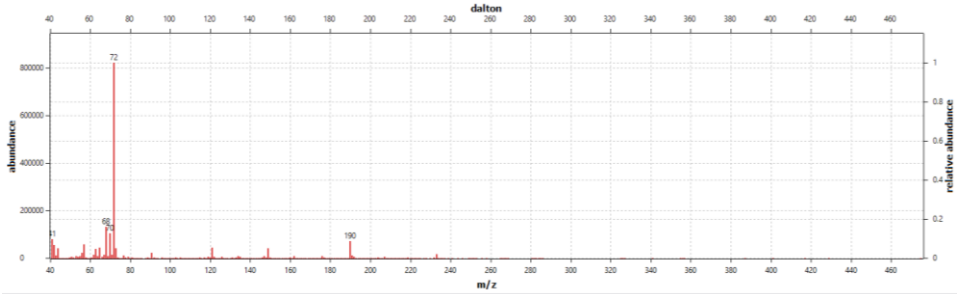


25T7-NBOMe mass spectrum after TFAA derivatisation

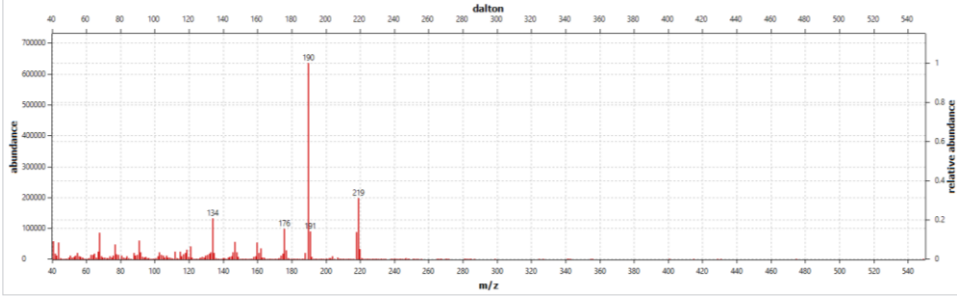
Appendix 4: Mass spectra of analytes after TMSI derivatisation.



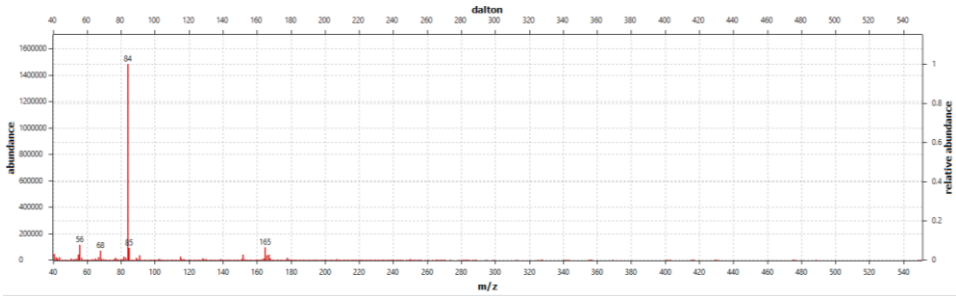
3-MeO-PCE mass spectrum after TMSI derivatisation



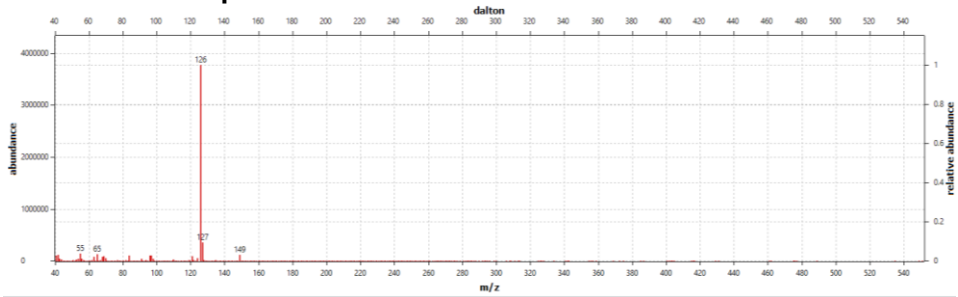
3-MeO-PCP mass spectrum after TMSI derivatisation



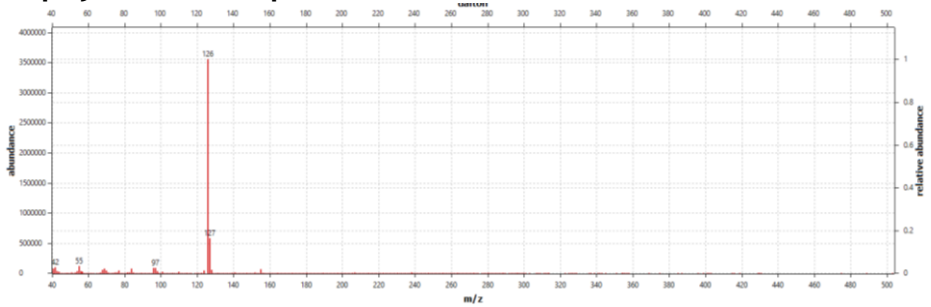
Methoxetamine mass spectrum after TMSI derivatisation



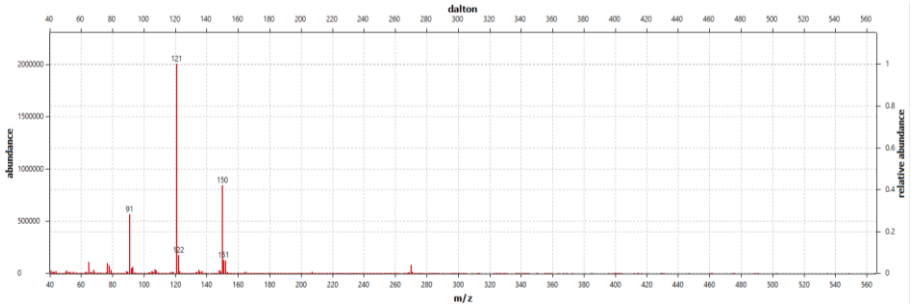
2-DPMP mass spectrum after TMSI derivatisation



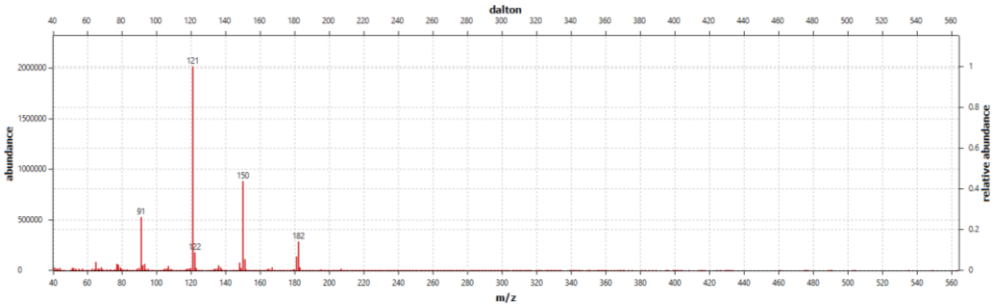
Naphyrone mass spectrum after TMSI derivatisation



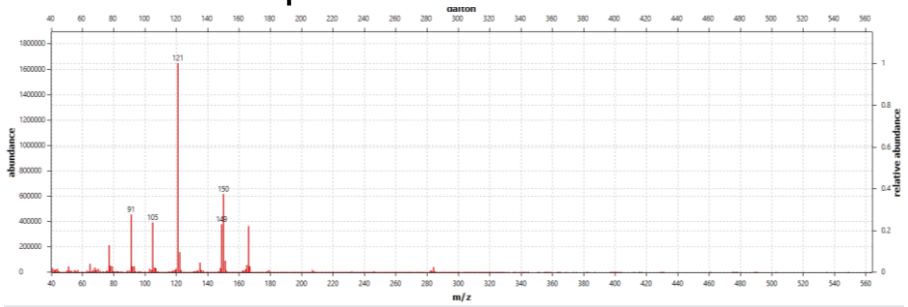
MDPV mass spectrum after TMSI derivatisation



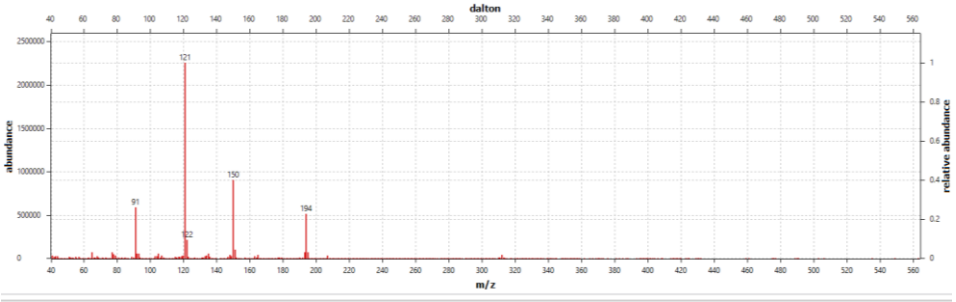
25H-NBOMe mass spectrum after TMSI derivatisation



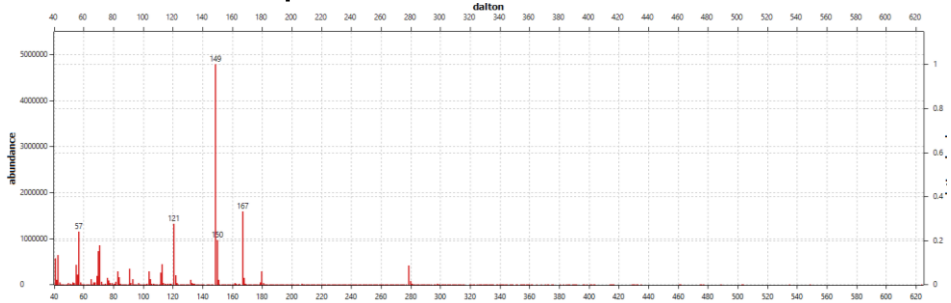
Mescaline-NBOMe mass spectrum after TMSI derivatisation



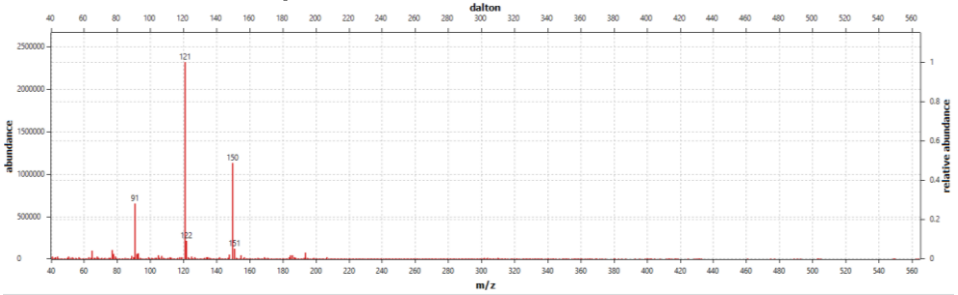
25D-NBOMe mass spectrum after TMSI derivatisation



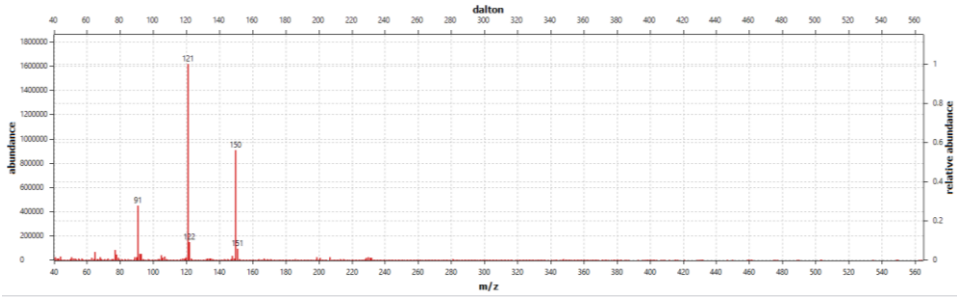
25P-NBOMe mass spectrum after TMSI derivatisation



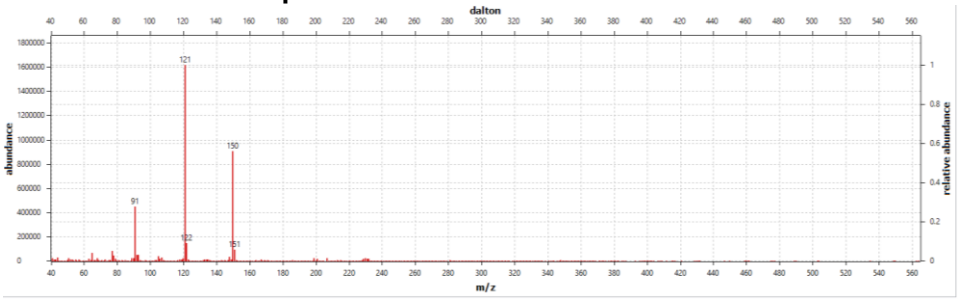
25E-NBOMe mass spectrum after TMSI derivatisation



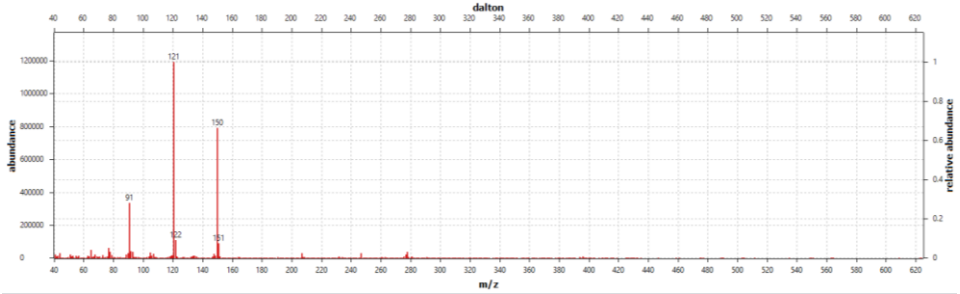
25C-NBOMe mass spectrum after TMSI derivatisation



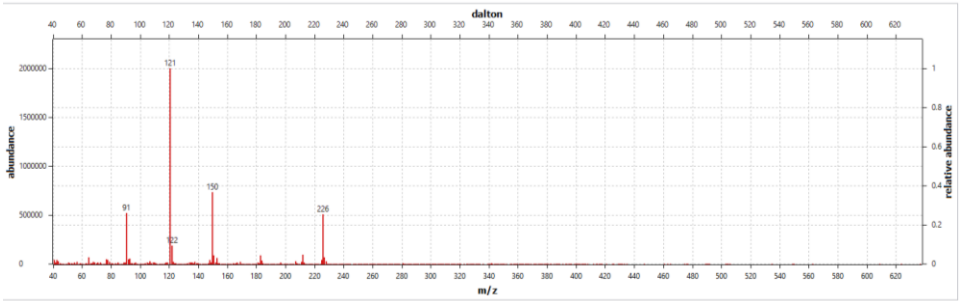
25B-NBOMe mass spectrum after TMSI derivatisation



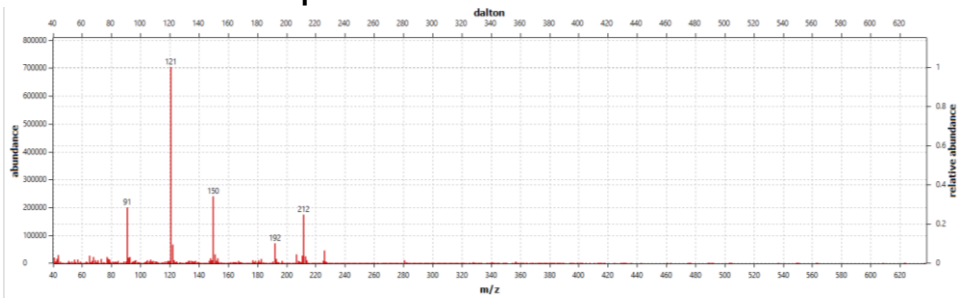
25I-NBOMe mass spectrum after TMSI derivatisation



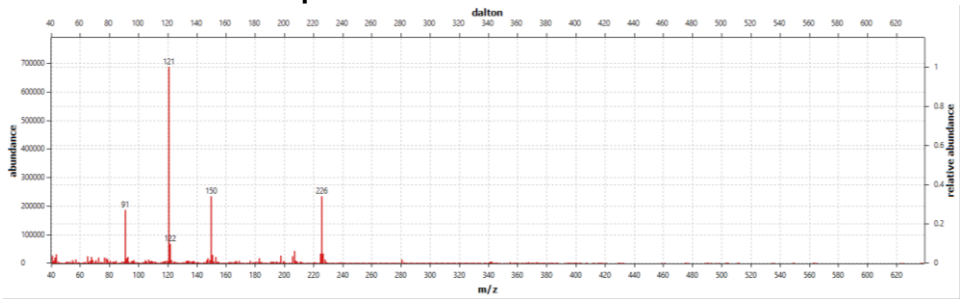
25N-NBOMe mass spectrum after TMSI derivatisation



25T4-NBOMe mass spectrum after TMSI derivatisation



25T2-NBOMe mass spectrum after TMSI derivatisation



25T7-NBOMe mass spectrum after TMSI derivatisation

Appendix 5: Average peak areas of analytes after derivatisation at each temperature and each incubation time

PFPA.

TEMPERATURE	24°C				37°C				50°C				70°C			
INCUBATION TIME	20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 mins	
	AVG	%CV	AVG	%C.V.	AVG	% C.V.	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV
25B-NBOME	2105701	3.6	2504570	5.2	4244681	13.3	3762591	5.4	3670253	7.8	2707283	5.4	3200421	28.4	1524970	11.6
25C-NBOME	3513788	2.4	4925203	10.4	5503196	7.5	4133332	9.5	3994884	6.7	3076087	3.2	4403348	6.1	2836258	11.2
25D-NBOME	3932827	6.5	6399257	12.6	8116595	23.8	5799487	7.4	5423849	12.4	4230023	2.7	6204035	4.6	3866235	12.7
25E-NBOME	3687305	9.8	6564222	1.8	6364609	12.9	5532153	7.0	5211808	12.8	3993611	1.8	5938545	3.6	3632574	12.3
25H-NBOME	4262327	2.7	7005366	8.2	7061705	16.9	6233248	9.2	5804158	11.6	4631684	4.5	6682308	5.8	4239265	12.5
25I-NBOME	915670	4.9	721080	5.7	5491689	20.1	2973294	5.6	2902121	11.6	2504135	17.3	774536	6.9	466842	12.1
25N-NBOME	457853	13.7	870257	15.3	1002858	1.3	996294	21.5	1105198	12.2	816689	14.0	1612858	8.6	853920	15.5
25P-NBOME	4192535	2.8	5393820	8.9	5822820	13.1	5339110	5.0	5108221	7.3	3658583	4.2	5545259	4.7	3370650	13.7
25T2-NBOME	1165422	2.1	876312	15.1	1866410	8.8	1108390	36.5	949575	4.0	573127	6.3	1224907	23.1	563990	20.7
25T4-NBOME	546985	8.0	434885	4.2	1868777	8.6	1364033	26.9	1058152	1.6	703060	1.1	1133597	17.1	325853	23.5
25T7-NBOME	936758	6.6	592659	17.3	1188352	14.8	849238	46.9	712458	7.5	387116	5.3	992144	31.4	400034	23.5
2-DPMP	581515	10.6	947793	4.1	930176	17.9	868722	4.3	752819	11.6	624772	0.0	847342	6.3	564644	12.5
3-MEO-PCE	3157441	1.7	5064590	5.2	5476374	32.4	4411894	12.4	4049420	12.3	3398859	1.8	4379442	13.5	2991923	13.0
3-MEO-PCP	1365264	9.5	1403293	6.7	2806468	83.3	1154145	7.7	1137819	11.7	926989	1.4	1306895	4.9	834494	13.5
5-APB	4463953	11.4	7344962	3.5	6298220	6.9	6298220	6.9	5852911	12.3	4761858	5.5	6876632	3.2	4311728	9.5
6-APB	4812617	3.7	7630914	6.7	7238264	12.8	6005884	6.1	5926034	8.5	4863066	2.3	6489215	3.7	4208685	12.5

BENZEDRONE	3667164	13.1	7268245	11.0	7599427	33.7	6976481	8.4	6298832	10.8	5230642	0.9	7931314	7.2	4921790	9.5
BUTYLONE	5836308	14.3	9917665	5.5	8380222	3.9	8233994	10.7	7984216	11.1	6556727	0.1	9497740	5.0	6209998	17.6
ETHYLONE	1686385	10.2	2823908	6.1	2390671	3.3	2401559	12.6	2289297	6.5	1941727	0.0	2831936	5.6	1831142	17.4
FLEPHEDRONE	1982662	15.3	2767969	12.7	1752083	15.6	1535275	38.1	2270071	11.4	1717255	2.5	2635889	2.4	1524509	23.3
MDPV	4192048	4.1	8579133	3.5	7516640	6.3	7332130	2.8	6919086	11.8	5843390	6.2	8215615	5.6	5205325	13.8
MEPHEDRONE	584318	5.1	1079169	10.2	837729	7.7	822358	29.7	959918	11.0	787525	8.7	1148247	2.0	683280	16.8
MESCALINE NBOME	3437569	10.1	5361762	2.6	5662009	13.9	5228510	6.9	5048671	8.2	3732599	3.1	5656966	4.5	3387991	15.5
METHIOPROPAMINE	1178785	9.8	2916088	7.9	2019404	12.3	1496617	25.7	2352143	13.4	1508280	5.4	2668527	3.8	1435544	29.1
METHOXETAMINE	1781058	5.9	3509882	8.1	2604987	11.5	2799352	14.0	2746721	12.9	2321266	4.8	3330469	11.8	2063738	19.8
METHYLONE	593104	4.5	1867611	5.9	1731194	11.9	1674569	15.4	1625026	14.1	1283936	0.5	1892738	1.9	1162741	11.5
NAPHYRONE	2644897	12.4	6654953	6.1	4358618	47.6	5469075	10.5	5754016	11.1	4838097	7.8	7107451	4.5	4432547	14.6

MSTFA

TEMPERATURE	24ºC				37ºC				50ºC				70ºC			
INCUBATION TIME	20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 mins	
	AVG	%CV	AVG	%C.V.	AVG	% C.V.	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV
25B-NBOME	937856	1	369536	2	1074240	2	951265	8	1099776	7	1000015	8	985164	10	801541	19
25C-NBOME	1089888	2	1003456	2	975936	5	946125	7	1099776	8	1010545	9	954156	11	821451	18
25D-NBOME	1835520	4	2080256	1	1958912	1	2215612	5	2302976	9	2212345	9	2015211	15	195124	16
25E-NBOME	1705472	5	2003456	4	179072	2	1652152	6	2108928	10	2100981	7	1954121	16	2015412	20
25H-NBOME	270656	2	374464	1	1153541	4	1572135	6	2195968	5	2015641	4	1851261	16	1901521	15
25I-NBOME	205248	5	1389056	5	1280512	5	1502136	6	1747968	5	1601451	5	1520412	15	1015412	17
25N-NBOME	389632	4	285376	3	320008	5	365201	4	420480	5	384516	6	360152	15	301541	21
25P-NBOME	880896	4	1836544	4	1840440	5	1851264	5	2041856	4	1984513	6	1541241	19	104124	16
25T2-NBOME	362515	5	410215	5	317376	6	401562	5	420480	6	381541	3	321564	12	251241	15
25T4-NBOME	157056	3	1147904	2	1051951	1	951621	3	1181184	9	1167541	5	1100001	12	100145	20
25T7-NBOMe	83052	4	365779	1	388800	2	402152	1	423040	6	401254	2	384512	12	301245	10
2-DPMP	960120	1	1246720	2	1177344	3	974160	2	845126	11	801541	1	764512	18	678454	11
3-MEO-PCE	873408	2	971392	1	890515	2	691904	2	551251	5	541645	4	501425	16	489154	11
3-MEO-PCP	200992	5	387648	3	378208	1	209612	5	201564	8	195461	5	150241	15	101541	14
5-APB	121136	5	845681	3	807168	5	751215	5	701564	1	701564	6	605184	16	541245	15
6-APB	743104	6	1523712	3	1283072	1	901536	2	912115	5	891542	3	701564	19	645124	19
BENZEDRONE	449968	5	455424	5	393600	1	340736	1	320315	8	281567	4	255554	15	200154	21
BUTYLONE	124130	4	139712	4	132451	1	110125	1	112025	8	102451	5	95145	15	78451	22
ETHYLONE	105692	5	676800	1	135104	2	105102	2	120125	9	134651	6	112456	10	101245	16
MDPV	1256640	6	1329664	2	1005504	3	1325792	5	995152	7	874561	9	851645	11	764154	19
MEPHEDRONE	557568	7	561088	3	460160	4	451233	4	420152	6	384569	9	356481	12	281541	13

MESCALINE NBOME	985702	5	977344	2	956736	5	991520	3	1088512	9	984555	8	874512	14	701541	11
METHOXETAMINE	203008	4	351168	2	217600	6	221520	5	191522	9	177775	5	184574	16	161245	18
METHYLONE	809088	5	905728	3	801521	7	759152	6	684152	4	568749	6	554785	17	501245	19
NAPHYRONE	1927168	3	2045440	1	457216	1	630464	4	548645	5	504516	5	454545	18	401245	21

TFAA

TEMPERATURE	24°C				37°C				50°C				70°C			
INCUBATION TIME	20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 mins	
	AVG	%CV	AVG	%C.V.	AVG	% C.V.	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV
25B-NBOME	880576	3	813971	4	893312	8	829184	5	867200	6	943432	9	787521	10	652312	15
25C-NBOME	1146368	5	292800	5	770112	4	1096192	4	945472	7	1277952	10	1150496	11	782152	15
25D-NBOME	1042304	1	531776	5	1150464	5	1149280	6	1121120	8	1189888	12	1015360	16	801234	19
25E-NBOME	703872	2	734910	1	66960	1	719040	2	541560	8	754488	13	565696	16	402512	16
25H-NBOME	1023200	6	417280	3	1126080	1	1045888	3	1144320	8	1187328	10	1078176	18	901254	15
25I-NBOME	85080	9	71488	2	421952	3	283712	4	685681	5	746481	9	672128	19	546212	14
25N-NBOME	133664	5	90112	5	106752	6	274816	5	214790	6	292736	9	254251	21	201512	16
25P-NBOME	957568	2	277760	3	1071616	4	1075458	6	1108480	9	1161216	8	1114304	15	1052311	19
25T2-NBOME	44774	1	225746	1	221952	5	282081	4	241632	11	292736	6	215404	15	201520	21
25T4-NBOME	198976	2	185748	5	142472	6	202081	5	192265	9	207744	5	201856	19	182512	20
25T7-NBOME	26246	3	144192	7	185552	1	94656	5	186720	8	190720	4	133440	16	126530	25
2-DPMP	311756	5	1804288	5	1836896	2	1907712	6	1816704	6	1499648	3	1307648	16	1835520	16
3-MEO-PCE	257042	1	618448	1	584512	3	621056	5	584156	9	185760	2	124256	10	611264	16
3-MEO-PCP	110752	2	87072	3	75812	3	76812	5	135744	5	141440	1	126592	10	115212	15
5-APB	3688629	3	312832	3	1064448	2	1000688	3	1097728	8	1027840	3	1329664	9	1147904	14
6-APB	3761448	1	707072	3	855872	1	909312	2	740928	5	941696	3	1266176	9	1100288	10

TEMPERATURE	24ºC				37ºC				50ºC				70ºC			
INCUBATION TIME	20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 mins	
	AVG	%CV	AVG	%C.V.	AVG	% C.V.	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV
BENZEDRONE	1318421	2	1539072	3	1861120	8	2001920	1	1343488	6	1611264	8	1241664	8	1204480	19
BUTYLONE	1067458	2	1364480	2	1547456	1	1661440	1	1660144	6	1332736	9	1223680	10	1004448	18
ETHYLONE	1158369	3	1600448	1	758400	5	1603072	5	1562816	5	1472000	10	1591328	10	1569280	19
FLEPHEDRONE	441135	1	373056	1	58992	5	520128	4	388224	5	441920	15	224960	15	201664	23
MDPV	1551299	1	1612541	2	2164736	4	2387968	6	2005568	5	2191360	10	1951621	17	1907200	20
MEPHEDRONE	409762	2	775296	1	401920	6	886208	6	881968	5	841408	9	795584	19	740880	21
MESCALINE NBOME	185216	1	214720	5	1093312	2	1000215	5	1062400	6	1156608	8	951201	21	861400	19
METHIOPROPAMINE	227978	1	248192	1	31584	3	307264	4	156928	3	308240	9	99416	16	131712	18
METHYLONE	2648561	2	1294848	4	400128	5	330816	5	1958912	3	810880	9	1722368	19	1081856	17
METHOXETAMINE	648260	3	298176	4	441720	4	1285623	4	700672	2	132416	8	564912	20	1245256	16
NRG-1	858004	6	415488	5	888576	3	914496	2	904272	5	841516	6	90360	16	820768	15

TMSI

TEMPERATURE	24°C				37°C				50°C				70°C			
INCUBATION TIME	20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 MINS		20 MINS		40 mins	
	AVG	%CV	AVG	%C.V.	AVG	% C.V.	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV	AVG	%CV
25B-NBOME	1418240	5	770816	2	1570304	3	580736	4	921984	5	616160	6	829440	10	560000	16
25C-NBOME	1016768	6	1086144	2	1144834	4	1093824	5	1128448	4	1123328	3	1096736	11	879040	15
25D-NBOME	1290272	4	1135104	3	1333760	1	1015488	1	897088	6	1020032	7	1296736	15	954240	15
25E-NBOME	331776	7	1184256	1	1756672	1	310144	3	375424	9	877632	10	611840	16	632384	18
25H-NBOME	1012672	1	928000	4	1421312	1	586688	4	672832	10	787520	11	942016	11	748480	19
25I-NBOME	1078336	3	328384	3	1193472	2	507200	6	688640	2	879424	5	809920	9	503104	21
25N-NBOME	1010688	3	744576	3	1637376	3	1406848	2	1374912	6	931264	9	1038080	15	1114112	22
25P-NBOME	319872	2	471488	1	535616	1	400544	5	418304	4	289792	2	400320	15	345344	16
25T2-NBOME	1045120	4	633600	4	1637376	1	830720	1	1305088	3	1200640	3	551744	18	411712	13
25T4-NBOME	711792	2	727648	4	790656	2	472576	2	243968	3	554624	4	454208	20	293504	14
25T7-NBOME	515648	1	540704	5	612736	4	591042	2	44568	2	424576	6	536512	21	568640	15
2-DPMP	1413504	1	1380192	3	1475072	1	830656	5	1214976	1	1133056	6	817984	13	122928	15
3-MEO-PCE	594240	6	190784	1	794624	1	296640	1	170688	1	700640	8	233216	13	281920	18
3-MEO-PCP	1032544	5	980800	1	1052160	4	985615	6	885617	5	559744	9	869216	19	770560	21
MDPV	2068992	3	2405376	5	2707456	3	2661888	6	2613248	1	2431488	9	154280	18	1514496	20
MESCALINE NBOME	1476096	4	1031936	4	4419072	2	1174016	1	1345024	1	1364800	10	2436608	15	2564608	19
METHOXETAMINE	389440	2	345344	8	648260	5	536064	2	336448	2	602176	11	630784	16	600064	17
NRG-1	2017792	2	1362944	2	2280448	1	175616	3	1616896	4	805184	10	1847808	20	1478464	10

Appendix 6: SPE blood, urine serum and plasma cartridge comparison data

BLOOD	CSDAU		ZSDAU		XCEL I		OASIS		206		502	
	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
25B-NBOMe	86	11	76	8	43	1	6	12	26	14	2	7
25C-NBOMe	83	7	49	2	31	2	6	7	47	13	2	2
25D-NBOMe	102	4	86	4	59	1	4	13	85	12	36	2
25ENBOMe	110	6	80	8	55	2	2	5	77	15	101	6
25H-NBOMe	99	10	53	5	76	1	10	2	41	14	38	3
25I-NBOMe	90	8	63	18	50	1	23	4	49	6	14	9
25N-NBOMe	17	18	81	5	16	6	2	13	18	12	17	3
25P-NBOMe	39	11	22	15	27	1	2	9	69	13	25	9
25T2-NBOMe	69	7	79	14	53	2	2	10	111	10	27	5
25T4-NBOMe	105	9	74	13	22	2	0	3	61	10	36	8
25T7-NBOMe	101	11	95	9	106	1	2	7	83	4	40	9
2-DPMP	88	6	39	11	18	12	30	7	39	9	18	6
3-MeO-PCE	37	3	40	5	30	17	27	14	45	11	25	3
5-APB	49	2	45	9	35	2	16	11	47	3	32	2
6-APB	22	7	27	11	12	2	14	12	26	2	31	4
BENZEDRONE	4	3	32	11	3	8	28	9	47	3	41	5
BUTYLONE	43	6	42	17	40	5	11	8	53	4	34	2
ETHYLONE	24	1	115	15	92	2	13	6	40	10	33	5
FLEPHEDRONE	65	5	16	14	68	9	9	9	47	4	33	3
MDPV	106	8	17	8	52	14	15	8	41	2	45	13
MEPHEDRONE	112	9	29	1	92	4	27	12	45	9	65	16
Mescaline-NBOMe	5	11	2	5	49	12	7	13	52	2	46	3
METHIOPROPAMINE	26	8	35	7	57	15	52	5	26	8	24	7
METHOXETAMINE	53	9	19	13	49	11	7	5	56	2	45	6
NAPHYRONE	36	10	39	5	38	5	12	13	35	5	40	5

URINE	CSDAU		ZSDAU		XCEL I		OASIS		206		502	
	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
25B-NBOMe	85	3	82	6	49	1	12	1	65.0	2	5	4
25C-NBOMe	85	2	83	6	46	2	10	6	64.7	9	5	10
25D-NBOMe	102	2	103	7	66	1	11	8	109	1	21	14
25E-NBOMe	110	1	101	8	63	2	34	3	102	1	22	13
25H-NBOMe	139	10	99	7	96	3	40	16	67	6	14	12
25I-NBOMe	89	1	90	4	54	3	10	1	65	9	8	1
25N-NBOMe	55	5	98	9	18	6	26	7	39	59	9	14
25P-NBOMe	64	1	76	7	38	1	7	7	60	3	10	15
25T2-NBOMe	102	8	97	10	70	2	31	10	108	14	36	9
25T4-NBOMe	105	5	95	9	59	2	26	6	88	8	6	7
25T7-NBOMe	104	9	101	10	106	1	45	5	64	7	97	3
2-DPMP	115	11	105	5	27	19	107	6	23	6	12	10
3-MeO-PCE	93	7	86	6	96	10	43	10	50	4	78	10
5-APB	90	19	35	6	95	7	94	15	68	8	55	17
6-APB	85	15	35	7	94	6	90	12	71	7	57	12
BENZEDRONE	43	17	36	4	15	14	124	13	70	3	61	6
BUTYLONE	94	9	35	6	44	12	119	14	77	4	58	2
ETHYLONE	82	3	38	4	43	2	87	9	69	4	51	5
FLEPHEDRONE	81	4	34	3	32	17	76	12	63	5	50	4
MDPV	97	5	36	4	105	6	102	8	82	3	63	3
MEPHEDRONE	101	6	49	7	43	4	91	7	67	4	61	2
Mescaline-NBOMe	9	8	5	5	50	9	12	2	6	3	43	9
METHIOPROPAMINE	13	2	23	5	80	13	15	15	78	7	31	4
METHOXETAMINE	61	8	35	2	70	10	62	4	49	5	56	3
NAPHYRONE	37	7	95	3	113	2	63	5	77	6	50	4

PLASMA	CSDAU		ZSDAU		XCEL I		206		502	
	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
25B-NBOMe	30	1	117	4	25	10	42	19	1	6
25C-NBOMe	25	1	76	3	46	8	45	18	2	5
25D-NBOMe	40	1	117	3	77	11	77	16	3	6
25E-NBOMe	37	1	111	3	76	8	71	16	37	6
25H-NBOMe	24	1	37	2	51	6	52	15	16	5
25I-NBOMe	45	1	36	4	47	12	45	16	39	3
25N-NBOMe	10	2	105	3	1	13	28	18	8	8
25P-NBOMe	23	3	63	5	56	17	43	12	19	6
25T2-NBOMe	38	8	66	5	37	10	79	10	27	6
25T4-NBOMe	37	6	64	6	30	8	59	12	56	6
25T7-NBOMe	28	8	115	4	28	5	112	14	86	5
2-DPMP	89	3	79	5	20	13	10	8	15	6
3-MeO-PCE	18	11	21	4		14	60	3	56	6
5-APB	63	5	12	3	6	11	45	8	48	10
6-APB	3	12	45	2	4	13	47	10	38	6
BENZEDRONE	39	13	18	2		11	48	3	38	5
BUTYLONE	64	1	45	3	101	12	57	14	44	6
ETHYLONE	35	1	67	4	79	13	62	3	41	5
FLEPHEDRONE	67	5	27	1		5	52	12	38	4
MDPV	57	2	35	8	109	2	56	4	34	6
MEPHEDRONE	88	3	62	7		3	47	18	41	6
Mescaline-NBOMe	2	4	41	4	3	7	4	16	38	5
METHIOPROPAMINE	15	2	17	5	27	6	17	15	18	4
METHOXETAMINE	56	9	19	6	12	8	66	16	56	3
NAPHYRONE	27	10	40	2	37	6	39	10	42	4

SERUM	CSDAU		ZSDAU		XCEL I		206		502	
	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV	% Recovery	% CV
25B-NBOMe	51	1	43	16	71	9	45	19	2	4
25C-NBOMe	42	1	85	20	52	9	46	20	3	3
25D-NBOMe	61	1	111	13	84	15	81	18	37	5
25ENBOMe	59	2	108	14	81	16	73	15	25	2
25H-NBOMe	4	1	81	12	52	7	38	16	41	6
25I-NBOMe	59	2	118	16	80	9	46	16	15	6
25N-NBOMe	8	2	118	14	20	3	16	15	18	9
25P-NBOMe	36	1	62	13	42	9	39	14	29	6
25T2-NBOMe	46	3	65	15	81	7	71	17	69	6
25T4-NBOMe	46	8	61	15	70	10	55	13	37	5
25T7-NBOMe	53	6	107	16	94	12	102	14	41	6
2-DPMP	97	8	95	13	11	12	40	16	36	4
3-MeO-PCE	47	3	50	14	36	14	42	14	32	2
5-APB	71	5	24	16	69	11	32	12	48	5
6-APB	51	12	27	15	49	13	41	14	37	5
BENZEDRONE	35	13	25	12	40	11	38	10	47	4
BUTYLONE	65	1	65	11	87	12	64	9	28	5
ETHYLONE	74	2	64	7	79	13	67	15	25	10
FLEPHEDRONE	63	5	52	4	50	4	68	16	37	4
MDPV	76	2	46	15	100	19	52	19	32	5
MEPHEDRONE	126	3	52	16	101	4	67	16	40	6
Mescaline-NBOMe	3	4	13	10	10	6	4	10	90	10
METHIOPROPAMINE	33	2	43	9	34	8	26	16	38	9
METHOXETAMINE	53	10	58	8	20	5	41	12	40	12
NAPHYRONE	28	7	23	12	33	6	45	10	47	11

Appendix 7: Comparison of SLE and SPE recovery rates for various NPS from blood, urine, plasma and serum.

	BLOOD			URINE			PLASMA			SERUM		
	SLE (%)	SPE (%)	DIF (%)	SLE (%)	SPE (%)	DIF (%)	SLE (%)	SPE (%)	DIF (%)	SLE (%)	SPE (%)	DIF (%)
25B-NBOMe	78	67	+11	73	81	-8	71	82	-11	71	89	-18
25D-NBOMe	78	71	+7	72	83	-11	69	89	-20	69	86	-16
25E-NBOMe	79	54	+25	73	79	-6	69	86	-17	70	85	-15
25H-NBOMe	77	72	+5	71	88	-17	72	89	-17	70	101	-30
25I-NBOMe	83	60	+23	72	77	-5	66	83	-16	67	127	-60
25N-NBOMe	99	102	-2	108	127	-19	142	143	-1	46	35	+12
25P-NBOMe	82	64	+18	75	74	+1	64	84	-20	68	112	-43
25T2-NBOMe	55	61	-5	73	53	+20	66	100	-34	66	109	-43
25T4-NBOMe	68	56	+12	73	55	+19	63	94	-31	63	124	-61
25T7-NBOMe	45	60	-15	68	49	+19	68	96	-28	69	145	-76
2-DPMP	90	103	-13	80	72	+8	70	70	+0	124	30	+94
3-MEO-PCE	74	51	+23	61	74	-13	43	70	-27	32	55	-23
3-MEO-PCP	75	49	+26	59	67	-8	39	69	-30	19	53	-33
5-APB	77	46	+31	49	81	-32	41	93	-52	40	84	-44
6-APB	81	40	+40	48	83	-35	34	98	-64	33	78	-45
BENZEDRONE	142	132	+10	10	86	+22	77	106	-29	100	21	+79
BUTYLONE	61	65	-4	62	56	+6	71	65	+6	83	29	+54
ETHYLONE-	20	65	-46	18	69	-51	15	74	-58	19	30	-11
FLEPHEDRONE	71	91	-21	108	63	+45	97	59	+39	120	85	+35
MDPV	79	63	+16	59	74	-14	64	75	-11	70	75	-5
MEPHEDRONE	82	43	+40	75	41	+34	22	65	-43	43	56	-13
MESCALINE-NBOMe	72	74	-2	73	88	-15	74	91	-17	69	63	+7
METHIOPROPAMINE	43	90	-47	101	54	+47	112	49	+62	145	88	+57
METHYLONE	79	56	+23	65	76	-11	52	67	-16	55	54	+1
MXE	88	58	+30	62	94	-32	23	87	-63	16	66	-50
NAPHYRONE	117	70	+47	69	93	-24	56	92	-36	34	66	-33

Appendix 8: Autosampler peak area for urine and blood samples.

Urine QC1

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	25512	28523	20807	**-18.4**
FLEPHEDRONE	71434	71307	54098	**-24.3**
MEPHEDRONE	61229	47538	45775	**-25.2**
5-APB	245030	199412	236767	**-3.4**
6-APB	400215	350964	358969	**-10.3**
METHYLONE	59909	48980	41712	**-30.4**
3-MeO-PCE	32671	31906	22913	**-29.9**
BUTYLONE	334874	279176	290230	**-13.3**
ETHYLONE	106179	103694	91652	**-13.7**
MXE	98012	103694	91652	**-6.5**
BENZEDRONE	522356	452960	482187	**-7.7**
3-MeO-PCP	32671	39882	30551	**-6.5**
2-DPMP	664817	679440	522369	**-21.4**
MDPV	617330	452960	442004	**-28.4**
NAPHYRONE	379895	362368	281275	**-26.0**
25B-NBOMe	1764	790	1120	**-36.5**
25C-NBOMe	3528	1975	2614	**-25.9**
25D-NBOMe	5292	2765	4108	**-22.4**
25E-NBOMe	4410	3160	3361	**-23.8**
25H-NBOMe	5734	2765	5228	**-8.8**
25I-NBOMe	8380	8295	6348	**-24.2**
Mescaline-NBOMe	3528	2370	2988	**-15.3**
25P-NBOMe	2205	1185	1494	**-32.3**
25T4-NBOMe	441	395	373	**-15.3**
25T7-NBOMe	441	790	373	**-15.3**
Mephedrone-D3	510241	475381	416137	**-18.4**
Ethylone-D5	816765	797646	763764	**-6.5**
Methylone-D3	399395	376767	320865	**-19.7**
MDPV-D8	4748692	4529598	4018221	**-15.4**
NBOMe-D3	44104	39501	37344	**-15.3**

Urine QC2

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	131275	110213	124378	**-5.3**
FLEPHEDRONE	432115	482838	350101	**-19.0**
MEPHEDRONE	317249	251916	271789	**-14.3**
5-APB	1327570	1037252	995384	**-25.0**
6-APB	1843848	1590452	1526255	**-17.2**
METHYLONE	883868	777683	748186	**-15.4**
3-MeO-PCE	1305444	1265447	1154645	**-11.6**
BUTYLONE	1696340	1452152	1327178	**-21.8**
ETHYLONE	442523	484051	398153	**-10.0**
MXE	685911	684586	637045	**-7.1**
BENZEDRONE	4467169	3805045	3406747	**-23.7**
3-MeO-PCP	191760	200535	179169	**-6.6**
2-DPMP	2680302	2147401	1937170	**-27.7**
MDPV	2639691	1846012	1903770	**-27.9**
NAPHYRONE	3248850	2787855	2939154	**-9.5**
25B-NBOMe	7268	5126	5212	**-28.3**
25C-NBOMe	17928	10719	13230	**-26.2**
25D-NBOMe	18897	13515	14032	**-25.7**
25E-NBOMe	16474	9787	11626	**-29.4**
25H-NBOMe	22773	18175	16437	**-27.8**
25I-NBOMe	48939	43341	42095	**-14.0**
Mescaline-NBOMe	14536	13515	13230	**-9.0**
25P-NBOMe	13083	7923	8820	**-32.6**
25T4-NBOMe	1454	1864	1604	**10.3**
25T7-NBOMe	1938	1864	1203	**-37.9**
Mephedrone-D3	546981	524824	460659	**-15.8**
Ethylone-D5	737539	691501	663589	**-10.0**
Methylone-D3	496555	471323	434992	**-12.4**
MDPV-D8	4061063	3767371	3339948	**-17.8**
NBOMe-D3	48454	46603	40090	**-17.3**

Urine QC3

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	255121	266111	197302	**-22.7**
FLEPHEDRONE	954151	877180	713645	**-25.2**
MEPHEDRONE	1663386	1769145	1456675	**-12.4**
5-APB	4511622	40835747	3674266	**-18.6**
6-APB	5540589	49833454	5221325	**-5.8**
METHYLONE	1267979	1220370	1128906	**-11.0**
3-MeO-PCE	4015128	35298696	3287501	**-18.1**
BUTYLONE	6202581	49141322	5027942	**-18.9**
ETHYLONE	1784501	13842626	1289216	**-27.8**
MXE	2345756	20763939	1998285	**-14.8**
BENZEDRONE	18794764	16972690	15711315	**-16.4**
3-MeO-PCP	575646	6090755	489902	**-14.9**
2-DPMP	15785490	14233581	13829459	**-12.4**
MDPV	16471815	12912940	13348054	**-19.0**
NAPHYRONE	15574313	12423813	11422433	**-26.7**
25B-NBOMe	59455	53834	43701	**-26.5**
25C-NBOMe	69618	82709	48279	**-30.7**
25D-NBOMe	58438	58728	39123	**-33.1**
25E-NBOMe	61487	58728	42036	**-31.6**
25H-NBOMe	72667	83198	60765	**-16.4**
25I-NBOMe	60471	50408	45366	**-25.0**
Mescaline-NBOMe	49800	60196	34545	**-30.6**
25P-NBOMe	59455	64111	44533	**-25.1**
25T4-NBOMe	23375	21534	16648	**-28.8**
25T7-NBOMe	22867	17618	13735	**-39.9**
Mephedrone-D3	512798	492798	419791	**-18.1**
Ethylone-D5	719557	6921313	644608	**-10.4**
Methylone-D3	489567	453669	427616	**-12.7**
MDPV-D8	5279428	4891265	4376411	**-17.1**
NBOMe-D3	50816	48940	41620	**-18.1**

Blood QC1

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	18501	22649	18320	**-1.0**
FLEPHEDRONE	40703	29121	36639	**-10.0**
MEPHEDRONE	51803	42063	45799	**-11.6**
5-APB	204627	183312	183469	**-10.3**
6-APB	424412	366624	366938	**-13.5**
METHYLONE	73800	83915	57699	**-21.8**
3-MeO-PCE	30315	29330	27181	**-10.3**
BUTYLONE	386518	337294	332962	**-13.9**
ETHYLONE	106103	102655	95132	**-10.3**
MXE	121260	102655	101927	**-15.9**
BENZEDRONE	511632	442960	408222	**-20.2**
3-MeO-PCP	37894	36662	33976	**-10.3**
2-DPMP	604656	575848	571511	**-5.5**
MDPV	558144	620144	530689	**-4.9**
NAPHYRONE	372096	310072	285756	**-23.2**
25B-NBOMe	1726	1798	1650	**-4.4**
25C-NBOMe	3453	2158	2310	**-33.1**
25D-NBOMe	5179	4675	3301	**-36.3**
25E-NBOMe	4316	4315	3631	**-15.9**
25H-NBOMe	5611	6113	3961	**-29.4**
25I-NBOMe	8201	8271	6931	**-15.5**
Mescaline-NBOMe	3453	3596	2971	**-14.0**
25P-NBOMe	2158	2158	1650	**-23.5**
Mephedrone-D3	370024	323563	305329	**-17.5**
Ethylone-D5	757878	733248	679515	**-10.3**
Methylone-D3	670907	599395	443840	**-33.8**
MDPV-D8	4651200	4429598	4082222	**-12.2**
NBOMe-D3	43161	35959	33006	**-23.5**

Blood QC2

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	101638	71518	77204	**-24.0**
FLEPHEDRONE	300848	259723	201401	**-33.1**
MEPHEDRONE	260193	218318	191331	**-26.5**
5-APB	1112299	834532	871852	**-21.6**
6-APB	1809247	1353926	1405288	**-22.3**
METHYLONE	1081451	1078344	712055	**-34.2**
3-MeO-PCE	1295336	1266388	1233212	**-4.8**
BUTYLONE	1809247	1353926	1433968	**-20.7**
ETHYLONE	506871	461035	435926	**-14.0**
MXE	999662	887055	889060	**-11.1**
BENZEDRONE	12984599	11185990	10150188	**-21.8**
3-MeO-PCP	183037	151733	137661	**-24.8**
2-DPMP	3528424	3150340	2557847	**-27.5**
MDPV	3340241	2693769	2436045	**-27.1**
NAPHYRONE	3998880	4474396	3735269	**-6.6**
25B-NBOMe	6086	6810	5480	**-10.0**
25C-NBOMe	15013	15323	12787	**-14.8**
25D-NBOMe	15825	10897	10656	**-32.7**
25E-NBOMe	13796	8513	9743	**-29.4**
25H-NBOMe	19071	13280	13701	**-28.2**
25I-NBOMe	36518	33711	26488	**-27.5**
Mescaline-NBOMe	12173	10897	8525	**-30.0**
25P-NBOMe	10955.52	12258.72	9133.8	**-16.6**
Mephedrone-D3	406551	376410	335669	**-17.4**
Ethylone-D5	703987	583589	573587	**-18.5**
Methylone-D3	597487	573587	400031	**-33.0**
MDPV-D8	4704565	4565710	4060075	**-13.7**
NBOMe-D3	40576	34052	30446	**-25.0**

Blood QC3

Analyte		P.A. (n=3)		
	t=0	t=24	t=48	% Recovery
METHIOPROPAMINE	308427	281260	201196	**-34.8**
FLEPHEDRONE	807786	797603	732657	**-9.3**
MEPHEDRONE	1405057	1288758	1097087	**-21.9**
5-APB	3815088	3146785	2856737	**-25.1**
6-APB	4538164	3761946	3816466	**-15.9**
METHYLONE	1584732	1270633	1025044	**-35.3**
3-MeO-PCE	2768349	2472474	2273042	**-17.9**
BUTYLONE	5157944	4075442	4040964	**-21.7**
ETHYLONE	1983295	1313132	1391888	**-29.8**
MXE	2382708	1537903	1610773	**-32.4**
BENZEDRONE	13703712	130852868	11343415	**-17.2**
3-MeO-PCP	564688	425881	381647	**-32.4**
2-DPMP	14025611	121662533	11301711	**-19.4**
MDPV	11358446	137417393	10634451	**-6.4**
NAPHYRONE	14209553	133478678	12803045	**-9.9**
25B-NBOMe	54452	52441	49583	**-8.9**
25C-NBOMe	63760	56187	45964	**-27.9**
25D-NBOMe	53521	44950	40173	**-24.9**
25E-NBOMe	56313	54938	52478	**-6.8**
25H-NBOMe	66552	55771	50669	**-23.9**
25I-NBOMe	55383	52441	44516	**-19.6**
Mescaline-NBOMe	45609	46614	31125	**-31.8**
25P-NBOMe	54452	43701	33659	**-38.2**
Mephedrone-D3	489567	419791	379615	**-22.5**
Ethylone-D5	688644	591501	561245	**-18.5**
Methylone-D3	628862	512352	401978	**-36.1**
MDPV-D8	4598561	43763501	4170373	**-9.3**
NBOMe-D3	46540	41620	36192	**-22.2**

Appendix 9: Amphetamine ELISA instruction sheet.

IMMUNALYSIS

AMPHETAMINE DIRECT ELISA KIT

REF Immunalysis Corporation:
Catalog Number 209-0192 2 x 96 well plates
Catalog Number 209-0480 5 x 96 well plates
Catalog Number 209-4800 50 x 96 well plates

THE IMMUNALYSIS AMPHETAMINE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Amphetamine Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GC-MS) is the preferred confirmatory method (1). Professional judgment should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Amphetamine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of d-amphetamine in forensic samples such as whole blood, oral fluids, serum, plasma and urine. While the assay will detect amphetamine use, interference by l-amphetamine and pseudo-ephedrine is virtually nonexistent.

Amphetamine is a potent central nervous system stimulant. The (+)-isomer also referred to as d-amphetamine is three to four times more potent than the (-)-isomer, l-amphetamine (2). Amphetamine may be metabolized and excreted as the p-hydroxy isomer. Amphetamines act by inducing euphoria, irritability, anxiety and paranoia. Urinary excretion rates are influenced by the urinary pH with acidic urine favoring the excretion of unchanged drug (2). Up to 80% of a given dose may be excreted unchanged, especially in acid urine. Alkaline urine reduces the excretion of unchanged amphetamine to less than 5% of the dose.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Amphetamine Direct ELISA Kit (for d-amphetamine measurement) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled d-amphetamine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis Amphetamine Direct ELISA Kit avoids extraction of urine sample for measurement. It employs a d-amphetamine directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Amphetamine Direct ELISA Kit are itemized below:

- 12x75 mm Disposable Glass or Plastic Culture Tubes to pre-dilute samples (if required)
- Manual or electronic micropipets (single channel or multi channel) or automated pipetting stations
- Refrigerator (for kit storage)
- Interval Timer
- Wash bottle or Plate Washer
- Micro-plate reader capable of reading at 450 nm and 650 nm

REAGENTS

CONTENTS Immunalysis Amphetamine Direct ELISA Kit Contents

Component	192 Test Kit Cat# 209-0192	480 Test Kit Cat# 209-0480	4800 Test Kit Cat# 209-4800
96 well Micro-plate	2	5	50
d-Amph-Conjugate	25 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate The micro-plate is coated with polyclonal anti-d-amphetamine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Amphetamine-Enzyme Conjugate The conjugate solution contains d-amphetamine labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing azide free preservatives. (Colored Red)

Negative Standard This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxide in buffer.

Stop solution This contains 1 N hydrochloric acid.

Precautions

1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle Acid stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate strips and desiccant is sealed well, if only a few strips are used.

General Precise pipetting is the essence of successful radio immunoassay. Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 8° C.

Indications of Deterioration A drop of greater than 50% in the A_0 value (zero standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions
The Immunalysis Amphetamine Direct ELISA Kit is to be used with human forensic samples, such as whole blood, oral fluids, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. **Cutoff criteria are important in deciding the sample dilution.**

Additives
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions
Urine samples should be stored at 2 - 4° C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence, using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:20 for an Amphetamine cutoff of 500 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.

3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25^0 C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

d-amphetamine ng/ml	Absorbance
0	2.459
10	0.891
25	0.431
50	0.255

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for amphetamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for amphetamine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
Forty whole blood samples and 40 urine samples collected from presumed non-users were tested in the Immunalysis Amphetamine Direct ELISA Kit. One hundred percent of these normal samples measured negative at 50 ng/ml for whole blood and 500 ng/ml for urine. Thirty five whole blood samples which were previously confirmed positive for amphetamine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Immunalysis Amphetamine Direct ELISA Kit. All of the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

Precision
The precision of the Immunalysis Amphetamine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0, 10, 25 and 50 ng/ml standard was assayed five times in the same assay. The results are tabulated in Table 1.

Table 1

Amphetamine (ng/ml)	Mean Abs.	S.D.	C.V.%
0	2.399	0.115	4.8
10	0.897	0.095	10.6
25	0.458	0.061	13.32
50	0.271	0.022	8.12

Sensitivity
Assay sensitivity based on the minimum amphetamine concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

Specificity
The specificity of the Immunalysis ELISA for Amphetamine was determined by generating inhibition curves for each of the compounds listed below. The antisera cross-reactivities are listed in Table 2.

Table 2

Compound	Approx. ng/ml equivalent to 25ng amphetamine	Cross-reactivities percentage
l-Amphetamine	865	2.9
Hydroxyamphetamine HCl	57	44
l-Methamphetamine HCl	1250	2
dl-MDA (methylenedioxyamphetamine)	10	250
d-Methamphetamine HCl	417	6.5
dl- HMA (hydroxy methoxyamphetamine)	100	25
Phenteramine	28	89
Fenfluramine	>2500	<1
d-Ephedrine	>2500	<1
l-Ephedrine	>2500	<1
d-Phenylpropanolamine	>2500	<1
l-Phenylpropanolamine	>2500	<1
dl-MDMA (methylene dioxymethamphetamine)	>2500	<1
dl-MDEA (methylene dioxyethylamphetamine)	>2500	<1
d-Pseudoephedrine	>2500	<1
l-Pseudoephedrine	>2500	<1
dl-MBDB (N-methyl-3,4methylene dioxyphenyl-2-butanamine)	>2500	<1
Tyramine	>2500	<1
Methylphenidate	>2500	<1

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 5,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (1 ng/ml).
Acetaminophen, Acetylsalicylic acid, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Benzoylecgonine, Butabarbital , Caffeine , Cocaine, Carbamazepine, Codeine , Chloroquine , Chloropromazine, Carbromal , Desipramine, Dextromethorphan, Dextropropoxyphene , 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazepine , Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide , Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone , Methadone-primary metabolite, Methaqualone, Methbarbital , Mephenytoin, "-Methyl-"-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine , Meperidine , Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

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Revision: C
Revision Date: 06/2011

Appendix 10: Methamphetamine ELISA instruction sheet.

IMMUNALYSIS

METHAMPHETAMINE DIRECT ELISA KIT

REF **Immunalysis Corporation :**
Catalog Number 211-0192 2 x 96 well plates
Catalog Number 211-0480 5 x 96 well plates
Catalog Number 211-4800 50 x 96 well plates

THE IMMUNALYSIS METHAMPHETAMINE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Methamphetamine Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GC-MS) is the preferred confirmatory method (1). Professional judgment should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Methamphetamine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of d-methamphetamine in forensic samples such as whole blood, oral fluids, serum, plasma and urine. While the assay will detect amphetamine use, interference by l-methamphetamine and pseudo-ephedrine is virtually nonexistent.

Methamphetamine is a potent central nervous system stimulant(1) with less peripheral actions than amphetamine. The (+)-isomer also referred to as d-methamphetamine is ten times more potent than the (-)-isomer, l-methamphetamine. Amphetamines act by inducing euphoria, irritability, anxiety and paranoia Methamphetamine is metabolized to its active metabolite amphetamine (via N-demethylation) and is further metabolized by hydroxylation and deamination of amphetamine. Urinary excretion rates are influenced by the urinary pH with acidic urine favoring the excretion of unchanged drug(2). Alkaline urine reduces the excretion of unchanged methamphetamine to less than 5% of the dose.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Methamphetamine Direct ELISA Kit (for d-methamphetamine measurement) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled d-methamphetamine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis Methamphetamine Direct ELISA Kit avoids extraction of urine sample for measurement. It employs a d-methamphetamine directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size, reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Methamphetamine Direct ELISA Kit are itemized below:

- 12x75 mm Disposable Glass or Plastic Culture Tubes to pre-dilute samples (if required)
- Manual or electronic micropipets (single channel or multi channel) or automated pipetting stations
- Refrigerator (for kit storage)
- Interval Timer
- Wash bottle or Plate Washer
- Micro-plate reader capable of reading at 450 nm and 650 nm

REAGENTS

CONTENTS Immunalysis Methamphetamine Direct ELISA Kit Contents

Component	Test Kit Cat#211-0192	480 Test Kit Cat#211-0480	4800 Test Kit Cat#211-4800
96 well Micro-plate	2	5	50
d-Meth-Conjugate	25 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate The micro-plate is coated with polyclonal anti-d-methamphetamine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Methamphetamine-Enzyme Conjugate The conjugate solution contains d-methamphetamine labeled with horseradish peroxidase in a buffered protein solution with stabilzers, pH 7.6 containing azide free preservatives. (Colored Red)

Negative Standard This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxide in buffer.

Stop solution This contains 1 N hydrochloric acid.

Precautions

1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate and desiccant is well sealed, if only a few strips are used.

General Precise pipetting is the essence of successful radio immunoassay. Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at $2 - 8^0$ C.

Indications of Deterioration A drop of greater than 50% in the A_0 value (zero standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions
The Immunalysis Methamphetamine Direct ELISA Kit is to be used with human forensic samples, such as whole blood, oral fluids, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. **Cutoff criteria are important in deciding the sample dilution.**

Additives
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions
Urine samples should be stored at 2 - 4 degrees centigrade until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE

All reagents must be brought to room temperature (20-25^0 C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:20 for a methamphetamine cutoff of 500 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.

3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25^0 C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

d-methamphetamine ng/ml	Absorbance
0	1.519
10	0.649
25	0.471
50	0.359

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for methamphetamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for methamphetamine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
Sixty whole blood samples and 40 urine samples collected from presumed non-users were tested in the Immunalysis Methamphetamine Direct ELISA Kit. One hundred percent of these normal samples measured negative at 50 ng/ml for whole blood and 500 ng/ml for urine. Fifty five whole blood samples which were previously confirmed positive for methamphetamine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Immunalysis Methamphetamine Direct ELISA Kit . All of the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

Precision
The precision of the Immunalysis Methamphetamine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0,10, 25 and 50 ng/ml standard was assayed five times in the same assay. The results are tabulated in Table 1.

Table 1

Methamphetamine (ng/ml)	Mean Abs.	S.D.	C.V.%
0	1.652	0.089	5.4
10	0.697	0.055	7.9
25	0.504	0.0614	12.2
50	0.366	0.0391	10.7

Sensitivity
Assay sensitivity based on the minimum methamphetamine concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

Specificity

The specificity of the Immunalysis ELISA Methamphetamine was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

Table 2
Methamphetamine Antiserum Cross-reactivities

Compound	Approx. ng/ml equivalent to 50ng/ml d-methamphetamine	Cross-reactivities
d,l-methamphetamine	77	65
l-methamphetamine	625	8
d,l-MDMA	37	135
d-amphetamine	2500	2
l-amphetamine	1500	3.4
p-hydroxyamphetamine	>5000	<1.0
d,l-MDA	>5000	<1.0
d,l-MDEA	500	10
d,l- MBDB	1000	5
d,l-HMA	>5000	<1.0
fenfluramine	1000	5
d-ephedrine	4300	1.2
l-ephedrine	>5000	<1.0
d,l-ephedrine	>5000	<1.0
beta - phenethylamine	>5000	<1.0
phentermine	>5000	<1.0
d-phenylpropanolamine	>5000	<1.0
d,l-phenylpropolamine	>5000	<1.0
d-pseudoephedrine	>5000	<1.0
l-pseudoephedrine	>5000	<1.0
tyramine	>5000	<1.0

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (1 ng/ml).

Acetaminophen, Acetylsalicylic acid, Aminopyrine, Ampicillin Amobarbital, Ascorbic acid, Atropine, Barbital, Benzoylecgonine, Butabarbital, Caffeine , Cocaine, Carbamazepine, Codeine, Chloroquine, Chloropromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro carbamazepine, Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Metharbital , Mephenytoin, "-Methyl-"-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital, Phensuximide,PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH.

REFERENCES

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Revision: C
Revision Date: 06/2011

Appendix 11: Ketamine ELISA instruction sheet.

KETAMINE DIRECT ELISA KIT

Version: 11/2007

Immunalysis Corporation:
Catalog Number 240-0096 1 x 96 well plates
Catalog Number 240-0480 5 x 96 well plates

THE IMMUNALYSIS KETAMINE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Ketamine Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method. Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis KETAMINE Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of Ketamine in forensic samples such as whole blood, serum, plasma and urine.

Ketamine is a dissociative anesthetic use in human and veterinary medicine. The hydrochloride salt is sold as Ketanest, Ketaset, and Ketalar. Ketamine is classified as an N-Methyl d-Aspartate (NMDA) receptor antagonist (1). Another drug in this class is Phencyclidine (PCP). Ketamine induces a state referred to as "dissociative anesthesia" As with other pharmaceuticals of this type, ketamine is used illicitly as a recreational drug. It sometimes referred to as Special K.

Ketamine is usually administered intravenously or intramuscularly. It is also effective if taken orally, smoked or insufflated.

PRINCIPLES OF THE PROCEDURE

The Immunalysis KETAMINE Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled Ketamine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 500 pg/mL.

The Immunalysis KETAMINE Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs a KETAMINE directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate, much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size, reducing matrix effects and interference with binding proteins(s) or other macromolecules.

Materials and Equipment
Materials and equipment required but not supplied with the Immunalysis Ketamine Direct ELISA Kit are itemized below:

12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. and 650 nm.

REAGENTS
Immunalysis Ketamine Direct ELISA Kit Contents.

Component	96 TestKit Cat#240-0096	480 Test Kit Cat#240-0480
96 well Micro-plate	1	5
Ketamine Conjugate	15 ml	60 ml
Positive Ref. Std	2 ml	5 ml
Neg Std	2 ml	5 ml
TMB Substrate	30 ml	2 x 30 ml
Stop Reagent	25 ml	55 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-Ketamine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a dessicant.

Ketamine-Enzyme Conjugate The conjugate solution contains a Ketamine derivative labeled with horseradish peroxidase in a stabilized protein buffer solution, pH 7.6 containing azide free preservatives

Positive Reference Standard. This contains 25 ng/ml of Ketamine dissolved in a synthetic urine containing azide free preservatives. This is to be diluted to the laboratory cutoff.

Negative Standard. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating

chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle Acid stop reagent with care, since it is dilute acid.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation. Automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4^0 C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.

The Immunalysis KETAMINE Direct ELISA Kit is to be used with human forensic samples, such as whole blood, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. **Cutoff criteria are important in deciding the sample dilution.**

Additives.

Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.

Urine samples should be stored at 2 – 4^0 C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25 C) before use.

BLOOD

For a blood cutoff level of 10 ng/ml of Ketamine, dilute all controls and samples 1:10 in Phosphate Buffer Saline and use 50 µl of the diluted sample in each well.

URINE

For a urine cutoff level of 50 ng/ml of Ketamine, dilute all controls and samples 1:20 in Phosphate Buffer Saline and use 20 µl of the diluted sample in each well.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range. The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. or the appropriate volume of the diluted calibrators and standards to each well in duplicate (recommended).
3. Add 10 µl. or the appropriate volume of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25^0 C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled or de-ionized water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

Ketamine pg/well	Absorbance
0	2.283
50	1.006
100	0.782
250	0.581
500	0.364

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Ketamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Ketamine.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy

30 urine samples collected from presumed non-users were tested in the Immunalysis Ketamine Direct ELISA Kit. All these normal samples measured negative at 50 ng/ml for urine. 10 urine samples were spike with Ketamne levels greater than 50 ng/mL were tested in the Immunalysis Ketamine Direct ELISA Kit. All these samples were positive at a 50 ng/mL cutoff.

Precision

The precision of the Immunalysis Ketamine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision

Intra-assay precision was determined with reference controls. A 0, 50, 100 and 250 pg/well of Ketamine spiked urine was assayed sixteen (16) times in the same assay. The results are tabulated in Table 1.

Table 1

Ketamine (pg/well)	Mean Abs.	S.D.	C.V.%
0	2.214	0.100	4.53
50	1.062	0.044	4.13
100	0.814	0.048	5.91
250	0.539	0.028	5.16

Inter-assay Precision

Inter-assay precision was determined with reference controls. A 50, 100 and 250 pg/well of Ketamine spiked urine was assayed seven independent assays over 5 days with eight replicates in the each assay. The results are tabulated in Table 2

Table 2

Ketamine (pg/well)	B/B0%	S.D.	C.V.%
50	44.13	2.21	5.00
100	33.94	1.33	3.92
250	22.31	1.91	8.54

B/B0 is the Absorbance of the individual well divided by the Absorbance of the Zero Standard expressed as a percentage

Sensitivity

Assay sensitivity based on the minimum Ketamine concentration required to produce a two standard deviation from assay zero absorbance is 500 pg/ml.

Specificity

The specificity of the Immunalysis ELISA for Ketamine was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in

Table 3.

Cross Reactivities with Related Drugs

All controls and related drug spikes were diluted 1:10 in PBS and 10 uL of the diluted sample added to each well

TABLE 3

Compound	Conc ng/mL	Approx ng/mL equivalent to Ketamine	% Cross reactivity
Ketamine	5	5	100
Norketamine	100	5	5
PCP	1000	8	0.8
Dextromethorphan	10000	ND	ND
Amitriptyline	10000	8	0.08
Desipramine	10000	5	0.05
Imipramine	10000	8	0.08
Proptiyptyline	10000	5	0.05
Trimipramine	10000	5	0.05
Nortriptyline	10000	25	0.25

Cross-Reactivity with Unrelated Drugs

A human urine matrix was spiked with the following compounds at a concentration of 10,000 ng/ml. None of these compounds gave values in the assay that are equal to or greater than the assay sensitivity level (500 pg/ml).

Alprazolam, Amphetamine , Amobarbital, Barbital, Benzylpiperazine, Benzoylecgonine, Bromazepam, Butabarbital, 2C-B, Caffeine , Cocaine, Δ-9-THC , Carbamazepine, Chlorpromazine, Carbromal, Clonazepam, Dextromethorphan, Diazepam, 10,11-Dihydorcarbamazepine, Diphenhydramine, Doxepin, Ephedrine, Ethosuximide, Ethotoin, Flunitrazepam, Flurazepam, Glutethimide , Hexobarbital, Lidocaine, Lorazepam, MDA, MDMA, MDEA, Medazepam, Meprobamate, Methaqualone, Methadone Metabolite, Metharbital, Mephenytoin, Methyl-α-propylsuccinimide, Mephobarbital, Metharbital, Methyl PEMA, Methsuximide, 4-Methylprimidone, Methamphetamine, Methaqualone, Methylphenidate, Methylprylon, Nitrazepam, N-Normethsuximide, Nordoxepin, Oxazepam, Phenobarbital, Phenytoin, Phensuximide,Phetermine, PEMA ,Primidone, Quinine, Secobarbital, Temazepam, TFMPP

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Appendix 12: IACUC letter of approval.

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Glenside, PA 19038
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
(Animal Welfare Assurance Number A4424-01)

April 14, 2014

Protocol #: 14-01-06
PI: Nisbet
Title: Evaluation of Behavioral and Pharmacological Parameters of new recreational drugs known as NB2OMe's

Dear Dr. Nisbet,

Thank you for your revised submission of the above referenced protocol. Your revisions were reviewed by the Institutional Animal Care and Use Committee (IACUC) and approved on **April 14, 2014**. **You are required to submit yearly progress reports to maintain approval from the IACUC for the length of this protocol.** This letter constitutes official notification of the approval, and you are authorized to continue the research as of the date of approval.

Under the provisions of Arcadia University's Animal Welfare Assurance for compliance with the Office of Laboratory Animal Welfare Regulations, the principle investigator is directly responsible for submitting to the IACUC any change in the use of animals. Note: All changes must be reviewed and approved by the Committee prior to implementation. In addition, any unanticipated, unusual, unexpected hazards or adverse events involving risk to the subject or others must be reported immediately to the Committee giving detail and your assessment of the occurrence.

This approval expires on **April 14, 2015**. Prior to that time, please consult the IACUC requirements for a progress report or termination report, as appropriate, and submit all completed paperwork to the Chair of COPRS. Note that the committee recommends submitting progress reports 90 days prior to expiration in order to allow adequate time for review. If a research project lasts longer than three years, at the completion of the third year the investigator must submit a complete packet (e.g. cover letter, IACUC protocol submission form, protocol summary, etc.) for review at the same level as the original proposal.

We wish you the best in your research endeavors!

Sincerely,

Scott Stackhouse, PhD
Chair, Institutional Animal Care and Use Committee
Chair, Committee for the Protection of Research Subjects

TEL: 215.572.2900 WWW.ARCADIA.EDU
450 S. Easton Road, Glenside, PA 19038-3295
WISDOM TO GROW ON, WORLDS TO EXPLORE

Appendix 13: IACUC protocol.

To be filled in by IACUC Office ---IACUC Form 2011
IACUC Number _____ Use Level _____
Date Received _____ Date Reviewed: _____ Date Approved: _____

All protocols must be typed. Submit **(1)** an original, clipped, **single-sided** signed copy of the protocol to the Secretary, IRB/IACUC,38 Heinz Hall; and**(2**) electronic copy **(PDF)** to IRB_IACUC@arcadia.edu.Written approval from the IACUC must be obtained before initiating any research, teaching, or testing involving vertebrate animals or animal by-products.

Protocol Title: Evaluation of Behavioral and Pharmacological Parameters of new recreational drugs known as NB2OMe's

Estimated dates of protocol: From: __Aug 2013_____ to _May 2014_ (not to exceed 3 years)

New proposal: __YES__

or Revision of IACUC Number __ _____ approval date ___ ____

or 3-year Resubmission of IACUC Number____ _____ approval date __ _____

Funding Sponsor/Agency__None_____

Principal Investigator (Arcadia faculty only): ___Lorna A Nisbet, BSc, MSc, _____

Department: ____ Chemistry & Physics_____ extension: __4098_____

E-mail address: __nisbetl@arcadia.edu _____ emergency contact #:__(267)-475-7335

Co-Investigator ____Karen S Scott, PhD._____

Status (student/staff/faculty): __Faculty_____ extension: __2675 _____

E-mail address: ____scottk@arcadia.edu _____ emergency contact #:(267)401-4627_____

Co-Investigator _____Joshua Blustein, PhD _____

Status (student/staff/faculty): __Faculty_____ extension: ___4002_____

E-mail address: ____blustein@arcadia.edu_____ emergency contact #:__(215)939-2332_____

Co-Investigator _____Alysha Andrews, BS_____
_____

Status (student/staff/faculty): __Student_____ extension: ___N/A_____

E-mail address: _____Aandrews@arcadia.edu____ emergency contact #:_(717)799-4323_____

Co-Investigator _____Alysia Kosmach, BS _____

Status (student/staff/faculty): __Student_____ extension: ___N/A_____

E-mail address: ____Akosmach@arcadia.edu_____ emergency contact #:__(570)814-3022_____

Co-Investigator _____Alex Krotulski, BS_____

Status (student/staff/faculty): __Student_____ extension: ___N/A_____

E-mail address: ____Akrotulski@arcadia.edu _____ emergency contact #:__(610)468-5616_____

IACUC Face Sheet/Animal Submission Proposal

Co-Investigator _____Aileen Lu, BS_____

Status (student/staff/faculty): __Student_____ extension: __N/A_____

E-mail address: ____Alu@arcadia.edu _____ emergency contact #:__(914)325-2500_____

Co-Investigator _____Kevin Picirilli , BS_____

Status (student/staff/faculty): __Student_____ extension: ___N/A_____

E-mail address: ____Kpicirilli@arcadia.edu _____ emergency contact #:__(440)539-0408_____

Species of Animals: ___Ratusrattus_____weight _**50g**_____ age**___3-4 weeks_____**

Strain: ___Long Evans_____ Source: __Harlan Laboratories_____

Number of animals in each category:

Category C: No pain or distress beyond that involved in the restraint, injections, or collection of samples.	Category D: potential for pain or distress but relief is provided by analgesics and/or sedatives as appropriate.	Category E: pain or distress not relieved by sedatives or analgesics.
525		

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Committee Action:
Tabled: _____ Approval Pending: _____Approved: _____ Disapproved _____
Ayes: _____ Nays: _____ Abstentions: _____ Absent: _____

CFurperson of IACUC: _____ Date: _____

1. Does the study involve animals in Humane Use Category D or E? **IF YES**, Complete and attach IACUC Form B – Alternatives.	☐ Yes
2. Does the study involve anesthetic, analgesic, tranquilizing, or neuromuscular blocking agents? **IF YES**, Complete and attach IACUC Form C – Anesthetic and analgesic agents	☒ Yes
3. Does the study involve a survival surgery? **IF YES**, Complete and attach IACUC Form D - Surgery:	☐ Yes
4. What will be the final disposition of animals? ☐ a. transfer to different protocol: Provide protocol IACUC approval number _____ ☒ b. Euthanasia-complete and attach IACUC Form E-Euthanasia ☐ c. Adoption – complete and attach IACUC Adoption release Form	
5. Do you or any member of your research group, spouses or any dependent children have any interest (i.e. any property of financial interest including stock in the sponsor company, patents, trademarks, copyrights or licensing, supplemental research grants or consulting arrangements) in the test drug/product, device, or research procedure that is the subject of this study? • **IF YES, please complete a Conflict of Interest Disclosure Form available through the Arcadia University Grants Office.** Please discuss how these conflicts will be managed during the period of the trial. 5a. In addition, for industry-sponsored trials, please attach the documentation submitted to the sponsor as required by 21CFR54.1, if applicable.	☐ Yes
6. Has the Principal Investigator and all co-investigators completed the animal CITI training modules? • **IF YES,** attach copy of certification.	☒ Yes
7. Has the Principal Investigator and all co-investigators completed the Instructor Training form (if appropriate)? **IF YES**, attach copy of certification.	☒ Yes
8. Has the Principal Investigator and all co-investigators signed the Animal Health Emergency Protocol document?	☒ Yes
9. Does the study involve using controlled substances? **IF YES**, have DEA rep sign below DEA representative signature if study involves controlled substances _____	☒ Yes

		Check all attachments that apply:

Y? Principal Investigator (PI) Signature**(Must be Arcadia faculty member)**		☒ Form A - Protocol Summary
Printed Name of PI: Lorna Nisbet	Date:	☐ Form B – Alternatives (if Item #1 checked)
Co-Investigator's Signature		
Printed Name of Co-Investigator: Dr Karen Scott	Date:	☒ Form C – Anesthetic and analgesic agents (If #2checked)
Co-Investigator's Signature		☐ Form D – Surgery (If Item #3 checked)
Printed Name of Co-Investigator: Dr Joshua Blustein	Date:	
Co-Investigator's Signature		☒ Form E – Euthanasia (If item 4b checked)
Printed Name of Co-Investigator: Alysha Andrews	Date:	☐ Conflict of Interest Disclosure Form (If Item checked)
Co-Investigator's Signature		☒ Copy of CITI Lab Animal Course certificatio
Printed Name of Co-Investigator: Alysia Kosmach	Date:	☐ Copy of grant application (minus appendices)
Co-Investigator's Signature		
Printed Name of Co-Investigator: Alex Krotulski	Date:	
Co-Investigator's Signature		
Printed Name of Co-Investigator: Aileen Lu	Date:	
Co-Investigator's Signature		
Printed Name of Co-Investigator: Kevin Picirilli	Date:	

STUDENT INVESTIGATOR'S ASSURANCE

I certify that the information provided is complete and correct.

I understand that as Student Investigator, I have responsibility for the care and use of the animals in these proposed research/teaching activities. I agree to comply with all Arcadia University policies and procedures, as well as with all applicable federal, state, and local laws regarding the protection of animals in research, teaching, and testing including, but not limited to, the following:

- the project will be performed by qualified personnel according to the research project/protocol,
- obtain necessary review by the AU IACUC if changes are made in the research project/protocol, and
- I agree to meet with my faculty advisor (PI of the study)on a regular basis to review study progress.

I acknowledge that the completion of this work occurs within the oversight of the Arcadia University Institutional Animal Care and Use Committee. This oversight includes, but is not limited to, the following:

- written IACUC approval must be obtained prior to any animal use or initiation of the project,
- **ANY**revision to the protocol must be approved by the IACUC **PRIOR** to implementing changes,
- renewal of the protocol is required on an annual basis (completion and submission of IACUC Form F),
- on-going protocols must be approved de novo every three years,
- submission of a termination report is required at the completion of the project, and
- failure to provide proper animal care or follow the approved protocol or AU IACUC guidelines may result in the suspension of the project or loss of Arcadia University animal use certification.

I have read and understand the above.

_____ Alysha Andrews _____

Student Investigator Signature Printed Name
Date

_____ Alysia Kosmach _____

Student Investigator Signature Printed Name
Date

_____ Alex Krotulski _____

Student Investigator Signature Printed Name
Date

_____ Aileen Lu _____

Student Investigator Signature Printed Name
Date

_____ Kevin Piccirilli _____

Student Investigator Signature Printed Name
Date

PRINCIPAL INVESTIGATOR(FACULTY) ASSURANCE

By my signature as Principle Investigator on this research application, I certify that the student investigators are knowledgeable about the regulations and policies governing use of animals and have sufficient training and experience to conduct this particular study in accord with the approved project/protocol. In addition,

- I agree to meet with the student investigators on a regular basis to review study progress.
- Should problems arise during the course of the study, I agree to be available to supervise the student investigators in solving them.
- I assure that the student investigators have completed all required educational IACUC and Occupational Health and Safety programs as required (CITI Training).
- I agree to abide by regulations that govern use of controlled substances (if applicable).
- I agree to assume responsibility for the final disposition of the animals.
- If I will be unavailable (such as on sabbatical, leave or vacation), I will arrange for an alternate faculty member to assume responsibility during my absence, and I will advise the IRB by letter of such an arrangement. This alternate faculty member will need to have passed all appropriate IACUC and Occupational Health and Safety programs (CITI Training).
- I am responsible for documentation of any adverse events related to the research project and notification of the faculty advisor (if primary investigator is a student), Animal Care Representative, and the CFur of AU IACUC by e-mail IRB_IACUC@arcadia.edu**within 24 hours of incidence**.
- **Any failure to provide proper animal care or follow the approved protocol or AU IACUC guidelines may result in the suspension of the project or loss of Arcadia University animal use certification.**

_____ Lorna A Nisbet_____ _____

Principal Investigator(Faculty) signature Printed Name Date

The Principal Investigator must be a member of the Arcadia University faculty. The Principal Investigator is considered the responsible party for legal and ethical performance of the project.

ANIMAL HEALTH EMERGENCY PROTOCOL

If an animal is found dead:

1. Immediately remove animal from cage and:
    a. Place it in a bag labeled with Animal ID, Date/Time found, Name of individual who found animal
    b. Place bag in small necropsy refrigerator
2. Visually inspect all of the other animals in the facility/room
3. Immediately contact one of the following (must speak with person):
    a. Dr. Blustein (215-939-2332)
    b. Dr. Scott (267-401-4627)
    c. If unable to reach contact Dr. Hoffman (215) 572-2195
4. Must also immediately notify the Faculty Animal Care Representative
    a. Dr. Wright (wrightm@arcadia.edu, Office:267-620-4827, Cell: 267-241-8241)

If an animal appears to be in distress (e.g., bloody nose, wheezing/panting/labored breathing, lying on side/lethargy, rapid weight loss >20%, self mutilation, abnormal vocalization, diarrhea):

1. Check food and water access.
2. Visually inspect all of the other animals in the facility/room.
3. Immediately contact one of the following (must speak with person)
    a. Dr. Blustein (215-939-2332)
    b. Dr. Scott (267-401-4627)
    c. If unable to reach contact Dr. Hoffman (215) 572-2195
4. Must also immediately notify the Faculty Animal Care Representative
    a. Dr. Wright (wrightm@arcadia.edu, Office:267-620-4827, Cell: 267-241-8241)

My signature below indicates that I have read and agree to abide by the above Animal Health Emergency Protocol:

_____ Lorna A Nisbet_____ _____
Principal Investigator(Faculty) signature Printed Name Date

_____ Karen S Scott_____
Co- investigator Signature Printed Name Date

_____ Joshua Blustein_____ _____
Co-Investigator Signature Printed Name Date

_____ Alysha Andrews_____ _____
Co-Investigator Signature Printed Name Date

_____ Alysia Kosmach_____ _____
Co-Investigator Signature Printed Name Date

_____Alex Krotulski

Co-Investigator Signature Printed Name Date

_____Aileen Lu_____

Co-Investigator Signature Printed Name Date

_____Kevin Piccirilli_____

Co-Investigator Signature Printed Name Date

List any drugs or medications the animals will receive. If it is a controlled drug, put (c) next to the drug. For all drugs, include dose (mg/kg), concentration (mg/ml) and mode of administration (i.e., oral, subcutaneous injection, intraperitoneal injection, intramuscular injection, etc.) Provide a reference for the use of the drug at this dose.

Drug	dose	concentration	route	reference
25T2-NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25T4- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25T7- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25H- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25I- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E(Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25B- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25E- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25D- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25C- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25N- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
25P- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)
Mescaline- NB2OMe	30µg/kg	30 µg/ml	IP	A-E (Below)
	100µg /kg	100µg/ml	IP	A-E (Below)
	300µg/kg	300 µg/ml	IP	A-E (Below)

Methiopropamine	5mg/kg	5mg/ml	IP	G (Below)
	10mg/kg	10mg/ml	IP	G(Below)
	20 mg/kg	20 mg/ml	IP	G(Below)
Fluoroephedrone	5 mg/kg	5 mg/ml	IP	I (Below)
	10 mg/kg	10 mg/ml	IP	I (Below)
	20 mg/k	20 mg/ml	IP	I (Below)
5-APB	6mg/kg	6mg/ml	IP	M(Below)
	18 mg/kg	18 mg/ml	IP	M(Below)
	30 mg/kg	30 mg/ml	IP	M(Below)
6-APB	6mg/kg	6mg/ml	IP	M(Below)
	18 mg/kg	18 mg/ml	IP	M(Below)
	30 mg/kg	30 mg/ml	IP	M(Below)
MDAI	0.5 mg/kg	0.5 mg/ml	IP	H(Below)
	3 mg/kg	3 mg/ml	IP	H(Below)
	5 mg/kg	5 mg/ml	IP	H(Below)
Methedrone	0.5 mg/kg	0.5 mg/ml	IP	C(Below)
	3 mg/kg	3 mg/ml	IP	C(Below)
	5 mg/kg	5 mg/ml	IP	C(Below)
3-Me-O-PCE	25 mg/kg	25 mg/ml	IP	N(Below)
	40 mg/kg	40 mg/ml	IP	N(Below)
	60 mg/kg	60 mg/ml	IP	N (Below)
Camfetamine	2mg/kg	2mg/ml	IP	O(Below)
	6 mg/kg	6 mg/ml	IP	O(Below)
	10 mg/kg	10 mg/ml	IP	O(Below)
5-IAI	0.5mg/kg	0.5mg/ml	IP	F (Below)
	1.0 mg/kg	1.0mg/ml	IP	F (Below)
	1.5mg/kg	1.5mg/ml	IP	F (Below)
Butylone	2mg/kg	2mg/ml	IP	P(Below)
	6 mg/kg	6 mg/ml	IP	P(Below)
	10 mg/kg	10 mg/ml	IP	P(Below)
Ethylone	2mg/kg	2mg/ml	IP	P (Below)
	6 mg/kg	6 mg/ml	IP	P (Below)
	10 mg/kg	10 mg/ml	IP	P (Below)
Methoxetamine	12.5 mg/kg	12.5 mg/ml	IP	I &N (Below)
	20 mg/kg	20 mg/ml	IP	I &N(Below)
	30 mg/kg	30 mg/ml	IP	I &N (Below)
Benzedrone	60µg/kg	30 µg/ml	IP	R (Below)
	0.4mg /kg	0.4mg/ml	IP	R (Below)
	1.2mg/kg	1.2mg/ml	IP	R (Below)
3-MeO-PCP	2.5mg/kg	2.5mg/ml	IP	N (Below)
	3mg /kg	3mg/ml	IP	N (Below)

	5mg/kg	5mg/ml	IP	N (Below)
Desoxypipradol	0.4 mg/kg	0.4 mg/ml	IP	L(Below)
	1.2 mg/kg	1.2 mg/ml	IP	L (Below)
	2 mg/kg	2 mg/ml	IP	L (Below)
MDPV	60µg/kg	60 µg/ml	IP	J(Below)
	0.4mg /kg	0.4mg/ml	IP	J(Below)
	1.2mg/kg	1.2mg/ml	IP	J(Below)
Naphyrone	0.4 mg/kg	0.4 mg/ml	IP	K(Below)
	1.2 mg/kg	1.2 mg/ml	IP	K(Below)
	2 mg/kg	2 mg/ml	IP	K(Below)
Azacylonol	2mg/kg	2mg/ml	IP	Q(Below)
	6 mg/kg	6 mg/ml	IP	Q(Below)
	10 mg/kg	10 mg/ml	IP	Q(Below)

References:

A. Barnes &Eltherington (1973). Drug Dosage in Laboratory Animals. University of California Press. 2nd Ed; 41-42, 158.
B. Ouagazzal A.-M.,Grottick A.J.,Moreau J.-L., Higgins G.A.**Effect of LSD on prepulse inhibition and spontaneous behavior in the rat: A pharmacological analysis and comparison between two rat strains,** (2001)*Neuropsychopharmacology*, 25 (4) , pp.565-575.
C. ZubaD,Sekuła K,Buczek A, **25C-NBOMe – New potent hallucinogenic substance identified on the drug market**, (2013)*Forensic Science International*, 227, (1–3), pp 7-14.
D. Sekula K, Zuba D, **Structural elucidation and identification of a new derivative of phenethylamine using quadrupole time-of-flight mass spectrometry**, (2013) Mass Spectom, 27, pp1-10.
E. Stellpflug S et al, **2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe): Clinical Case with unique Confirmatory Testing**, (2013) J. Med. Toxicol
F. M. Coppola, R. Mondola, **5-Iodo-2-aminoindan (5-IAI): Chemistry, pharmacology, and toxicology of a research chemical producing MDMA-like effects,** (2013), Toxicology Letters,218,pp24-29
G. Welter, J.; Meyer, M. R.; Wolf, E. U.; Weinmann, W.; Kavanagh, P.; Maurer, H. H., **2-methiopropamine, a thiophene analogue of methamphetamine: studies on its metabolism and detectability in the rat and human using GC-MS and LC-(HR)-MS techniques**. *Anal Bioanal Chem* **2013,** *405* (10), 3125-35.
H. Gallagher, C. T.; Assi, S.; Stair, J. L.; Fergus, S.; Corazza, O.; Corkery, J. M.; Schifano, F., **5,6-Methylenedioxy-2-aminoindane: from laboratory curiosity to 'legal high**'. *Hum Psychopharmacol* **2012,** *27* (2), 106-12; Hofer, K. E.; Grager, B.; Müller, D. M.; Rauber-Lüthy, C.; Kupferschmidt, H.; Rentsch, K. M.; Ceschi, A., Ketamine-like Effects After Recreational Use of Methoxetamine. *Annals of Emergency Medicine* **2012,** *60* (1), 97-99.
I. Meyer, M. R.; Vollmar, C.; Schwaninger, A. E.; Wolf, E.; Maurer, H. H., **New cathinone-derived designer drugs 3-bromomethcathinone and 3-fluoromethcathinone: studies on their metabolism in rat urine and human liver microsomes using GC-MS and LC-high-resolution MS and their detectability in urine**. *J Mass Spectrom* **2012,** *47* (2), 253-62.
J. Kriikku, P.; Wilhelm, L.; Schwarz, O.; Rintatalo, J., New designer drug of abuse**: 3,4-Methylenedioxypyrovalerone (MDPV). Findings from apprehended drivers in Finland.** *Forensic Science International* **2011,** *210* (1–3), 195-200.
K. Brandt, S. D.; Freeman, S.; Sumnall, H. R.; Measham, F.; Cole, J., **Analysis of NRG 'legal highs' in the UK: identification and formation of novel cathinones**. *Drug Test Anal* **2011,** *3* (9), 569-75.
L. James D A, P. S., Thomas SHL, Chincholkar VM, Clarke S, Dear J, Ramsey J, **Clinical Features Associated with Recreational Use of 'Ivory Wave' Preparations Containing Desoxypipradol.** *Clinical Toxicology* **2011,** *49* (3).
M. Dawson, P.; Opacka-Juffry, J.; Moffatt, J. D.; Daniju, Y.; Dutta, N.; Ramsey, J.; Davidson, C., **The effects of benzofury (5-APB) on the dopamine transporter and 5-HT2-dependent vasoconstriction in the rat**. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **2014,** *48* (0), 57-63.
N. Westwell, A. D.; Hutchings, A.; Caldicott, D. G. E**., The identification and chemical characterization of a new arylcyclohexylamine, methoxetamine, using a novel Emergency Department toxicosurveillance tool**. *Drug Testing and Analysis* **2013,** *5* (3), 203-207.

O. Kavanagh, P.; Angelov, D.; O'Brien, J.; Power, J. D.; McDermott, S. N. D.; Talbot, B.; Fox, J.; O'Donnell, C.; Christie, R. (2012). **The synthesis and characterization N-methyl-3-phenyl-norbornan-2-amine (Camfetamine™)**. *Drug Testing and Analysis* **5** (4)
P. Zaitsu K, Katagi M, Kamata HT, Kamata T, Shima N, Miki A, Tsuchihashi H, Mori Y (July 2009). **Determination of the metabolites of the new designer drugs bk-MBDB and bk-MDEA in human urine**. *Forensic Science International* **188** (1–3): 131–9.
Q. BRAUN DL, BROWN BB, FELDMAN RG (October 1956). ***The pharmacologic activity of alpha-(4-piperidyl)-benzhydrol hydrochloride (azacyclonol hydrochloride); an ataractive agent*** . *The Journal of Pharmacology and Experimental Therapeutics* **118** (2): 153–61.
R. Nic Daeid, N.; Savage, K. A.; Ramsay, D.; Holland, C.; Sutcliffe, O. B**., Development of gas chromatography–mass spectrometry (GC–MS) and other rapid screening methods for the analysis of 16 'legal high' cathinone derivatives.** *Science & Justice, (0).*

Example:

Drug	dose	concentration	route	reference
LSD	0.03, 0.1 and 0.3mg/kg		IP	B

Number of Animals, Species, and Humane use Categories:
The Animal Welfare Act requires annual reporting of animal use according to the following categories.

Estimate the total number of animals that will be used in each Humane Use Category in the proposed study.

Humane Use Categories

	Description:	*animals requested*
C	*No or minimal pain and/or stress (with or without the use of pain-relieving agents and techniques).* No pain or distress beyond that involved in the restraint, injections, or collection of samples. For comparison, no pain relieving drugs would be given under normal circumstances for a human patient going through the same procedure.	525
D	*Pain and/or stress that does not become intolerable and thereby distressful.* Potential for pain or distress but relief is provided by analgesics and/or sedatives as appropriate. The USDA regards survival and non-survival surgery to fall in this category. This category includes all procedures in which the animal may experience pain, discomfort, or distress which would be treated with the use of anesthetics, analgesics, or tranquilizers. Therefore, this category includes euthanasia via anesthetic overdose.	0
E	*Pain and/or stress that reaches the level of distress.* Pain or distress not relieved by sedatives or analgesics. This category includes procedures expected to cause pain, discomfort, or distress but the administration of normal anesthetics, analgesics, or tranquilizers cannot be used without adversely affecting the experimental results.	0
	Total Animals Requested	525

Description of Project:

1. How would you explain to a non-scientist the goals and objectives of the proposed work?

New psychoactive substances (NPS's) have become an integral part of the UK and USA recreational drug market. These substances are marketed as "legal" and not for human consumption to negate controls via medicines acts and are thus wrongly deemed by users to be safe. Mephedrone was the first such substance to be marketed in this way appearing in 2007, and IT gained rapid popularity amongst recreational drug users. Since the control of mephedrone both in the UK and USA many more substances such as aminoindanes, NBOMe's and additional synthetic cathinones have been rapidly produced to replace this substance on the "legal" market. NB2OMe's in particular are a relatively new class of drugs that are structurally similar and thought to potentiate similar behavior to 2C-I. They are often classified as "Legal Highs" due to these structural similarities and the fact that they were not classified as illegal. 2C-I is a Schedule I drug, due to the potential dangers and side effects experienced when recreationally abused. NBOMe'S are thought to potentiate similar behavioral and pharmacological effects as their 2C-I derivative such as analgesia, tolerance and metabolism.

A search on PubMed with the keywords NB2OMe's and analgesia revealed no published data on NB2OMe's producing analgesia. To date very few articles have been published on these drugs with the majority focusing on accident and emergency case reports and the medical treatment of individuals who have overdosed. Research has also shown that these N-benzyphenethylamine 2C derivatives are potent serotonin 2A (5-HT2A) receptor antagonists with similar effects to LSD and other potent hallucinogenic drugs. Due to their high potency NB2OMe's are typically administered in liquid form or on "blotters", again similar to LSD. Recent research has commented on the lack of animal studies or toxicological information for these drugs.

A search on PubMed with the keywords cathinone and analgesia revealed 4 results although these were for cathinone itself and not for any synthetic cathinones. Further searches using individual synthetic cathinone names alongside analgesia produced no results. As analgesia has been shown with cathinone it is likely that it will also be seen in these newer synthetic cathinones whose structures are based on cathinone.

A search on PubMed with the keywords aminoindanes and analgesia produced 1 result although this focused on 2-aminoindane and not the 5-aminoindane proposed in this application. This research was carried out on mice that developed tolerance and displayed signs of analgesia. It is likely that this would also be seen with the 5-aminoindane compound.

Overall, the present study will investigate the gaps within the existing research of NPSs and their ability to produce analgesia. Analgesia and tolerance will be tested using a tail-flick test (52^{o}C). If tail-flick latency is significantly longer than the baseline, analgesia is present. Tolerance will be defined when tail-flick latency returns to baseline for three consecutive days. Once tolerance is achieved, withdrawal effects of each rat will be observed, using the tail-flick test and behavior within the cage. The present study will collect fur and urine samples from the rats. The fur urine and feces samples will be analyzed to find different possible metabolites that may be present for all of the drugs stated above at three different doses. The use of Long Evans rats will allow further hair analysis work to establish if drug incorporation occurs at different rates depending if white or black hair is analyzed. The animals will then be euthanized and stored for further work determining whether drugs and their metabolites can be detected in various tissue samples including bone.

2. How would you explain to a non-scientist the ways the proposed animal use might benefit human or animal health, the advancement of knowledge, or the good of society?

The proposed work will help further the knowledge of drug tolerance, analgesia, withdrawal and metabolism. The study will examine the individual effects NPS's have on analgesia, tolerance, withdrawal in Long Evans rats and examine the presence of parent drugs and metabolites in both fur,

urine, feces and various other tissue samples collected during the study, allowing better pharmacological and toxicological understanding of these drugs.

3. **Are they any adverse events (such as death, excessive weight loss, body temperature fluctuation, heart rate/respiration changes, etc.) expected or documented in the literature relevant to this particular study? If yes, please describe and provide a detailed course of action to minimize adverse events. You may need to adjust your sample size accordingly.**

Yes. Increased heart rate may be encountered due to the stimulant and psychedelic nature of some of these drugs. The rats will not be receiving doses that will cause dangerous stimulation effects only enough to examine the behavioral effect the drugs may potentiate. To date no published animal studies exist although research has shown NBOMe drugs have similar potency to LSD, cathinone derivatives are similar to methamphetamine and cathinone and aminoinanes are similar to amphetamines. The rats will be examined by comparing with the control group (the group dosed with saline solution only) to assess if they exhibit signs of hyper-activity, agitation and anxiety. If a rat is experiencing a great deal of stimulation that may be dangerous, the rat will be no longer be dosed and no longer be included in the experiment.

Project Summary: **Provide enough information so that the IACUC members can review the rationale and purpose of** the proposed study. Detail of experimental procedures, justification of animal numbers, and training and experience of personnel completing the procedures should also be provided. Use additional pages if necessary.

Procedure:

Prior to commencing these experiments, full training in handling of rats and administration of drugs via i.p. injection will be received. In the initial stages of the research, Dr. Blustein will be on site to monitor all injections. Rats will be purchased about 3 weeks prior to commencing the experiment. In batches of 35 rats, 15 rats will be administered drug A, 15 rats will be administered drug B and 5 rats will be used as a control group. The rats will be randomly assigned to three groups of 5, a total of 15 rats per drug. Each group containing 5 rats will be dosed and given the tail flick testfor 10 days or until tolerance is achieved. The rats will be weighed prior to each injection to determine the volume of solution for injection. For example the first group containing 15 rats will be injected with 25B-NB2OMe; 5 rats will be individually injected with 30µg/kg i.p, a different set of 5 rats will be individually injected with 100µg/kgi.p, and the last set of 5 rats will be individually injected with 300µg/kg i.p. of 25B-NB2OMe alone. The second group containing 15 rats will for example be individually injected with 25C-NB2OMe; 5 rats will be individually injected with 30µg/kg i.p of 25C-NB2OMe alone, 5 rats will be individually injected 100µg/kg i.p of 25C-NB2OMe alone, and 5 rats will be individually injected with 300µg/kg i.p25C-NB2OMe alone. There will be 5 separate rats which will be individually injected with saline to serve as a control group. This protocol will be applied to each drug listed previously (page 8-11). This will result in a total of 15 experiments (30 drugs to be tested, testing two drugs during each experiment). Analyzing multiple drugs at once reduces the numbers of control rats needed as the same controls can be used for each drug set. All rats will be housed separately so that no stresses interfere with the study. All injections will be given every 24 hours and administered with a sterile needle, 27G x ½'' attached to a 1cc tuberculin syringe. All drugs will be administered via intraperitoneal injection. Figure 1 is a scheme representing one complete experiment.

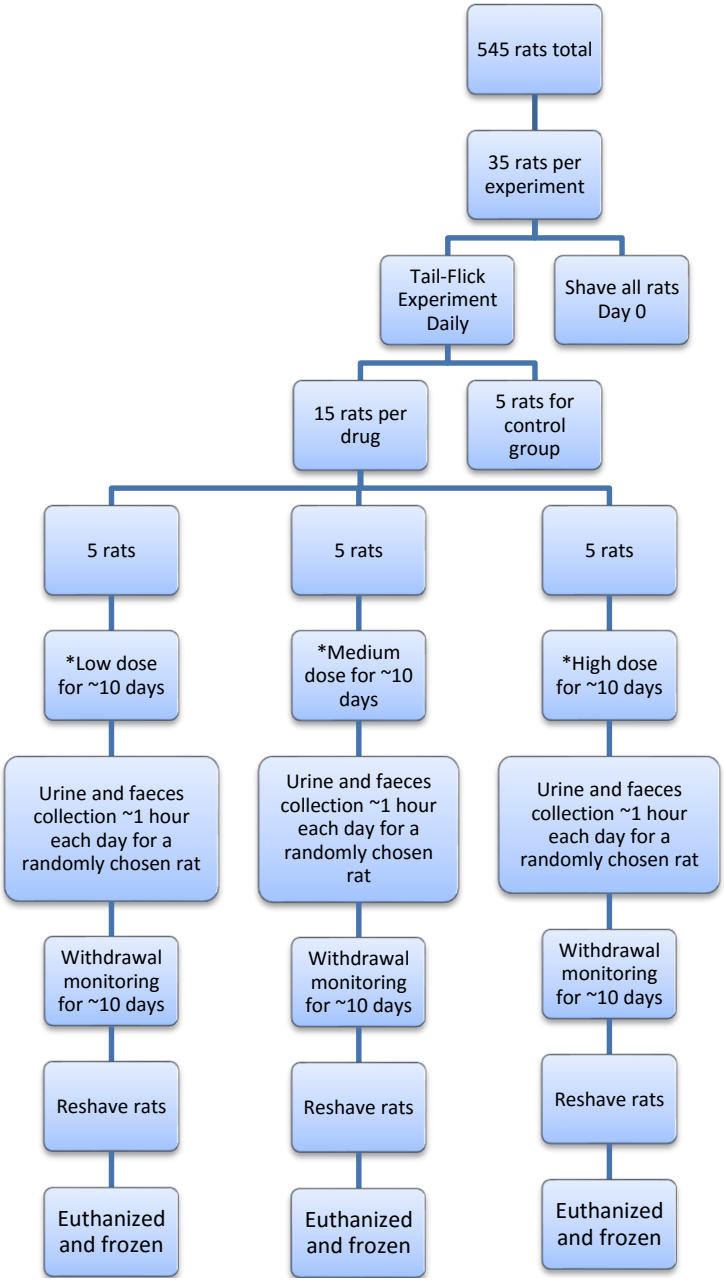

Figure 1: schematic representing one complete experiment.

Each rat will be weighed and given a tail-flick test using Fisher Scientific Isotope 220 warm water immersion bath (52ºC) to measure the baseline tail-flick latency. Rats will be weighed daily thought the duration of the study. The rats will be wrapped in a soft cloth and the first 8cm of their tail lowered into the 52ºC warm water while a stopwatch is started simultaneously. The first 8cm of each rats tail will be marked with permanent marker resulting to ensure consistency between days. Once the tail flicks out of the water, the latency will be recorded. If the tail does not flick out of the water in 20 seconds, the trial is ended and a maximum latency of 20 seconds is recorded. All of the rats throughout the experiment will

be placed in metabolism cages for collection of urine. There will be 6 metabolism cages in which randomly chosen rats will be placed in daily. The rats will be placed on a metal wire insert within the cage until they urinate. If they do not urinate within one hour, the rats will be placed back in their bedding. Feces will also be collected. Personal protective equipment (lab coat, gloves, and masks) will be worn when handling during urine and feces collection and cage cleaning. The collected urine/ feces will be used for analysis of any remaining unmetabolized parent drug plus metabolites by GC/MS for phase I metabolites and LC/MS/MS for phase II metabolites and using an UPLC-qTOF. The collected urine will be refrigerated prior to analysis. Fur samples will also be collected from the rats for analysis to determine the presence of both parent drug and phase I metabolites by GC/MS. For fur collection, the back of the rat will be shaved using standard clippers on Day 0 and Day 20 of the experiment. Long Evans rats where specifically chosen for this study as they have both black and white fur. The fur will be collected to ensure both black and white fur is obtained. From this an understanding of the processes of incorporation into the fur can be evaluated. The rats will not be under any physical pain or subjected to stress during the urine and fur collection procedures.

Table 1 is a conversion table that represents the amount of powdered drug needed to mix the desired concentrations explained below.

Table 1: Conversion Table					
Drug	**Drug free weight**	**Drug salt weight**	**Salt weight/Free weight**	**Weight required (mg)**	**Weight needed (mg)**
25B-NB2OMe	**380**	**416**	**1.1**	**5**	**5.5**
25C-NB2OMe	**335**	**371**	**1.1**	**5**	**5.5**
25D-NB2OMe	**315**	**351**	**1.1**	**5**	**5.5**
25E-NB2OMe	**329**	**365**	**1.1**	**5**	**5.5**
25H-NB2OMe	**301**	**337**	**1.1**	**5**	**5.5**
25I-NB2OMe	**427**	**463**	**1.1**	**5**	**5.5**
25N-NB2OMe	**346**	**382**	**1.1**	**5**	**5.5**
25P-NB2OMe	**343**	**379**	**1.1**	**5**	**5.5**
25T2-NB2OMe	**361**	**397**	**1.1**	**5**	**5.5**
25T4-NB2OMe	**375**	**411**	**1.1**	**5**	**5.5**
25T7-NB2OMe	**375**	**411**	**1.1**	**5**	**5.5**
Mescaline-NB2OMe	**331**	**367**	**1.1**	**5**	**5.5**
Methiopropamine	**155**	**191**	**1.2**	**275**	**330**
Fluoroephedrone	**181**	**217**	**1.2**	**275**	**330**
5-APB	**175**	**211**	**1.2**	**450**	**540**
6-APB	**175**	**211**	**1.2**	**450**	**540**
MDAI	**177**	**213**	**1.2**	**75**	**90**
Methedrone	**192**	**228**	**1.2**	**75**	**90**
3-MeO-PCE	**233**	**269**	**1.1**	**1000**	**1100**
Camfetamine	**201**	**237**	**1.2**	**150**	**180**
5-IAI	**259**	**295**	**1.1**	**75**	**82.5**
Butylone	**221**	**257**	**1.2**	**150**	**180**

Ethylone	**221**	**257**	**1.2**	**150**	**180**
Methoxetamine	**247**	**283**	**1.1**	**500**	**550**
Benzedrone	**253**	**289**	**1.1**	**15**	**16.5**
3-MeO-PCP	**273**	**309**	**1.1**	**100**	**110**
Desoxypipradol	**251**	**287**	**1.1**	**50**	**55**
MDVP	**275**	**311**	**1.1**	**15**	**16.5**
Naphyrone	**281**	**317**	**1.1**	**50**	**55**
AzacyClonol	**267**	**303**	**1.1**	**150**	**165**

Lorna nisbet will mix each drug from powder, and personal protective equipment (lab coat, gloves, and masks) will be worn when handling the drugs. Lorna Nisbet will mix each of the NB2OMe solutions using the following algorithm: xmg of each drug (where x stands for the weight needed in table 1 above) will be dissolved in 5ml of 0.9%NaCl saline to yield a ymg/5 ml solution (where y refers to the weight required column in table1). Reverse osmotic water will be added to the 5ml glass bottle and an orange rubber stopper will be inserted into the bottle before being inverted several times to ensure the drug is thoroughly mixed in solution. The bottle will then be placed on a test-tube shaker and shaken until all particulate matter has been dissolved into solution. This "stock solution" will then be used to make appropriate working solutions.

For example a 5mg NB2OMe per 5ml of saline stock solution will yield a solution of 1mg per ml. Therefore; a rat weighing 1000g will be injected with 0.3cc and 0.3mg of NB2OMe. And so in reality if a rat weighs 250g then the rat will be injected with 0.075cc of solution and receive 0.075 mg of NB2OMe. This solution will be use to dose the rats at the highest concentration.
1.66ml of the 5mg NB2OMe per 5 ml of saline solution will be transferred to a separate 5ml glass bottle. This solution will be further diluted by adding 5 ml saline solution to yield a 0.33 mg per ml solution. This solution will be used to administer each drug at the middle concentration. Therefore; a rat weighing 1000g will be injected with 0.33cc and 0.1mg of NB2OMe. And so in reality if a rat weighs 250g then the rat will be injected with 0.082cc of solution and receive 0.0025 mg of NB2OMe
1ml of the 5mg NB2OMe per 5 ml of saline solution will be transferred to a separate 10ml glass bottle. This solution will be further diluted by adding 10 ml saline solution to yield a 0.1 mg per ml solution. This solution will be used to administer each drug at the lowest concentration. Therefore; a rat weighing 1000g will be injected with 0.3cc and 0.03mg of NB2OMe. And so in reality if a rat weighs 250g then the rat will be injected with 0.075cc of solution and receive 0.0075 mg of NB2OMe.

This will be repeated for each other the other drugs in a similar manner.

Each individual vial containing a single drug, will be carried from the locked refrigerator (key held only by Dr. Blustein) housed in the locked Behavioral Neuroscience Lab to a digital analytical balance, with glass sliding doors to prevent fluctuations in atmospheric pressure which will ensure minimal variability in powder weight each time each drug is weighed. The analytical balance is accurate to 0.1mg. The balance is housed in Boyer Hall Room 323 of the Arcadia University Forensic Science Department. A weighing boat will be placed on the balance tray prior to adding the individual drug powder and the scale will be zeroed using the →0/T function. Then using a standard chemical spatula made of non-reactive metal each powder will be transferred to a plastic weighing boat and sliding glass doors will be closed and each powder will be weighed individually.

Prior to injection, the prepared drug solutions will be removed from the refrigerator and allowed to equilibrate to room temperature for a minimum of ten minutes. 15 minutes, 20minutes and 25 minutes after the injection, the rats will be given the tail-flick test to measure analgesia. Tolerance will be defined

when tail-flick latency returns to baseline for three consecutive days. Baseline measurements will be taken on day one of testing prior to injection. Any withdrawal symptoms will be observed once tolerance has been established, by observing any differences in analgesia using the tail-flick test. At this point in the experiment the rats will no longer be dosed with any drug and will not be in any physical harm or under any stress. When this study is complete all animals will be euthanized. We confirm that no high school students will be involved in the duration of the experiment.

The Institutional Animal Care and Use Committee should determine that "…*The principal investigator has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals, and has provided a written narrative description of the methods and sources… used to determine that alternatives were not available."* [Federal Animal Welfare Act (9 CFR 2.31(d)iii]

The investigator needs to examine recent scientific advances to determine if the experiment could be **refined** to make it less stressful through the use of better procedures or the use of anesthetics, analgesics, or tranquilizers. The investigator should also consider **reduction** in animal numbers, and/or **replacement** of the animal model with a species lower on the phylogenetic scale or a non-*in vivo* model.

1. Literature Search: The investigator should complete a literature search to determine that the proposed experiments do not unnecessarily duplicate previous experiments, minimize pain and distress, and alternative models are not available for the study. (Specify database(s), date searched, years covered, and keywords utilized.)
   Journals Cited: Journal of Pharmacology and Experimental Techniques, Psychopharmacology, Addiction Biology, Neuropsychopharmacology, Journal of Clinical Pharmacology, Toxicology Letters, Anesthesiology, Annals of Emergency Medicine, European Neuropsychopharmacology, Xenobiotica, British Journal of Pharmacology, Journal of Medical Toxicology, Anesthesia and Analgesia, PubMed, Dates Searched: June 1st through August 14th, 2013. Covered years spanning 1982 to 2013. Keywords:NB2OMe, NBOMe, 25I-NBOMe, 25C-NBOMe, 2C-I, Analgesia, Dosage, Metabolites in hair, Metabolites in urine.

2. Review of Scientific Journals: (specify which journals)
   Journal of Pharmacology and Experimental Techniques, Psychopharmacology, Addiction Biology, Neuropsychopharmacology, Journal of Clinical Pharmacology, Toxicology Letters, Anesthesiology, Annals of Emergency Medicine, European Neuropsychopharmacology, Xenobiotica, British Journal of Pharmacology, Journal of Medical Toxicology, and Anesthesia and Analgesia.

3. Discussion with colleagues: (specify which colleagues)

4. Are alternative models or methods available that would minimize the use of living animals?
   NOX / YES If yes, describe why these methods are not being used in the proposed study.

5. Does the proposed study duplicate previous work?
   NOX / YES If yes, explain why duplication is necessary.

The 3 R's of Animal Research first presented by William Russell and Rex Burch indicate the need for Refinement (minimize the suffering and distress of animals), Reduction (minimize the minimal number of animals used), and replacement (substitution for conscious living higher animals of insentient)
W.M.S. Russell and R.L. Burch. The Principles of Humane Experimental Technique
http://altweb.jhsph.edu/pubs/books/humane_exp/het-toc

The Institutional Animal Care and Use Committee should determine that "…*researchers consider alternatives to painful procedures and that, with regard to painful procedures, researchers must consult a veterinarian; use adequate tranquilizers, anesthetics, and analgesics; and provide for adequate pre- and post-surgical care. Moreover, exceptions to these standards may be made only when specified by research protocol and explained in a report mandated in the Act."* [Federal Animal Welfare Act]

Note: Undergraduate and graduate students, your faculty sponsor will be responsible for providing information for this section.

6. Will the animals be anesthetized? NO X YES
   If yes:
   A. Identify the name of the agent, dose of the agent (mg of drug/kg body weight); and route of administration.

   B. What will be the maximum duration of anesthesia?

   C. How will depth of anesthesia be monitored?

   D. How will you determine if supplemental doses of anesthetic are necessary? Provide dosage and frequency of administration.

   E. Describe post-anesthetic care.

7. Will the animals receive analgesics? NO**X** YES
   If yes
   A. Identify the name of the agent, dose of the agent (mg of drug/kg body weight); and route of administration.
8. B. Describe the procedure for determining supplemental doses of analgesics. Provide dosage and frequency of administration.

9. Will the animals receive tranquilizing agents (other than already listed under anesthesia)? NOX YES
   If yes, answer A and B.
   A. Identify the name of the agent, dose of the agent (mg of drug/kg body weight); and route of administration.

   B. Describe the procedure for determining supplemental doses of tranquilizing agents. Provide dosage and frequency of administration.

Will the animals receive neuromuscular blocking agents? NOX YES
If yes, answer A and B.
A. Identify the name of the agent, dose of the agent (mg of drug/kg body weight); and route of administration.

B. Describe the procedure for determining the appropriate level of anesthesia in animals exposed to the paralyzing agent.

Check Appropriate Surgery:

	Type of surgical Procedure
_____	Terminal (non-survival) surgery will be performed.
_____	Survival surgery will be performed once per animal
_____	Multiple survival surgery will be performed
_____	Non-survival second surgery will be performed

1. All survival surgery procedures must occur under aseptic conditions. Describe the physical location in the laboratory to be used for survival surgery and the methods employed to maintain aseptic conditions and technique.

2. Describe the personnel training and expertise in the surgical model, include pre-, intra-, and post-surgical experience.

3. Describe procedures for monitoring and administering appropriate levels of anesthetics and analgesics. Be sure to include how you will monitor the depth of anesthesia and how the level of anesthetic will be increased if necessary.

4. Describe the entire surgical procedure. Be sure to include preparation of the surgical site, anesthesia, surgical procedure, and post-surgical care.

5. Multiple survival Surgeries are not normally approved. Provide scientific justification for the need to conduct multiple survival surgeries.

Investigator: _____Veterinarian: _____
Signature Signature

ARCADIA UNIVERSITY
IACUC Animal Use Proposal

The Institutional Animal Care and Use Committee should determine that "…*researchers consider alternatives to painful procedures and that, with regard to painful procedures, researchers must consult a veterinarian; use adequate tranquilizers, anesthetics, and analgesics; and provide for adequate pre- and post-surgical care. Moreover, exceptions to these standards may be made only when specified by research protocol and explained in a report mandated in the Act."* [Federal Animal Welfare Act]

1. Will the animals be euthanized? **NO YES X**
   If yes:
   A. Identify the name of the agent, dose of the agent (mg of drug/kg body weight); and route of administration. Carbon dioxide is used to euthanize the rats that have not been adopted. The rats are placed in a large trashcan. The top of the can is fitted with a spout where the hose for the carbon dioxide is attached to the tank. The gas is administered for 10 minutes and then the rats remain in the can for 30 minutes.

2. How will euthanization be confirmed?
   After 30 minutes, the rats will be checked to make sure there are no signs of breathing. Paw pinch reflect and corneal blink reflex will also be checked.

3. All euthanized animals must be placed in a bag and grouped by treatment (for drug studies). The bag must be labeled with PI name, Study ID, Date of Death, and any corresponding drugs used during the course of the study. Bags must be placed in the freezer in the Animal Facility unless a necropsy (autopsy) has been ordered by either the PI, Animal Care Representative, or Veterinarian. If a necropsy has been ordered, animals should be placed in individual, labeled bags in the necropsy refrigerator.

Appendix 14: CITI PROGRAM Certificates

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)
COURSEWORK REQUIREMENTS REPORT*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** Ioma nisbet (ID: 3574352)
- **Email:** nisbeti@arcadia.edu
- **Institution Affiliation:** Arcadia University (ID: 740)
- **Institution Unit:** forensic science

- **Curriculum Group:** Working with the IACUC
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Basic Course

- **Report ID:** 10589596
- **Completion Date:** 08/22/2013
- **Expiration Date:** 08/21/2016
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED
Introduction to Working with the IACUC (ID: 1807)	07/02/13
Working with the IACUC (ID: 1808)	07/02/13
Federal Mandates (ID: 1809)	07/02/13
The Veterinary Consultation (ID: 1810)	07/02/13
Getting Started (ID: 1811)	07/02/13
Alternatives (ID: 1812)	07/02/13
Avoiding Unnecessary Duplication (ID: 1813)	07/02/13
USDA Pain/Distress Categories (ID: 1814)	07/02/13
Personnel Training and Experience (ID: 1819)	07/02/13
Occupational Health and Safety (ID: 1820)	07/02/13
Using Hazardous and Toxic Agents in Animals (ID: 1821)	08/22/13
Housing Social Animals (ID: 1822)	08/22/13
Housing Rodents on Wire Floors (ID: 1823)	08/22/13
Prolonged Restraint (ID: 1826)	08/22/13
Making Changes after You Receive Approval (ID: 1830)	08/22/13
Reporting Misuse, Mistreatment, or Non-Compliance (ID: 1831)	08/22/13
Final Comments (ID: 1832)	08/22/13

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

CITI Program
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Phone: 305-243-7970
Web: https://www.citiprogram.org

COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK REQUIREMENTS REPORT*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** loma nisbet (ID: 3574352)
- **Email:** nisbetl@arcadia.edu
- **Institution Affiliation:** Arcadia University (ID: 740)
- **Institution Unit:** forensic science

- **Curriculum Group:** Working with Rats in Research Settings
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Basic Course

- **Report ID:** 10589598
- **Completion Date:** 06/20/2013
- **Expiration Date:** 06/19/2016
- **Minimum Passing:** 80
- **Reported Score*:** 92

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED
Introduction to Working with Rats in Research Settings (ID: 1916)	06/13/13
Research Mandates and Occupational Health Issues (ID: 1917)	06/17/13
Alternatives Searches, Humane Standards, Housing, and Acclimation and Quarantine (ID: 1919)	06/20/13
Detecting Pain and Distress, Genetics, and Biological Features (ID: 1923)	06/20/13
Injections, Blood Collection, Antibody Production, and Pain Relief (ID: 1926)	06/20/13
Surgery, Supportive Care and Monitoring, Euthanasia, and References (ID: 1929)	06/20/13

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK REQUIREMENTS REPORT*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** loma nisbet (ID: 3574352)
- **Email:** nisbetl@arcadia.edu
- **Institution Affiliation:** Arcadia University (ID: 740)
- **Institution Unit:** forensic science

- **Curriculum Group:** Responsible Conduct of Research
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - RCR

- **Report ID:** 10589599
- **Completion Date:** 06/24/2013
- **Expiration Date:** N/A
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED
Introduction to the Responsible Conduct of Research Archived 1248 (ID: 1248)	06/23/13
Research Misconduct (RCR-Biomed) (ID: 1215)	06/23/13
Data Management (RCR-Biomed) (ID: 1308)	06/23/13
Authorship (RCR-Biomed) (ID: 1380)	06/23/13
Conflicts of Interest (RCR-Biomed) (ID: 1622)	06/24/13
Collaborative Research (RCR-Biomed) (ID: 1450)	06/24/13
Responsible Conduct of Research (RCR) Course Conclusion (ID: 1043)	06/24/13

For this Report to be valid, the learner identified above must have had a valid affiliation with the CITI Program subscribing institution identified above or have been a paid Independent Learner.

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COLLABORATIVE INSTITUTIONAL TRAINING INITIATIVE (CITI PROGRAM)

COURSEWORK REQUIREMENTS REPORT*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** loma nisbet (ID: 3574352)
- **Email:** nisbetl@arcadia.edu
- **Institution Affiliation:** Arcadia University (ID: 740)
- **Institution Unit:** forensic science

- **Curriculum Group:** Reducing Pain and Distress in Laboratory Mice and Rats
- **Course Learner Group:** Same as Curriculum Group
- **Stage:** Stage 1 - Basic Course

- **Report ID:** 10589597
- **Completion Date:** 06/24/2013
- **Expiration Date:** 06/23/2016
- **Minimum Passing:** 80
- **Reported Score*:** 100

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED
Introduction to Post-Procedure Care of Mice and Rats in Research: Minimizing Pain and Distress (ID: 1868)	06/24/13
Investigator Responsibility (ID: 1869)	06/24/13
Minimizing Sources of Nonexperimental Variation (ID: 1870)	06/24/13
Systematically Monitoring for Pain and Distress (ID: 1871)	06/24/13
Detecting Clinical Signs of Pain and Distress (ID: 1872)	06/24/13
Appearance and Behavior (ID: 1873)	06/24/13
Physical Exam for Clinical Condition (ID: 1874)	06/24/13
Body Weight (ID: 1875)	06/24/13
Fluid and Electrolyte Balance (ID: 1876)	06/24/13
Body Temperature (ID: 1877)	06/24/13
Tumors (ID: 1878)	06/24/13
Alleviation of Pain and Distress (ID: 1879)	06/24/13
Documentation of Post-Procedure Care (ID: 1880)	06/24/13
Summary (ID: 1881)	06/24/13

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COURSEWORK REQUIREMENTS REPORT*

* NOTE: Scores on this Requirements Report reflect quiz completions at the time all requirements for the course were met. See list below for details. See separate Transcript Report for more recent quiz scores, including those on optional (supplemental) course elements.

- **Name:** loma nisbet (ID: 3574352)
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- **Institution Affiliation:** Arcadia University (ID: 740)
- **Institution Unit:** forensic science

- **Curriculum Group:** Human Research
- **Course Learner Group:** Biomedical Research Investigators and Key Personnel
- **Stage:** Stage 1 - Basic Course

- **Report ID:** 10589595
- **Completion Date:** 10/15/2013
- **Expiration Date:** 10/14/2016
- **Minimum Passing:** 80
- **Reported Score*:** 92

REQUIRED AND ELECTIVE MODULES ONLY	DATE COMPLETED
Avoiding Group Harms - U.S. Research Perspectives (ID: 14080)	09/30/13
Introduction (ID: 757)	09/30/13
Students in Research (ID: 1321)	10/01/13
History and Ethics of Human Subjects Research (ID: 498)	10/01/13
Basic Institutional Review Board (IRB) Regulations and Review Process (ID: 2)	10/03/13
Informed Consent (ID: 3)	10/03/13
Social and Behavioral Research (SBR) for Biomedical Researchers (ID: 4)	10/03/13
Records-Based Research (ID: 5)	10/03/13
Genetic Research in Human Populations (ID: 6)	10/03/13
Research With Protected Populations - Vulnerable Subjects: An Overview (ID: 7)	10/03/13
Vulnerable Subjects - Research Involving Prisoners (ID: 8)	10/03/13
Vulnerable Subjects - Research Involving Children (ID: 9)	10/03/13
Vulnerable Subjects - Research Involving Pregnant Women, Human Fetuses, and Neonates (ID: 10)	10/03/13
International Studies (ID: 971)	10/03/13
FDA-Regulated Research (ID: 12)	10/03/13
Research and HIPAA Privacy Protections (ID: 14)	10/04/13
Vulnerable Subjects - Research Involving Workers/Employees (ID: 483)	10/04/13
Conflicts of Interest in Research Involving Human Subjects (ID: 488)	10/04/13
Arcadia University (ID: 1130)	10/15/13

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Appendix 15: Tail flick data.

		Tail Flick Time (seconds)								
Drug Type	Drug Dose	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
25I-NBOMe	300	1.53	1.82	2.37	1.62	1.78	2	2.16	2.31	2.22
		1.42	2.41	1.53	2.31	1.9	2.41	1.84	1.44	2.75
		1.58	2.22	1.81	3	2.81	2.81	2.31	2.87	2.68
		1.36	2	2.03	1.94	2.13	2.03	1.85	2.06	2.28
		1.83	2.75	2.44	3.4	3.72	2.47	2.62	2.59	2.81
		1.15	1.62	2.18	2.79	2.71	2.25	1.94	1.78	1.97
	100	2.99	2.33	3.13	1.75	3.75	3.25	3.56	3.03	3.5
		1.92	2.41	2.75	3.16	2.69	2.81	2.56	2.56	2.15
		1.86	1.91	2.75	3.03	2.25	2.19	2.22	2.91	2.83
		1.39	2.44	2.94	2.41	2.5	2.5	2.57	2.79	2.78
		1.87	2.16	2.38	2.32	2.22	2.34	2.56	3.03	2.57
		1.43	2.28	2.41	2.41	2.13	1.94	2.06	1.69	1.85
	30	1.36	1.72	1.91	1.37	2.22	2.22	1.81	2.16	2.44
		1.66	1.66	1.94	1.66	2.56	1.69	1.69	1.94	1.87
		1.77	1.56	1.71	1.97	2.09	2.25	2.18	1.75	2.87
		2.6	3.12	2.14	3.25	2.65	2.62	2.97	2.5	2.4
		1.76	3.03	2.38	2.53	2.13	2.13	2.19	1.85	2.16

		Tail Flick Time (seconds)								
Drug Type	Drug Dose	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
		1.62	1.84	2.53	2.18	2.34	1.81	3.12	3.47	2.19
25B-NBOMe	300	1.6	1.64	1.96	1.94	1.65	2	1.57	2.66	1.67
		1.74	1.78	2.06	2.28	2.25	2.5	2.38	2	2.1
		1.42	1.47	2	2.09	2.38	1.9	2.22	1.97	2.31
		1.73	1.97	2.53	2.66	2.47	1.81	2.38	2.25	2.59
		1.53	2.25	2.4	3.21	2.56	2.9	3.41	2.5	2.16
		1.39	1.69	2.41	2.97	2	2.25	2.62	2.88	2.31
	100	1.28	1.44	1.57	1.6	1.65	1.57	1.72	1.72	1.66
		1.52	1.62	1.97	1.46	2.32	1.8	2.16	2.09	1.84
		2.05	2.69	2.28	2.56	2.72	2.87	3.38	2.93	2.56
		1.52	2.1	2.28	2.34	2.35	2.31	2.5	2.41	2.5
		2.11	2.19	1.78	2.82	2.75	2.19	2.59	2.38	2.78
		1.65	2.41	1.97	2.6	2.69	2.53	2.25	2.19	2.66
	30	1.5	1.43	2.34	1.65	2.06	2.25	1.81	2.5	2.16
	30	1.78	2.35	2.5	2.9	2.12	2.31	1.69	2.16	2.56
		1.97	3.04	2.5	2.63	2.69	2.22	3.19	2.5	2.4
		2.48	1.81	2.16	2.09	2.31	2.07	2.44	2.44	1.85
		1.69	2.47	1.95	2.06	2.34	2.25	2.56	2.84	2.35

		Tail Flick Time (seconds)								
Drug Type	Drug Dose	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
25C-NBOMe	300	2.44	2.47	2.79	2.57	2.44	2.18	1.79	2.87	1.81
		2.48	2.35	2.81	3.25	1.88	3.32	2.87	2.97	2.19
		2.22	1.72	2.47	2.06	2.03	1.94	2.35	2.65	1.96
		1.15	2.44	2.03	1.85	1.91	2.18	2.35	2.94	1.94
		1.45	1.37	2.34	2.34	1.69	2.07	2.78	2.6	2.07
	100	1.86	2.21	1.62	1.82	2.56	1.81	1.68	2.06	2.97
		2.1	1.81	2.4	2.18	2.6	2.41	2.75	2.78	2.25
		1.47	1.88	2.18	2.53	2.25	1.91	2.03	2.72	2.35
		1.92	2.28	1.97	3.12	2.94	2.66	2.53	2.32	2.22
		1.75	2.19	1.79	2.12	2.38	2.19	2.6	2.44	2.19
	30	1.43	1.9	1.4	1.69	2.06	1.84	2.03	2.53	3.4
		1.32	1.66	1.72	2.16	1.85	1.97	2.69	2.03	1.91
		2.61	2.34	1.94	2.46	2.34	2.38	2.19	2.63	2.1
		1.2	1.9	1.65	2.44	1.91	1.97	2.38	2.12	1.75
		1.14	1.93	1.75	2.47	2.03	2.34	2.38	2.56	1.78
CONTROL	0	1.51	2.34	2.6	2.87	2.44	1.81	2.75	3.31	2.44
		1.81	2.1	1.53	1.87	1.66	1.78	2.06	1.94	1.69
		1.95	2.25	2.22	2.34	2.06	2.53	3.03	2.22	2.04

		Tail Flick Time (seconds)								
Drug Type	Drug Dose	Baseline	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
		1.92	2.47	1.84	2.38	2.41	2.13	2.38	2.66	1.97
		1.12	1.43	1.66	1.97	1.31	2.65	1.53	2.03	1.47

Appendix 16: Presentations, posters and awards in support of this thesis.

Posters

- Lorna A Nisbet, and Karen S Scott: Comparison of SPE cartridges for the extraction of 25 new psychoactive substances**, Society of Forensic Toxicology (SOFT) - Orlando, October 2014.**

- Lorna A Nisbet, Jakub Klobut and Karen S Scott: Optimization of derivatization for new psychoactive substances (cathinones & NBOMe's) **United Kingdom and Ireland Association of Forensic Toxicology (UKIAFT)-Leicester, September 2014.**

- Lorna A Nisbet, Fiona M Wylie and Karen S Scott: Short term stability of mephedrone in blood and the impact of storage conditions on concentrations detected by GC-MS**, American Academy of Forensic Science (AAFS)- Seattle, February 2014.**

- Lorna A Nisbet, and Karen S Scott: Comparison of SPE cartridges for the extraction of 25 new psychoactive substances**, Society of Forensic Toxicology (SOFT) - Orlando, October 2014.**

Presentations

- Lorna A Nisbet, Rafael Venson, Fiona M Wylie and Karen S Scott: Analysis and detection of 25B, 25C and 25I-NBOMe in rat hair using SPE/LE/MSMS**, SOFT- Atlanta, October 2015.**

- Lorna A Nisbet, and Karen S Scott: Comparison of SPE and SLE columns for the extraction of 23 novel psychoactive substances from blood and urine**, AAFS- Orlando, February 2015.**

- Lorna A Nisbet, and Karen S Scott: Comparison of SPE cartridges for the extraction of 25 new psychoactive substances**, UKIAFT- Leicester, September 2014.**

Awards

- **Excellence in SPE Award** –SOFT annual meeting, Orlando, USA, October 2014.

  Lorna A Nisbet, Fiona M Wylie and Karen S Scott (2014). Short term stability of mephedrone in blood and the impact of storage conditions on concentrations detected by GC-MS**.**

- **UKIAFT Student Poster Award** –UKIAFT annual meeting, Leicester, UK, August 2014.

  Lorna A Nisbet, Jakub Klobut and Karen S Scott (2014), Optimization of derivatization for new psychoactive substances (cathinones & NBOMe's)

- **Toxicology Section Best Poster Award** - AAFS annual meeting, Seattle, USA, February 2014.

  Lorna A Nisbet, Fiona M Wylie and Karen S Scott (2014). Short term stability of mephedrone in blood and the impact of storage conditions on concentrations detected by GC-MS**.**

AAFS 2014 - SEATTLE

SHORT TERM STABILITY OF MEPHEDRONE IN BLOOD AND THE IMPACT OF STORAGE CONDITIONS ON CONCENTRATIONS DETECTED BY GC-MS

Lorna A.Nisbet*, [1,2] BSc.(hons), MSc , Dr Fiona M. Wylie[1], BSc (hons), PhD, Dr Karen S. Scott[2] BSc (hons.), PhD
[1] Forensic Medicine & Science, University of Glasgow, Scotland, UK
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After attending this presentation, attendees will understand the implications of different storage conditions upon mephedrone concentrations in blood.

This presentation will impact the forensic science community by offering forensic toxicologists and pathologists more information about optimum sample storage conditions to reduce potential sample degradation and loss between the time of sample collection and sample analysis.

Introduction: The time between sample collection and sample analysis varies greatly from case to case; therefore knowledge of analyte stability is of extreme importance. Not only does it help the toxicologist select the most suitable sample for analysis, it also ensures optimum storage conditions and preservatives are used to limit any sample degradation. This in turn aids the interpretation of concentrations of any drugs detected and their significance.

Mephedrone first appeared on sale to the public in 2007, and remains one of the most routinely detected "bath salts" or novel psychoactive substances (NPS) in the UK and the US. At the time, the majority of toxicology laboratories were not equipped to test for this compound, however since developing methods for its analysis many have performed retrospective testing on samples to investigate the presence of this drug. Previous work has shown mephedrone to be unstable in biological matrices. This research was intended to ascertain the effects of various storage temperatures and preservatives in preventing analyte degradation and determine the optimum conditions.

Method: Preservative free bovine blood was spiked with of mephedrone (1mg/L). This was divided into separate aliquots (1mL) enabling the examination of the stability of the drug in blood stored 1) without preservative;2) with citric

acid preservative (8%); and 3) with sodium fluoride (1.67%)/potassium oxalate (0.20%) at three different temperatures (-20^{o}C, -4^{o}C and 20^{o}C). Aliquots were tested daily for 7 days and then weekly over a period of 10 weeks. Samples were analysed in triplicate and calibrations and controls were run during each analysis. Samples were extracted using solid phase extraction (SPE) prior to derivatization with PFPA:EtOAc (2:1) and analysed using a Bruker GC-MS/MS with a DB5 column (30m x 0.25mm, 0.25 µm) in splitless mode. The total run time was 25 minutes, and mephedrone-D_3 was used as the I.S..

Results: Samples stored at 20^{o}C were the most effected with mephedrone becoming undetectable after a period of 21 days regardless of any preservative present. After 1 day samples stored at 20^{o}C saw a loss of on average 19% ranging from 17% with citrate solution to 21% with fluoride oxalate. Refrigerated samples preserved with fluoride/oxalate and citrate preservatives were initially stable however fluoride/oxalate rapidly decreased after 5 weeks, with a total loss of 96% over the 10 week period. Refrigerated samples preserved with citrate solution showed no significant decrease over the 10 week period. Refrigerated samples stored with no preservative saw a 41% drop after 5 weeks and a 74% reduction in concentrations across the 10 week period. Samples stored at -20^{o}C were stable under all conditions over the 10 week period.

Conclusion: To maximize stability of mephedrone, samples should be stored at -20^{o}C and preserved using citrate solution as this prevents any oxidative losses occurring. Although fluoride/oxalate was shown to preserve samples when stored at -20^{o}C, degradation was still problematic at 4^{o}C after 5 weeks seeing a 36% decrease. This in turn would affect reported concentrations from laboratories which store samples at 4^{o}C prior to analysis before archiving them at -20^{o}C. Regarding retrospective analysis, analysts should be cautious in interpreting negative results in cases where the history indicates mephedrone use. The sample history must be taken into consideration. Further work is underway to investigate potential degradation products; cross validation with human blood is also being planned.

STABILITY, MEPHEDRONE, PRESERVATIVES

SOFT 2014- GRAND RAPIDS

COMPARISON OF SPE CARTRIDGES FOR THE EXTRACTION OF 25 NEW PSYCHOACTIVE SUBSTANCES

Lorna A.Nisbet*, [1,2]MSc, Karen S. Scott[2] PhD
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[2] Forensic Science, Arcadia University, Glenside, PA 19038

Introduction: New psychoactive substances (NPS) have appeared on the recreational drug market at an unprecedented rate. Sample clean-up is a critical step in toxicological analysis; not only does it improve sensitivity and selectivity of results, but it also increases the lifetime of the instruments.

Objective: The aim of this study was to determine the most suitable SPE cartridge to use for the extraction of various NPS from a range of different matrices: blood, urine, plasma and serum.

Method: Blank methanol, urine, blood, plasma and serum samples (1 mL) were all spiked with 200µL of 10µg/mL solutions of various different NPS (methiopropamine, flephedrone, mephedrone, MDPV, 2-DPMP,butylone, ethylone, naphyrone, 5-APB, 6-APB, 3-MeO-PCE, methoxetamine, benzedrone, 25B-NBOMe , 25C-NBOMe , 25D-NBOMe , 25E-NBOMe , 25H-NBOMe, 25I-NBOMe, Mescaline-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe and 25T7-NBOMe). To each sample 1mL of 0.1M phosphate buffer (pH6) was added before centrifugation for 10 minutes at 4000rpm. Samples were then extracted using various different solid phase extraction cartridge; UCT's XCEL1, ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z as well as WatersOasis. ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z cartridges were conditioned using CH_3OH, dH_2O and phosphate buffer before loading samples. Columns were washed using dH_2O, 0.1M acetic acid and CH_3OH. Samples were eluted using methylene chloride; iso-propanol; ammonium hydroxide (78:20:2). Samples were loaded directly to the XCEL I cartridges and washed with 2%acetic acid/ 98% methanol before elution with methylene chloride; iso-propanol; ammonium hydroxide (78:20:2). Oasis cartridges were conditioned with MeOH and dH_2O

prior to loading samples. These were then washed using 2% acetic acid and MeOH prior to elution with 95% MeOH with 5% NH_4OH. Internal standards (mephedrone-D_3, methylone-D_3, ethylone-D_5, MDPV-D_8 and 25I-NBOMe-D_3) were added to the collection tubes prior to elution. Post extraction, samples were evaporated using a stream of nitrogen, derivatized using 50µL of PFPA:ethyl acetate at 70^oC for 40 minutes, before being evaporated again and reconstituted in 100µL of ethyl acetate. Samples were analysed by GC-MS.

Results: No drugs were detected (LOD <5 – 30 ng/mL) using the XRPCH50z cartridge. For urine samples, overall the UCT CSDAU cartridge had the best recovery with an average recovery rate of 71% (ranging 9 - 163% excluding methoxetamine and benzedrone which were undetected). Overall, blood samples had the best recovery rates with CSDAU SPE cartridges, averaging 60% (ranging 8 - 112% (excluding methoxetamine, benzedrone and mescaline-NBOMe which were undetected). Plasma and serum samples were best extracted using the ZSDAU cartridges with average recovery rates of 71% (ranging 5%- 156%) and 70% (ranging 12-167%) respectively.

Conclusion: This study has shown that when analysing urine and blood samples the smaller bed size of theCSDAU cartridges is preferable and when analysing plasma and serum samples ZSDAU cartridges should be used. XRPCH50z cartridges, using the method tested, are not recommended for sample clean-up of NPS compounds.

KEYWORDS: SPE, Novel psychoactive substances, NBOMes, Cathinones

AAFS 2014- ORLANDO
UKIAFT 2014 – LEICESTER

COMPARISON OF SPE AND SLE COLUMNS FOR THE EXTRACTION OF 23 NOVEL PSYCHOACTIVE SUBSTANCES FROM BLOOD AND URINE

Lorna A.Nisbet*, [1,2]MSc, Karen S. Scott[2] PhD
[1] Forensic Medicine & Science, University of Glasgow, Scotland, UK
[2] Forensic Science, Arcadia University, Glenside, PA 19038

After attending this presentation, attendees will be able to compare supported liquid extraction (SLE) and solid phase extraction (SPE) columns and identify the correct choice for optimum recovery of cathinones and NBOMes from a variety of biological matrices.

This presentation will impact on the forensic community by increasing awareness of extraction options for the determination of novel psychoactive substances in forensic toxicological samples.

Supported liquid extraction columns are now commercially available and pose an alternative to solid phase extraction (SPE).SLE has commercial benefits over SPE in that it does not produce the same amount of solvent waste and can be carried out in fewer steps depending on the type of SPE column being compared.

Recently there has been an influx of new synthetic substances to the recreational drug market with laboratories struggling to keep apace in this “cat and mouse” game. To improve detection rates it is vital that laboratories are using the optimum sample preparation technique to allow to maximum analyte recovery and sample throughput.

The aim of this research was to determine whether SLE+ columns can be used as a possible alternative sample extraction method for the detection of synthetic cathinones and NBOMes and to evaluate which clean up method produces the maximum recovery across a range of 25 drugs.

Blank methanol, urine and blood samples (1 mL) were spiked with 100µL of 10µg/mL solutions of various different NPS (methiopropamine, flephedrone, mephedrone, MDPV, 2-DPMP,butylone, ethylone, naphyrone, 5-APB, 6-APB, 3-

MeO-PCE, methoxetamine, benzedrone, 25B-NBOMe , 25C-NBOMe , 25D-NBOMe , 25E-NBOMe , 25H-NBOMe, 25I-NBOMe, Mescaline-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe and 25T7-NBOMe). Urine samples were pH adjusted to 10.8 using 1% ammonium hydroxide (NH_4OH). To each sample prepared for SPE, 1mL of 0.1M phosphate buffer (pH6) was added before centrifugation for 10 minutes at 4000rpm. UCT's ZDSAU020 columns were conditioned using MeOH, dH_2O and phosphate buffer before loading samples. Columns were washed using dH_2O, 0.1M acetic acid and methanol. Samples were eluted using methylene chloride; iso-propanol; NH_4OH (78:20:2). For SLE, following pH adjustment with 1% ammonium hydroxide, samples were loaded directly to Biotage's SLE+ columns. The sample was held on the column for 5 minutes before being eluted with 2x4mL of ethyl acetate. Internal standards (mephedrone-D_3, methylone-D_3, ethylone-D_5, MDPV-D_8 and 25I-NBOMe-D_3) were added to the collection tubes prior to elution. Post extraction, samples were evaporated using a stream of nitrogen, derivatized using 50µL of PFPA:ethyl acetate at 70^oC for 40 minutes, before being evaporated again and reconstituted in 100µL of ethyl acetate. Samples were analysed by GC-MS with the SLE and SPE results being compared directly to unextracted methanolic standards at the same concentration.

All drugs were successfully extracted from each matrix using both SPE and SLE columns. For blood, SLE+ columns provided a higher recovery rate of drug than the SPE columns, with an average increase of 10% (recovery ranging from -47% to 80%). SPE extracted urine samples more efficiently providing an average of 5% increase in recovery rates (recovery ranging -47% to 92%).

In conclusion when analysing blood samples SLE+ should be used whereas SPE is more efficient for the extraction of these analytes from urine.

KEYWORDS: SPE, SLE Novel psychoactive substances

SOFT 2015- ATLANTA

ANALYSIS AND DETECTION OF 25B, 25C and 25I-NBOMe IN RAT HAIR USING SPE/LC/MSMS

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[1] Forensic Medicine & Science, University of Glasgow, Scotland, UK
[2] Forensic Science, Arcadia University, Glenside, PA 19038

Introduction: NBOMes are phenethylamine derivatives of the 25C-X series, first mentioned in Ralf Heim's PhD thesis in 2004. These were further developed by David Nichol with the addition of a 2-methoxybenzyl (MeOB) onto the nitrogen (N) of the phenethylamine, hence the term NBOMe. The first mention of recreational abuse appeared in 2010, with NBOMe's now routinely associated with the "club drug" scene. They are administered either in liquid form or on blotters due to their high potency and similarities to LSD. (*119*)

Due to NBOMes' toxicity, adverse effects and fatalities have been reported. To date, analytical methods have been published for blood, urine, vitreous humour, brain, liver, bile and gastric contents; however no hair analysis has been published. (*124*)

Objective: The objective of this research was to extract 25B-NBOMe, 25C-NBOMe and 25I-NBOMe from rat hair by using a phosphate buffer incubation and SPE clean up method. Additional objectives including assessing any dose response relationship and determining whether the colour of hair affects concentration as seen with other phenethylamines.

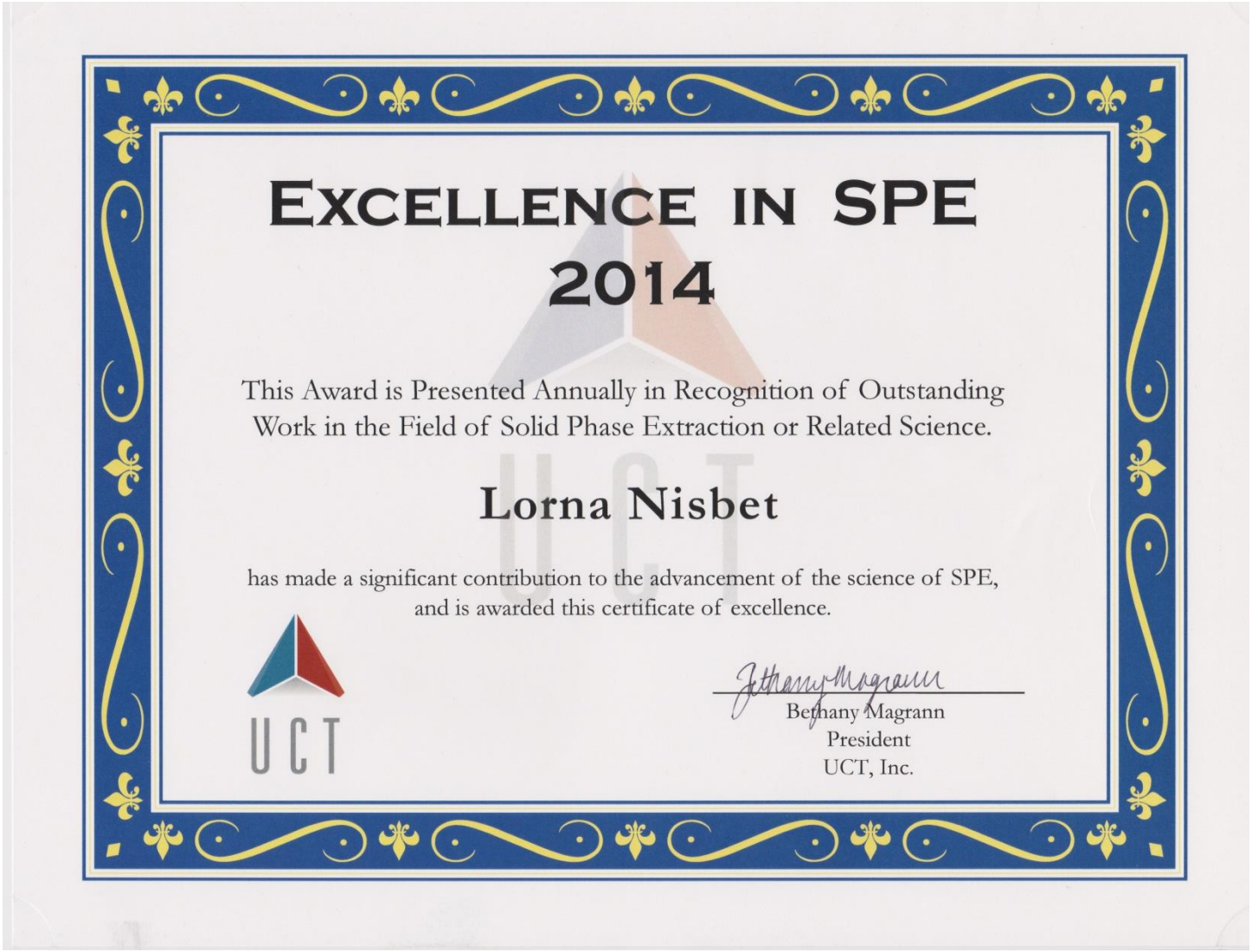
Methods: Long Evans rats (59) were partially shaved prior to first dose. The rats were shaved along their backs ensuring that both white and black hair was collected separately. Rats were then split into 4 groups, receiving saline, 25B-NBOMe, 25C-NBOMe and 25I-NBOMe respectively. Rats receiving NBOMes were subdivided into dose groups (5-6 rats per dose group), receiving 0.03 mg/kg, 0.1 mg/kg or 0.3 mg/kg. Each rat was dosed for a period of 10 consecutive days before being re-shaved; with white and black hair collected separately for analysis.

Hair was washed with dH_2O and Ch_2Cl_2, cut into small segments, and 40mg was transferred into a 7 mL vial. Next,100 µl of 0.01 µg/mL 25B-NBOMe-D3 was added to the vial along with 2 mL of 0.1 M pH 7.4 phosphate buffer. Vials were sonicated for 60 minutes and incubated 12hrs overnight at 40ºC. Sample vials were then centrifuged at 4000rpm and the supernatant was transferred to SPE cartridges conditioned with 2 mL MeOH and 1 mL 0.1M pH7.4 phosphate buffer. Cartridges were then washed using 3 mL dH_2O, 1 mL 1M acetic acid and 3 mL MeOH before eluting with 3 mL of CH_2Cl_2/IPA/NH_4 (78:20:2). Eluents were evaporated to dryness with nitrogen before being reconstituted in mobile phase and analysed by LC-MSMS.

Results: All drugs were successfully detected in black hair regardless of dose. Black hair incorporated all drugs to a higher degree than the white hair, with only the white hair from rats receiving the 300 mg/kg dose testing positive. Only the white hair from the 25C-NBOMe and 25I-NBOMe 300 µg/kg rats could be quantitated (4 pg/mg each). The concentration increase in black hair was dose-dependent, (2-30 pg/mg). 25I-NBOMe incorporated into the hair the greatest extent, approximately 80% more than 25C-NBOMe and 250% more than 25B-NBOMe.

Conclusion: The use of SPE followed by LC-MSMS analysis allowed for the detection of NBOMes in rat hair. To our knowledge this is the first example of NBOMes being successfully analysed in hair samples, despite the low concentrations administered.

KEYWORDS: NBOMe, hair testing, LC/MSMS.



EXCELLENCE IN SPE 2014

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