THE ANALYSIS AND LONG-TERM STABILITY OF AMPHETAMINE-TYPE STIMULANTS AND SYNTHETIC CATHINONES IN URINE USING NOVEL EXTRACTION METHODS AND GC–MS

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By

Khalid Alsenedi

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Abstract

Over the last few years stimulant substances, such as amphetamine-type stimulants (ATS) and synthetic cathinones (SC), have dramatically increased the frequency of lethal intoxications. The spread of these stimulant drugs has caused a complex challenge to the forensic toxicology community. Most analytical methods focus on detection and quantification of a specific class of drugs instead of an extensive variety of compounds. A method for the detection and quantification of 29 ATS and SC drugs in a single procedure was carried out using solid-phase extraction (SPE), pentafluoropropionic anhydride (PFPA) agent and gas chromatography—mass spectrometry (GC–MS). The method was validated in accordance with SWGTOX guidelines using human urine samples. The limits of detection (LOD) and lower limits of quantification (LLOQ) were between (0.5 and 10) ng mL^{-1}, and (5 and 50) ng mL^{-1}, respectively. The linearity range was between 50 and 2000 ng mL^{-1} with a R^2 >0.990 for 20 compounds. The bias and RSD were ≤20%, and no interferences or carryover were observed. The recovery was 80 to 120% for the majority of analytes.

Prior to testing the substances, the GC–MS was initially optimised in terms of the oven and injector port temperatures. The sensitivity and selectivity of the GC–MS were then improved using acidified methanol and derivatisation agents. Six acylation reagents were compared and investigated using PFPA, trifluoroacetic anhydride (TFA), chlorodifluoroacetic anhydride (CLF_2AA), heptafluorobutyric anhydride (HFBA), acetic anhydride (AA) and propionic anhydride (PA). The derivatisation method was optimised by modifying incubation time and temperature during the reaction and evaporation stages. Several parameters were used to evaluate the performance of the reagents, including the number of ions, relative ion ratio, peak area values, number of unique ions with some validation parameters. The reagents were further inspected using recovery through SPE in whole blood. The results of the comparison study showed that PFPA was the favoured reagent. All the derivatisation reagents were suitable for use on cathinones.

Long term stability was investigated for the 29 stimulant compounds in human urine specimens over a period of 381 days at room temperature (RT), refrigerator (4°C) and freezer (−20°C) conditions. ATS were stable under all conditions, and all

tested substances were stable at freezer conditions. Most SC at RT had lost more than 20% of the compound after two days, and had completely disappeared after a month. Most SC's at refrigerator temperatures were unstable after day 21, and gradually decreased until undetected between days 77 and 349. The substances were stable on the autosampler for three days. No concentration-dependent variations were observed. Half-lives of selected drugs were briefly discussed.

A sample preparation method that meets green analytic chemistry (GAC) requirements is desirable. Therefore, a method using solid phase microextraction (SPME) tips were initially developed via 13 processing steps and then validated using GC−MS in urine for eight ATS and SC substances. LOD and LLOQ were (5−25) ng mL^{-1}, and (25−100) ng mL^{-1}, respectively. The bias and RSD were <15% error with R^2 ≥0.992 for all analytes. Applying green analytical chemistry (GAC) parameters, the procedure had minor effects on health, waste and safety proportionate to LLE and SPE by adding the only microscale amounts of methanol and salt.

Attention to the prevalence of new psychoactive substances (NPS) such as SC, is significant to the justice system and the forensic toxicology community. The prevalence of SC was studied using 273 urine specimens collected from Riyadh City in Saudi Arabia. The cathinone compound estimation prevalence rate was 1.01%. No other cathinones were identified. Further prevalence studies should be conducted in the future using a larger sample size and incorporating more drug substances and metabolites.

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Author's declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution"

Signature _____

Printed name _____

List of abbreviations

3-BMC	3-Bromomethcathinone
3-FMC	3-Fluoromethcathinone
3-TFMPP	3-Trifluromethylphenylpiperazine
4-EMC	4-Ethylmethcathinone
4-FMC	Flephedrone
4-MEC	4-Methylethcathinone
4-MEC metabolite	4-METHYL-N-ETHYL-NOREPHEDRINE
4-M-N-E-NOREPHEDRINE	4-METHYL-N-ETHYL-NOREPHEDRINE
4-MTA	4-Methylthioamphetamine
AA	Acetic anhydride
ACMD	Advisory Council on the Misuse of Drugs
ADHD	Attention-deficit/hyperactivity disorder
ADS	Alkyl-diol-silica
ALS	Amphetamine like stimulants
ASE	Accelerated solvent extraction
ATS	Amphetamine-type stimulants
B.R	Bad response
BA	Butyric anhydride
BP	Boiling point
Buphedrine	Buphedrone ephedrine Metabolite
BZN	1-benzylpiperazine
BZP	Benzylpiperazine
C18	18 Carbon atoms
CAR	Carboxen
CCD	Central composite design
CLF_2AA	Chlorodifluoroacetic anhydride
CNS	Central nervous system
CPA	Cyproterone acetate
CW	Carbowax
d. H2O	Deionised water
DAD	Diode array detection
DART	Direct analysis in real time
DCM	Dichloromethane
DESs	Deep eutectic solvents
DFU	Drug-free urine
DI-SPME	Direct immersion solid-phase microextraction
DLLME	Dispersive liquid-liquid microextraction

DMC	N,N-dimethylcathinone
DOA	Drugs of Abuse
DS	Dilute-and-shoot
DSP	Dexamethasone sodium phosphate
D-SPE	Dispersive solid-phase extraction
DVB	Divinylbenzene
DYD	Dydrogesterone
ECD	Electron capture detector
EF	Efficiency factor
EHCC	Extrahepatic cholangiocarcinoma
EIC	Extracted ion chromatogram
EI-MS	Electron ionization mass spectrometry
El	Electron ionisation
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Electro membrane extraction
EMIT	Enzyme multiplied immunoassay technique
ENB	1-Ethyl-2-nitrobenzene
ESI	Electrospray ionization
EtCAT	N-ethylcathinone
EtOAc	Ethyl acetate
eV	Electron-volt
EWA	Early Warning Advisory
EWDTS	European Workplace Drug Testing Society
FMS	Forensic Medicine and Science
FTIR	Fourier transform infrared spectroscopy
FZ	Freezer
GC−MS	Gas chromatography-mass spectrometry
GDNC	General Directorate of Narcotics Control
HCl	Hydrochloric acid
HF-LPME	Hollow fibre membrane-liquid-phase microextraction
HFPA	Heptafluorobutyric anhydride
HPLC	High-performance liquid chromatography
HPLC-FLD	High-performance liquid chromatography-fluorescence detection
HPLC–UV	High performance liquid chromatography separation and ultraviolet detection
HR-MS	High-resolution mass spectrometry
HS-SDLLM	Headspace single-drop-liquid-liquid microextraction
HS-SDME	Head space in a single-drop microextraction technique
HS-SPME	Headspace-solid-phase microextraction
INCB	International Narcotics Control Board

IPA	Isopropanol
ISD	Internal standers
IR	Infrared spectroscopy
IUPAC	International Union of Pure and Applied Chemistry
K_{fs}	Distribution constant
Khat plant	Leaves of *Catha edulis*
LC–HRMS	Liquid chromatography–high resolution mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-QQQ-MS-MS	Liquid chromatography-triple quadrupole mass mass spectrometry
LC–QTOF-MS	Liquid chromatography in combination with hybrid quadrupole time-of-flight mass spectrometry
LC-TOF-MS	Liquid chromatography time-of-flight mass spectrometry
LLE	Liquid-liquid extraction
LLLME	Liquid-liquid-liquid micro-extraction
LLOQ	Lower limit of quantification
LME	Liquid-microextraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase-microextraction
M.W	Molecular weight
m/z	Ion ratios
MAE	Microwave-assisted extraction
MALDI-MS	Matrix-assisted laser desorption/ionization mass
MBDB	N-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine
MDA	3,4-Methylenedioxyamphetamine
MDBP	1-(3, 4-Methylenedioxybenzyl) piperazine
MDEA	(±)-N-ethyl-3,4-methylenedioxyamphetamine
MDMA	(±)-3,4-Methylenedioxymethamphetamine
MDPPP	4-Methoxy-α-pyrrolidinopropiophenone
MDPV	Methylenedioxypyrovalerone
MeOH	Methanol
MeOPP	1-(4-Methoxyphenyl) piperazine
Mephedrone metabolite	4-Methylephedrine
min	Minutes
MIP	Molecularly imprinted polymer
MOPPP	4'-Methoxy-alpha-pyrrolidinopropiophenone
MPBP	4'-Methyl-α-pyrrolidinobutiophenone
MPE	Micro pulverized extraction technique
MPHP	4'-Methyl-α-pyrrolidinohexiophenone

MPPP	*1-Methyl-4-phenyl-4-propionoxypiperidine*
MRM	Multiple reaction monitoring
MSPD	Matrix solid phase desperation
MTBE	Methyl-tert-butyl-ether
Na2HPO4	Disodium hydrogen orthophosphate anhydrous
NaCl	Sodium chloride
NaH2PO4	Sodium dihydrogen orthophosphate monohydrate
NFLIS	National Forensic Laboratory Information System
NH4OH	Ammonium hydroxide
NIST	National Institute of Standards
NMR	Nuclear magnetic resonance
NPS	New psychoactive substances
PA	Propionic anhydride
PCA	Perchloric acid
PDMS	Polydimythyl silocoxane
PEG	Polyethylene glycol
PFPA	Pentafluoropropionic anhydride
PLE	Pressurised-liquid extraction
PMA	Para-methoxyamphetamine
PMMA	Para-methoxy-N-methylamphetamine
PP	Polypropylene
PPP	Pyrrolidinopropiophenone
PTFE	Polytetrafluoroethylene
PV	Pyrovalerone
PVDF	Polyvinylidene fluoride
QCs	Quality controls
R^2	Correlation of coefficient
RF	Refrigerator
rpm	Rounds per minute
RSD	Relative standard deviation
RT	Room temperature
SA	Saudi Arabia
SAMHSA	Substance Abuse and Mental Health Service Administration
SBME	Solvent bar microextraction
SC	Synthetic cathinones
SD	Standard deviation
SD-LLLME	Single-drop-liquid-liquid-liquid microextraction
SDME	Single-drop-microextraction technique
SEM	Scanning electron microscopy
SFC	Supercritical fluid chromatography

SFDA	Saudi Food and Drug Authority
SFE	Supercritical fluid extraction
SFH	Security forces hospital
SIM	Selected ion monitoring
SLLE	Solid-supported liquid–liquid extraction
SLM	Supported liquid membrane
SOLA-SCX	Solid-phase cation exchange extraction
S/N	Signal−to−noise
SPA	Saudi Press Agency
SPDE	Solid phase dynamic extraction
SPE	Solid-phase extraction
SPME	Solid Phase Microextraction
SRM	Selected Reaction Monitoring
STD	Standard/ standards
SWGDRUG	The Scientific Working Group for the Analysis of Seized Drugs
temp.	Temperature
TFA	Trifluoracetic acid
TFME	Thin film microextraction
TFMPP	3-Trifluoromethylphenylpiperazine
THC	Tetrahydrocannabinol
TIC	Total ion chromatogram
TMA	Trimethoxyamphetamine
TMS	Trimethylsilyl
TPR	Templated resin
$_{t}R$	Retention time
UAE	Ultrasonic assisted extraction
UF	Ultra-filtration technique
UHPLC	Ultra-high-pressure liquid chromatography
UK	United Kingdom
UNODC	United Nations Office on Drug and Crime
UPLC-MS-MS	Ultra-performance liquid chromatography tandem mass spectrometry
USAEME	Ultrasound-assisted emulsification microextraction
v/v	Volume-to-volume ratio
WADA	World Anti-Doping Agency
WB	Whole blood
w/v	Weight-to-volume ratio
α-PVP	Pyrrolidinopentiophenone

1. Introduction

1.1 Statement of the problem

Drugs such as amphetamine-type stimulants (ATS) and synthetic cathinones (SC) have caused many intoxications and fatalities. These new stimulants drugs, for instance SC's, have become a significant class of new psychoactive substances (NPS) within European countries. The propagation of the NPS and the effects on abusers present a complex challenge to the forensic toxicology community. Not all laboratories have the ability to confirm these new stimulant drugs, even though use may cause significant problems in the health and security sectors. The detection of these drugs is difficult because routine immunoassay screening methods cannot fully detect them. This partly due to cost-effectiveness or unavailability of reagents and partly because it is difficult to confirm in spectrometry instruments (for example GC–MS), due to either the lack of reference standards or the fact that some substances have isomers with similar masses. The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), Zuba and Joshi et al. have all discussed the above problem based on mass spectrometry reporting the structural classes of related compounds and correspondent determination [1-3].

SC are a subgroup of NPS that are used as recreational drugs, due to amphetamine-like effects. These drugs are derived from the cathinone compound. The active ingredients of cathinones were originally found in the khat plant (leaves of *Catha edulis*). Khat was traditionally used for several hundreds of years, and when chewed it increased energy, made users more talkative and increased sexual drive [4, 5]. The SC made up 23% of the global usage of individual NPS, as reported in the Early Warning Advisory (EWA) from 2008 to 2015 [6]. Internationally, ATS are the second most commonly abused drugs, and often exceed heroin and cocaine use [7, 8].

Although the market has more sensitive instrumentation available, the most popular technique used in forensic laboratories is GC–MS. SC are well known to have relatively poor sensitivity and detection in electron ionisation (EI) and yield very few fragmentation ions and/or the quality of the mass spectra is relatively

weak. More specifically, the general rule for interpreting results in GC–MS is that at least three mass-to-ion ratio (m/z) must be selected and evaluated; the higher the ratio, the higher the sensitivity and specificity [3]. Therefore, the relative ion ratio intensities play an essential role in proper interpretation. However, cathinones have either poor detection as a result of the thermal decomposition of products in the injector port or having low ion ratios with sometimes only one ion. Consequently, the poor quality fragmentation patterns for derivative or underivative cathinones present a challenge; especially the pyrrolidine classes. MDVP, as an example, has only one ion (126 m/z) as a base peak, hence the residual mass spectra ions in the background of the electron ionisation has a very low abundance of ions with less than 5% relative intensities of the base peak.

Derivatisation reagents are normally used to solve the above problem. The sensitivity and selectivity of the GC–MS for the detection of SC substances can be improved by applying these reagents. Therefore, six acylation reagents were studied to improve the quality of fragmentation patterns using GC–MS and applied to nine SC for the evaluation. This selection covered a wide variety of SC groups. The GC–MS method and incubation time and temperature of each reagent were optimised to confirm excellent evaluation of the fragmentation patterns, including the quality of ions and the ion number. Besides the derivatisation agents, the sensitivity of the instrument can also be improved by using acidified methanol to concentrate the SC substance before evaporation. The use of acidified methanol was also demonstrated in this study, because of its contribution to the sensitivity of GC–MS.

The GC–MS itself was optimised until excellent responses were obtained with adequate separation for all studied compounds. Thermal degradation and decomposition of the SC in the injector port was minimised. Chapter 3 describes the above in more detail.

The matrix of the sample causes contamination when directly added to separation methods, such as GC–MS. In addition to its contribution to decreasing the chromatographic resolution, it also reduces the ionisation efficiency of MS and increases detection noise, which limits the detection level. This problem can be solved by applying sample preparation techniques that eliminate certain elements

in the biological samples, while keeping the target of analytes. Therefore, precise sample preparation techniques are fundamental. In this project, two sample preparation methods were developed for the detection and quantification of SC and ATS using GC–MS in biological matrices within a single procedure (see chapters 4 and 6).

Knowledge of the stability of the compounds is also very important. The degradation of the analyte is dependent on the time, concentration and storage conditions of the biological samples. This can influence the interpretation of the results and may provide a false negative. The stability of SC is a concern in the forensic toxicology field. In addition, although there are several published papers on the stability of SC, no single article has studied the stability of SC for more than six months in urine samples. This stability study lasted for 381 days (see chapter 5).

The achievement of long-term stability in urine samples required a sample preparation technique for the extraction of 29 selected drugs in a single procedure. Accordingly, the solid-phase microextraction (SPME) fibre tip was initially chosen over traditional techniques, such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), due to its simplicity and the low volume of solvent and chemicals required. Although the SPME and green analytical methods were undoubtedly desirable, the SPE extraction method was used for the stability work. This was because the SPE successfully provided high recoveries and responses for 29 selected drugs that were examined and fully validated. The SPME fibre tip was still in the development stage during that period.

The literature review showed numerous papers on SC and ATS in GC–MS, however there was a distinct gap, showing a lack of a method for the determination and quantification of a wide range of chemical groups (SC and ATS) in GC–MS in a single procedure. Additionally, the speed with which SC appeared on the recreational drug market meant that laboratories are much more likely to use a single procedure for the screening of any new compound that may appear under the scan method.

The development of a new method using SPME fibre tip was conducted, not only because of the publication gap, but also due to the need for green analytical chemistry (GAC). The SMPE and GAC are discussed in more detail in chapter 6.

Due to the harmful effects of SC products, many countries have announced that SC should be controlled using emergency powers. In Saudi Arabia (SA), there is no data regarding the use of products containing SC among Saudi people. Therefore, the prevalence of SC in SA was examined in this project (see chapter 7).

Indicators suggesting the presence and use of these products in SA were:

- SA reported in the UNODC questionnaire on NPS having to use emergency scheduling to temporarily ban NPS ‘‘while the legislative process is being completed and/or a rigorous assessment of the risks is conducted’’. Khat was the most frequently plant-based substance reported by respondents to the questionnaire. The highest seizures in 2010 were made in SA at 374 metric tons [9].

- United Arab Emirates (UAE) Ministry of Health has added 33 synthetic cathinones to Schedule V drugs [10]. There are thousands of Saudi people travelling to the UAE daily, and this could increase the probability of the presence of these products in SA.

Urine samples were collected for the prevalence assessment of SC in SA. Hence, this project will help with identifying the prevalence of these new drugs (SC) and lead to developing a better understanding of the issue in SA.

1.2 Aims and objectives

1. To compare six derivatising agents (trifluoroacetic anhydride (TFA), acetic anhydride (AA), chlorodifluoroacetic anhydride (CLF_2AA), heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA) and propionic anhydride (PA)) for the determination of nine synthetic cathinones (mephedrone, flephedrone, pentedrone, methylone, ethylone, methedrone, MDPV, butylone and pyrovalerone) using gas chromatography-mass spectrometry.

2. To determine and quantify a wide variety of SC and ATS compounds (amphetamine, methamphetamine, PMA, MDA, MDMA, MDEA, PMMA, cathinone, methcathinone, buphedrine (buphedrone metabolite), flephedrone, 4–methylephedrine (mephedrone metabolite), 4–methyl–N–ethyl–norephedrine (4–MEC metabolite), buphedrone, N–ethylcathinone, mephedrone, pentedrone, methedrone, methylone, butylone, ethylone, pyrovalerone, 4–EMC, 4-MEC, α–PVP, pentylone, MDPPP, naphyrone and MDPV) in urine using SPE and GC–MS in a single procedure.

3. To address the need for green analytical methods using new SPME fibre tips as an alternative to traditional sample preparation techniques, such as SPE and LLE. This was after the SPME fibre tip method was developed and validated for detection and quantification of eight selected ATS and SC substances (amphetamine, methamphetamine, PMA, MDMA, mephedrone, buphedrone ephedrine metabolite, 4-methylephedrine (mephedrone metabolite) and pentylone) using GC-MS in urine. The green analytical methods were compared based on the selection of SPME fibre tip, SPE and LLE procedures.

4. To evaluate and investigate stability of the 29 SC and ATS and associated metabolites (named above) in urine using SPE and GC–MS under different concentrations and temperatures over a period of 381 days.

5. To estimate the prevalence rate of SC in specific population in Riyadh City based on 273 urine samples that were collected at Security Forces Hospital (SFH) in Saudi Arabia using GC-MS.

2. Background and literature review

As this thesis mainly focuses on the stimulants drugs such as SC and ATS, and includes the using some sample preparation techniques, therefore the chapter spotlights on the up to the date of the mentioned above topics.

2.1 New Psychoactive Substances (NPS)

New psychoactive substances are being introduced to the market and referred to as “new synthetic drugs”, “legal highs”, “designer drugs”, “spice”, “club drugs”, “bath salts”, “herbal highs”, “research chemicals”, “new drugs”, “plant food”, “plant feeders” and “plant-growth fertilizers”; labeled with tag warnings “not for human consumption” or “not tested for hazards or toxicity”. These substances have emerged to replicate the traditional drugs of abuse, such as amphetamines, cocaine and cannabis. These design drugs have similar or more potent effects than the traditional predecessors [11-15]. Historically, the “designer drugs” term was used for the first time by Dr. Gary Henderson, who worked as a pharmacologist at the University of California at Davis. This term was used to introduce the definition of NPS to the press in 1988 [16].

The NPS have been distributed among abusers since mephedrone was reproduced in 2003. Manufacturing chemists have designed these compounds to bypass current legislation and regulation for profit, while the abusers get similar effects to the banned drugs regarding purity, affordability and availability. It is not likely that the trend of NPS will diminish in the coming years [4, 5, 17-20]. The NPS are defined by the UK Advisory Council on the Misuse of Drugs (ACMD) as “psychoactive drugs which are not prohibited by the United Nations Single Convention on Narcotic Drugs or by the Misuse of Drugs Act 1971, and which people are seeking for intoxicant use” [21].

In the last decade, abusers could purchase NPS or related drugs through the deep web in darknet markets. This new internet technology applies encrypted communication or anonymisation services to facilitate untraceable payments with cryptocurrency services. This example of strategies exists in parallel with participants in legitimate online marketplaces, such as Amazon and eBay [22].

The synthetic drugs were divided to nine categories based on effects and harm: cannabinoids, stimulants, opioids, psychedelics, empathogens, depressants, dissociative, others and unknown [23]. These drugs can also be classified in terms of chemical structure into the following categories: tryptamines, synthetic cathinones, synthetic cannabinoids, plant-based substances, piperazines, phenethylamines, phencyclidine-type substances, aminoindanes and other substances [24].

In 2016, the NPS produced at least one substance on a weekly basis internationally [25]. The United Nations Office on Drug and Crime (UNODC) reported more than 644 NPS amongst 102 countries between 2008 and 2015. On the following UNODC report between 2009 and 2016, the number of new compounds increased to 739 NPS, 19% of which were SC. Five-hundred NPS with 80000 seizures were reported in 2015 alone. This figure shows a sharp increase in NPS between these years [26]. This spread of recreational drugs naturally puts agencies of law and/or laboratories behind the constant innovation in the market. Since proposing new reference standards or regulations, inventors have created new compounds, resulting in a cat-and-mouse chase [19, 22, 26, 27]. Very recently, however, the innovations in NPS have been continuous but at a slower pace [22, 24].

2.2 Synthetic cathinones (SC)

2.2.1 General view

Cathinone designer drugs are a subgroup of NPS derived from cathinone. The active ingredients of cathinone were originally found in the khat plant (leaves of *Catha edulis*). This plant has been used for several hundreds of years because of its central stimulant actions. Khat is mostly found in countries of the Arabian Peninsula and the Horn of Africa. Its first description in western literature was in 1697 when a French scientist was visiting Yemen. The identification of the centrally acting agents of cathinone were announced 30 years ago. It was the first compound identified as a stimulant phenylalkylamine, beyond its effects on the central nervous system [28, 29]. SC substances have monoamine alkaloids and β-ketophenethylamines that were consumed for recreational purposes at the beginning of this century [27].

Popular brand names of SC drugs include “Ivory Wave”, “White Lighting”, “Meow-Meow”, “Blow”, “Cloud 9”, “Bohemian”, “Research Chemicals”, “Columbian Odorizer”, “Serenity”, “Explosion” and “Recharge”. These substances are widely sold as “bath salts”, “plant nutrients”, “plant feed”, “plant feeders”, “stain removers”, “insect repellants”, “glass cleaners” or “room deodorizers” with printed warnings stating “not for human consumption” or “licensed by the Ministry of Health” to avoid any legal consequences. Currently, the labeling of SC compounds have been replaced by “bidet refreshers”, “conquerors of leeches”, “additives to sand” or “driver’s charms” [12, 13, 30-35].

2.2.2 Chemical structure of synthetic cathinones

Cathinone is formally named 2-amino-1-phenyl-1-propanone by the International Union of Pure and Applied Chemistry (IUPAC), but has also been named ß-keto amphetamine or 2-aminopropiophenone. The common names of cathinone compounds that were studied in this project, along with IUPAC, street and other names are shown in **Table 2-1**. The labile cathinone can be transformed into a di-phenyl-pyrasine dimer named 3,6-dimethyl-2,5-diphenylpyrasine. Cathinone derivatives are very similar to the phenethylamine group. SC differ from the phenethylamine group by the presence of the keto functional group, linking to the parent substance cathinone that occurs naturally in the khat plant as the S-enantiomer. SC have the ability to be formed into two isomers, which may change the effects. The majority of ring-substituted derivatives of SC consist of racemic mixtures that resulted from keto-enol tautomerisation [36, 37].

Any SC compound described in this study is related to cathinone (primary amine), alkyl-amines (secondary amines) or a nitrogen atom in a pyrrolidine ring (tertiary amines). In general, SC must have ketone and amine functional groups. The general structure of SC are classified into four groups: N-alkylated, 3,4-methylenedioxy-N-alkylated, N-pyrrolidinyl and 3,4-methylenedioxy-N-pyrrolidinyl derivatives [38]. However, nine of new SC were substituted through a carbonyl group as illustrated by Smolianitski (these are not protected by law in most countries) [39]. The substitution patterns of cathinone derivatives are illustrated in **Figure 2-1**.

Table 2-1: Common, chemical, street and other names of SC included in this thesis

Common name	IUPAC name	Street name	Other name (s)
CATHINONE	2-amino-1-phenylpropan-1-one	Chat, tohai, khat, oat, African salad, qat, bushman tea and Abyssinian tea	ß-Keto-amphetamine
METHCATHINONE	2-methylamino-1-phenylpropan-1-one	Intash, cat, catnip or jeff	Ephedrone
BUPHEDRONE	2-methylamino-1-phenylbutan-1-one	Mebuphedrone	α-Methylamino-butyrophenone (MABP), α-ethylmethcathinone
ETHCATHINONE	2-ethylamino-1-phenyl propan-1-one	Eth-cat	N-Ethylcathinone, ethylpropion
PENTEDRONE	2-methylamino-1-phenylpentan-1-one	Drone	α-Methylamino-valerophenone
4-MEC	2-ethylamino-1-4-methylphenylpropan-1-one	Boosting or bumping	4-Methylethcathinone
MEPHEDRONE	2-methylamino-1-4-methylphenylpropan-1-one	M-cat, meow meow or white magic	4-Methyl methcathinone (4-MMC)
METHEDRONE	1-4-methoxyphenyl-2-methylaminopropan-1-one	Bubbles, bristol, meth, dolley	Para-methoxymethcathinone, 4-methoxymethcathinone
FLEPHEDRONE	1-4-fluorophenyl-2-methylaminopropan-1-one	4-FMC, flephedrone	4-Fluoromethcathinone (4-FMC)
4-EMC	1-4-ethylphenyl-2-methylaminopropan-1-one	4-EMC	4-Ethylmethcathinone
ETHYLONE	1-1,3-benzodioxol-5-yl-2-ethylamino propan-1-one	ßk-MDEA	3,4-Methylenedioxy-N-ethylcathinone
BUTYLONE	1-1,3-benzodioxol-5-yl-2-methylamino butan-1-one	Ease, arlone	ß-Keto-N-methylbenzodioxolylbutanamine
METHYLONE	1-1,3-benzodioxol-5-yl-2-methylamino propan-1-one	M1, explosion	3,4-Methylenedioxy-N-methylcathi
PENTYLONE	1-1,3-benzodioxol-5-yl-2-methylamino pentan-1-one	Pentylone	ß-Keto-methylbenzodioxolylpentanamine
MDPV	1-1,3-benzodioxol-5-yl-2-pyrrolidin-1-ylpentan-1-one	Lunar wave, magic, vanilla, sky, super coke	Methylenedioxypyrovalerone
NAPHYRONE	1-naphthalen-2-yl-2-pyrrolidin-1-ylpentan-1-one	Energy-1, NRG-1	Naphthylpyrovalerone
PYROVALERONE	1-4-methylphenyl-2-pyrrolidin-1-ylpentan-1-one	Rave	4-Methyl-ß-keto-prolintane
MDPPP	1-1,3-benzodioxol-5-yl-2-pyrrolidin-1-ylpropan-1-one	MDPPP	3,4-Methylenedioxy-α-pyrrolidinopropiophenone
α-PVP	1-phenyl-2-pyrrolidin-1-ylpentan-1-one	Flakka	α-Pyrrolidinopentiophenone
4-MEC metabolite	2-ethylamino-1-4-methylphenyl propan-1-ol	Not applicable	4-Methyl-N-ethyl-norephedrine
Mephedrone metabolite	2-methylamino-1-4-methylphenyl propan-1-ol	Not applicable	4-Methylephedrine
BUPHEDRINE	2-methylamino-1-phenylbutan-1-ol	Not applicable	Buphedrone ephedrine metabolite

Abbreviations: 4-MEC (4-methylethcathinone), 4-EMC (4-ethylmethcathinone), MDPV (methylenedioxypyrovalerone), MDPPP (methylenedioxy-α-pyrrolidinopropiophenone), α-PVP (α-pyrrolidinopentiophenone), ßk-MDEA (ß-keto-methylenedioxy-N-ethylcathinone).

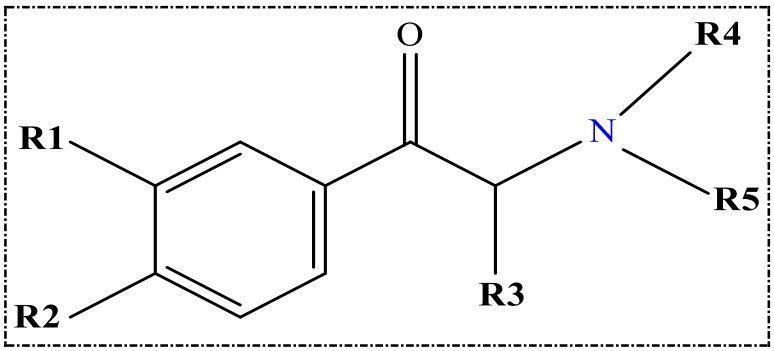


Figure 2-1: Substitution patterns of cathinone derivatives

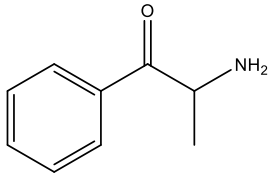
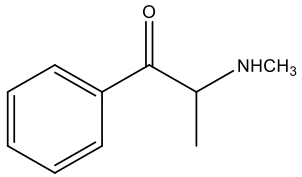
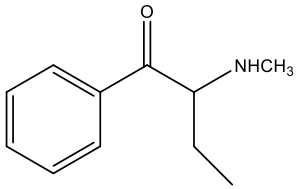
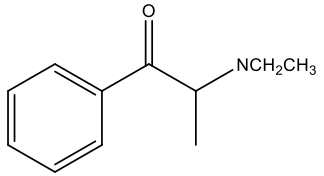
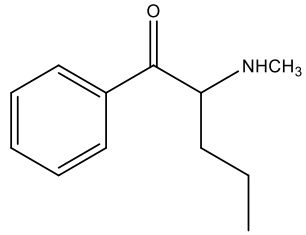
The history of the inherent chemistry of the SC substances is ambiguous and not well understood [33, 40]. The structure and the pathways of all SC studied in this thesis were conducted by several scientist groups using MS, nuclear magnetic resonance (NMR), infrared spectroscopy (IR) instruments [37, 41-50].

The fragmentation ions and the isomeric composition of SC tertiary amines were evaluated by Abiedalla using GC electron ionisation mass spectrometry (GC-EI-MS) and GC−MS-MS [51]. Reviews of chiral separation of SC and the stability study of isotopes in SC have been conducted in 2017 [52, 53].

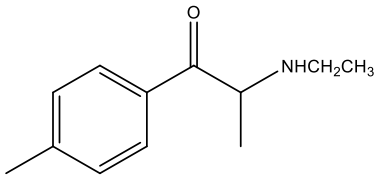
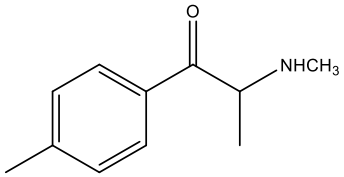
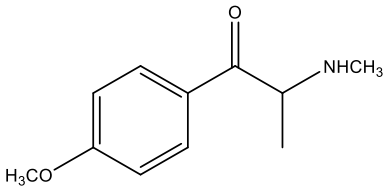
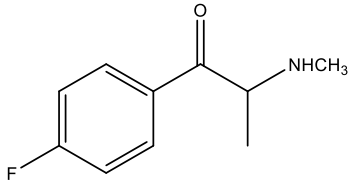
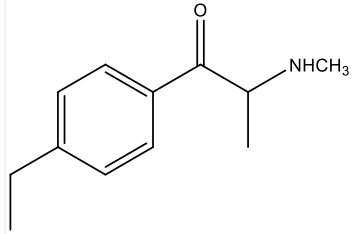
The chemical structures of cathinones discussed in the thesis are illustrated in **Table 2-2**. These structures were selected based on the most apparent availability in the market and trade. In addition, some substances from each group of cathinones were chosen to cover a wide range of SC structures.

Table 2-2: The chemical structures of SC and their metabolites included in this thesis

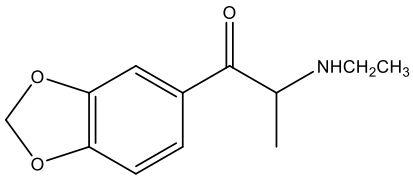
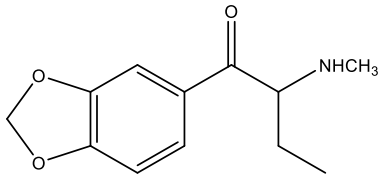
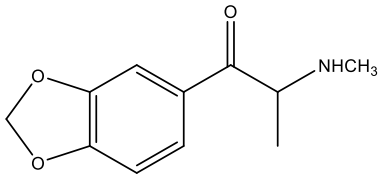
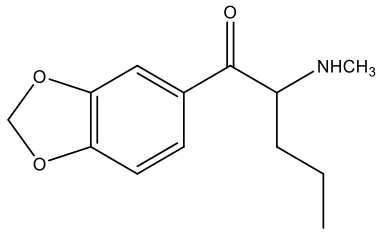
Non-ring substitute

CATHINONE	METHCATHINONE	BUPHEDRONE	N-ETHYLCATHINONE	PENTEDRONE
				

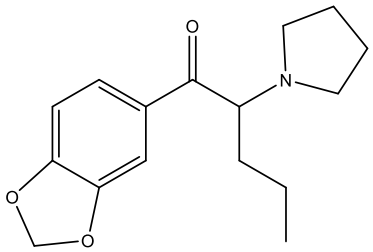
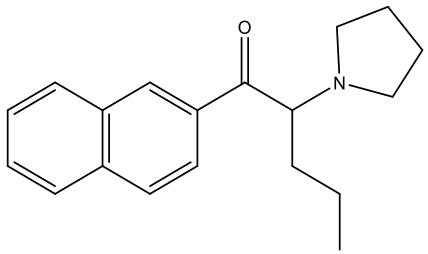
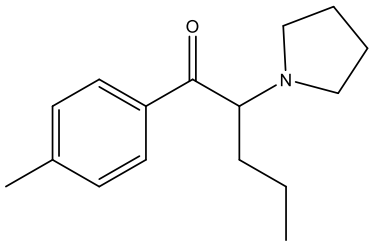
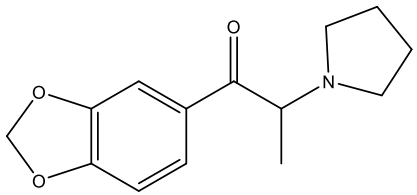
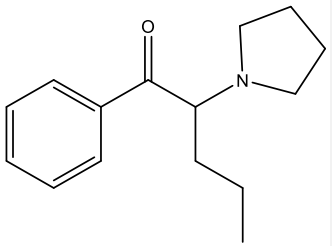
Ring substituted

4-MEC	MEPHEDRONE	METHEDRONE	FLEPHEDRONE	4-EMC
				

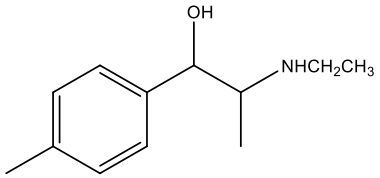
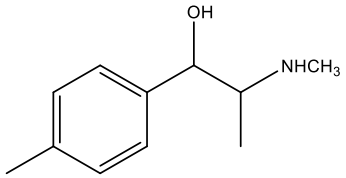
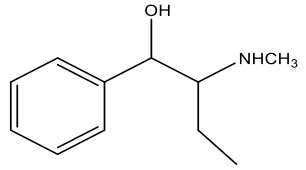
Methylenedioxy-substituted

ETHYLONE	BUTYLONE	METHYLONE	PENTYLONE
			

Pyrrolidine-type (tertiary amines)

MDPV	NAPHYRONE	PYROVALERONE	MDPPP	α-PVP
				

Cathinone metabolites

4-METHYL-N-ETHYL-NOREPHEDRINE (4-MEC metabolite)	4-METHYLEPHEDRINE (Mephedrone metabolite)	BUPHEDRINE (Buphedrone ephedrine metabolite)
		

2.2.3 Pharmacology and toxicology of synthetic cathinones

The research history of pharmacology and toxicology of cathinones and corresponding metabolites covers more than a century [40]. Many published review papers that have reviewed the cathinone derivatives are illustrated in **Table 2-3**. Even though the toxic effects of cathinones were well reported, the pharmacological and toxicological studies in humans is quite limited. The action of the agents pharmacologically was not equivalent in each cathinone product. These agents produce actions via serotonin, dopamine and/or norepinephrine transporters to either reuptake and/or release neurotransmitters. To date, no one paper has documented the logical relationships between structure activity of the mechanism and the behavioural actions in each SC compound. This is because each drug must be studied individually, or on a "case-by-case basis" [40].

The pharmacology, toxicology and treatment of intoxication from SC and metabolites have not yet been fully understood and further studies are required [40, 54, 55]. However, using these drugs can be very harmful and impair health [56]. The mechanism of action of SC and metabolites can be measured through in vitro experiments. SC enter the brain barrier through blood, and apply active stimulant agents to the central nervous system (CNS). This is due to the presence of side amines that are distributed in high concentrations on the synapses in the CNS. For that reason, the active stimulant agents of SC are regularly higher than ATS [57-59]. Both SC and ATS have somewhat similar effect actions since each exists in two stereoisomeric forms, each having a different potency [38, 57, 60-63].

Table 2-3: Review papers published on pharmacology and toxicology of SC

Title	Year	Ref.
Pharmacological aspects of the chewing of khat leaves	1985	[64]
Khat consumption: A pharmacological review	1989	[65]
Use and abuse of khat (*Catha edulis*): A review of the distribution, pharmacology, side effects and a description of psychosis attributed to khat chewing	1989	[66]
Amphetamine-like effects in humans of the khat alkaloid cathinone	1990	[67]
Adverse effects of khat: A review	2003	[68]
Effects of khat (*Catha edulis*) consumption on reproductive functions: A review	2003	[69]
Khat and synthetic cathinones: A review	2004	[60]
Khat (*catha edulis*)—An updated review	2005	[70]
Chronic khat use and psychotic disorders: A review of the literature and future prospects	2007	[71]
Risk assessment of khat use in the Netherlands: A review based on adverse health effects, prevalence, criminal involvement and public order	2008	[72]
Chemical composition of *catha edulis* (khat): A review	2008	[72]
A review of the neuropharmacological properties of khat	2008	[73]
Psychopharmacological aspects of *catha edulis* (Khat) and consequences of long term use: A review	2011	[74]
Pharmacology and toxicology of mephedrone	2011	[75]
Recently abused β-keto derivatives of 3,4-methylenedioxyphenylalkylamines: A review of their metabolisms and toxicological analysis	2011	[76]
Mephedrone toxicity in a Scottish emergency department	2011	[77]
Chemistry, pharmacology, and toxicology of khat: a review	2011	[78]
The chemistry, pharmacology and toxicology, synthetic cathinones	2011	[79]
The toxicology of bath salts: A review of synthetic cathinones	2012	[80]
Intoxication delirium following use of synthetic cathinone derivatives	2012	[12]
A brief review of the emergence of mephedrone use	2012	[81]
Bath salt use: A case report and review of the literature	2012	[82]
Resident journal review – synthetic cathinones ("bath salts") and herbal marijuana alternatives	2012	[83]
Khat as a risk factor for hypertension: a systematic review	2012	[84]
Miaow miaow: A review of the new psychoactive drug mephedrone	2012	[85]
Recently abused synthetic cathinones, α-pyrrolidinophenone derivatives: a review of clinical and pharmacological aspects of bath salt use: A review of the literature and case reports	2013	[86]
Forensic analysis of cathinones	2013	[87]
Khat: A widely used drug of abuse in the Horn of Africa and the Arabian Peninsula: Review of literature	2013	[28]
A review on hazards of khat chewing	2013	[88]
Mephedrone: Public health risk, mechanisms of action, and behavioural effects	2013	[89]
Methylenedioxypyrovalerone ("bath salts"), related death: Case report and review of the literature	2013	[90]
Khat: Social habit or cultural burden? A survey and review	2013	[91]
A review on synthetic cathinone and its derivatives: Prevalence and syntheses	2014	[92]
Behavioural pharmacology of designer cathinones: A review of the preclinical literature	2014	[93]
Recently abused synthetic cathinones, α-pyrrolidinophenone derivatives: a review of their pharmacology, acute toxicity, and metabolism. Forensic Toxicology	2014	[94]
"Not for human consumption": A review of emerging designer drugs	2014	[95]
Bath salts and synthetic cathinones: an emerging designer drug phenomenon	2014	[96]
Comprehensive review of the detection methods for synthetic cannabinoids and cathinones	2015	[97]
The psychostimulant drug khat (*Catha edulis*): A mini-review	2015	[98]
The effects and risks associated to mephedrone and methylone in humans: A review of the preliminary evidences	2016	[99]
Neurotoxicology of synthetic cathinone analogues	2016	[62]
Khat (*catha edulis*) and obesity: A scoping review of animal and human studies	2016	[100]
Khat use and mental illness: A critical review	2017	[101]
"Bath salts" the New York City medical examiner experience: A 3-year retrospective review	2017	[102]
A report of novel psychoactive substances in forensic autopsy cases and a review of fatal cases in the literature	2017	[103]
Toxic effect of khat (*catha edulis*) on memory: Systematic review and meta-analysis	2017	[104]
Neurotoxicity induced by mephedrone: An up-to-date review	2017	[105]

In general, pharmacological effects of SC on neurotransmission can be classified based on potency into three groups. First, cathinones that act similarly to cocaine and MDMA are named the mixed cathinone group. The action mechanism of SC related to this group includes non-selective reuptake inhibition of monoamines. For example, cocaine has more selectivity to dopamine than serotonin, with similar action for mephedrone, butylone, methylone and ethylone, or elevation and liberation of serotonin, such as in case of MDMA and naphyrone.

The second group is the SC that have a similar mechanism to methamphetamines, where actions increase liberation of dopamine and the reuptake inhibition of monoamines. Cathinones that belong to this group are flephedrone, methcathinone and clephedrone.

The third group is related to pyrovalerone structure compounds, such as MDPV and MDPPP. These substances do not have liberation effects on neurotransmission, but have very potent effects and selective inhibition of monoamine reuptake [38, 58, 59, 106-108].

The response, strength and extent of the mechanism of action of cathinones on the CNS vary depending on several factors, including mode of administration, type of drug, number of doses, duration of addiction, health conditions, age, mixture of drugs taken, onset action of addiction, alcohol consumption, tolerance, sex, medical treatments and body type. However, the desired feelings from taking SC are generally similar, including talkativeness, excitement, open mindedness, euphoria, concentration, mental awareness, positive feelings and sexual arousal. The effects of SC peak between 30 and 45 minutes after taking and last for three hours. SC produce a range of peripheral, central and mental effects. Examples include increased blood pressure, respiration and heart rate, anorexia, psychomotor agitation, hyperthermia and insomnia. Besides to the common psychostimulant effects, hallucinogenic effects approaching those of ecstasy can also result [38, 60, 109-114].

2.2.4 The metabolism of synthetic cathinones

The metabolism and pathways of most SC are fairly well known. For example, in 1926, studies found that cathinone was the metabolite of methcathinone [115], but cathine and ephedrine were the metabolites of cathinone which were pharmacologically identified as active central stimulants of khat in 1975 [40, 116]. The majority of SC metabolite and pathway studies were discussed in the 1960s [40]. The metabolism and pathways of SC substances discussed in this thesis have been demonstrated in many papers: cathinone [116, 117], methcathinone [116, 118], mephedrone [108, 119, 120], flephedrone [121], methylone [108, 122], α-PVP [123, 124], butylone [108, 125], MDPV [49, 126, 127], 16 SC metabolic profiles excreted from urine [128], buphedrone [129], 4-MEC [130], naphyrone [131], MDPPP [132, 133], methedrone [134] and ethylone [125]. Metabolism of mephedrone and its metabolites in urine and blood in vivo was discussed by Pedersen, Reitzel [119] using ultra-performance-liquid chromatography-tandem-mass spectrometry (UPLC-MS-MS) and ultra-performance-liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF). CYP2D6 was identified as the main enzyme responsible for mephedrone metabolites and any similar structures of NPS. See **Figure 2-2** for suggested metabolic pathways for mephedrone in rats and humans [108, 119].

2.2.5 Administration of synthetic cathinones

SC are commonly administered through nasal insufflation or oral ingestion. The compounds are fairly simple to synthesise and are sold as a white, brown or yellowish powder that is odourless and occasionally with coloured crystals. It is less often sold as tablets or capsules. Most SC were originally produced in China and South East Asian countries though less frequently in India, and were later distributed to Europe. These products often consist on a mixture of cathinones, diluted with caffeine, lidocaine and/or may other adulterants such as benzocaine or any synthetic byproduct [7, 31, 33, 38, 40, 60, 79, 134-136].

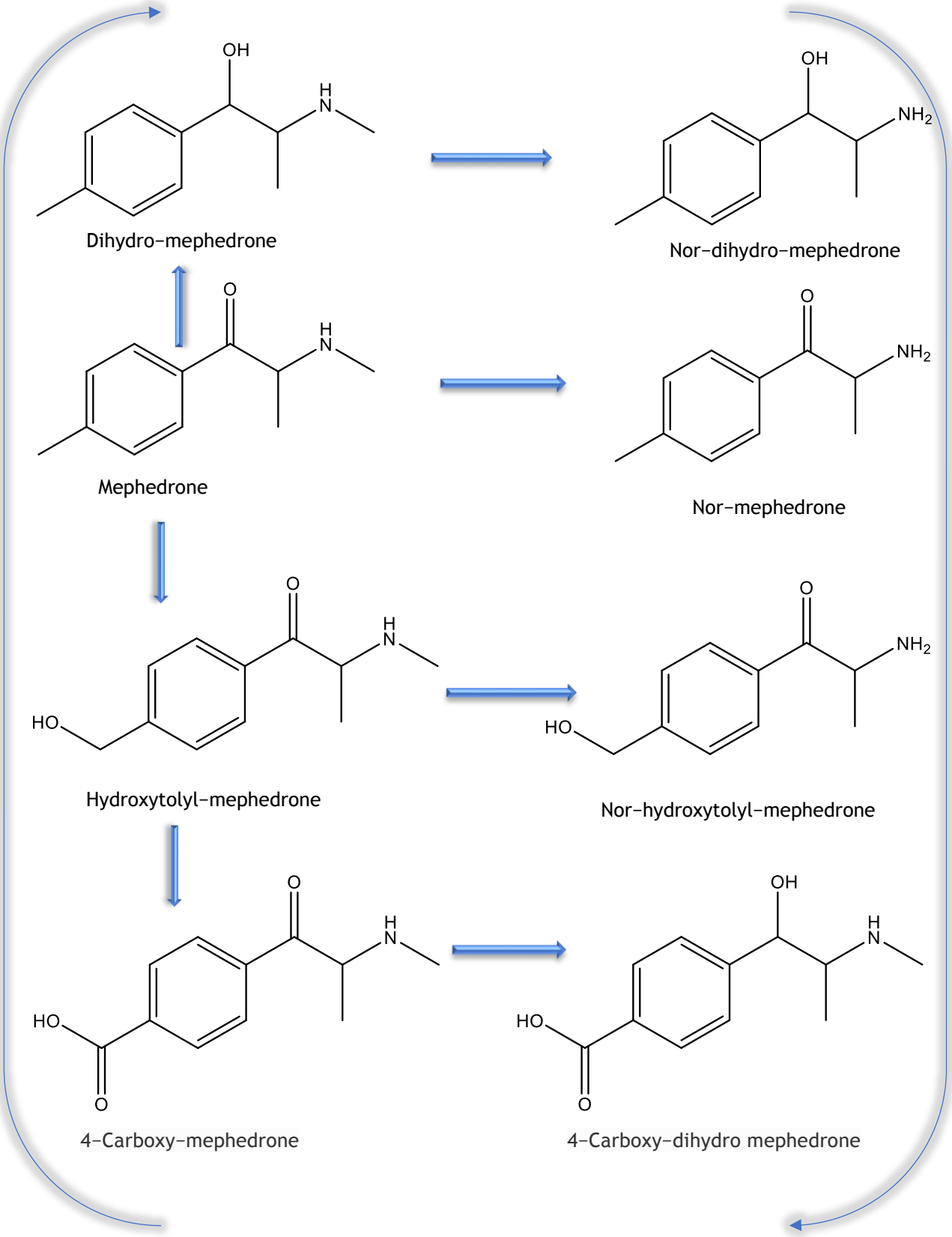


Figure 2-2: Metabolic pathways of mephedrone

2.2.6 Synthetic cathinones and fatal cases

SC have been related to acute toxicity, and as a consequence, several deaths have been documented [90, 130, 137-143]. For example, in the UK, in 2008, 59 deaths were reported because of mephedrone use only, and in 2011 that number increased to 90 fatal cases. Most users died because of being unaware of the mixtures taken [144, 145].

The determination of SC compounds in biological matrices in fatal cases depended on the type of case and the matrix. For examples, the concentrations of SC in blood in antemortem cases were reported between 10 and 1000 ng mL^{-1} and frequently higher in postmortem cases. In urine, mephedrone was found to be between 186 and 198 ng mL^{-1} (post mortem) [141, 146] and mephedrone in serum (antemortem) was reported as 150 ng mL^{-1} [147]. 4-MEC was 4.3 ng mg^{-1} (hair-antemortem) [148], while other fatal cases were reported related to 4 MEC by [50, 149]. In blood, methylone was 22 ng mg^{-1} [150], α-PVP was 654 ng mg^{-1} [151] and MDPV was 440 ng mg^{-1} [152]. In general, MDPV, mephedrone, 4 MEC, buphedrone, methylone, PVP, flephedrone and methedrone respectively were most reported in the literature for fatal cases. The variation of concentrations was large because of the effect of stability which was still not fully understood.

In England and Wales, there were 80 deaths between 2012 and 2016 reported by the Prisons and Probation Ombudsman, due to suspected NPS use. The number of deaths from using amphetamine compounds was 151 deaths in 2014, while MDMA was reported in 50 cases the same year, but amphetamine itself was the largest proportion of the total. 114 deaths related to NPS were seen in 2014, 49 of which were due to using cathinones and 44 of those were mephedrone. Most of the deaths were individuals under 40 years old, related to NPS, cocaine and amphetamine use. Almost one-third of deaths were because of using amphetamines and NPS by individuals under 30 years of age [153-155]. In Scotland, deaths related to NPS increased suddenly five years ago, with 123 deaths reported in 2016 (8% increase from 2015), and only 31 deaths in 2011 [156]. The reported deaths from amphetamine, ecstasy, NPS and mephedrone in Scotland, England and Wales in the years 2000, 2005, 2010 and 2015 are illustrated in **Figure 2-3**.

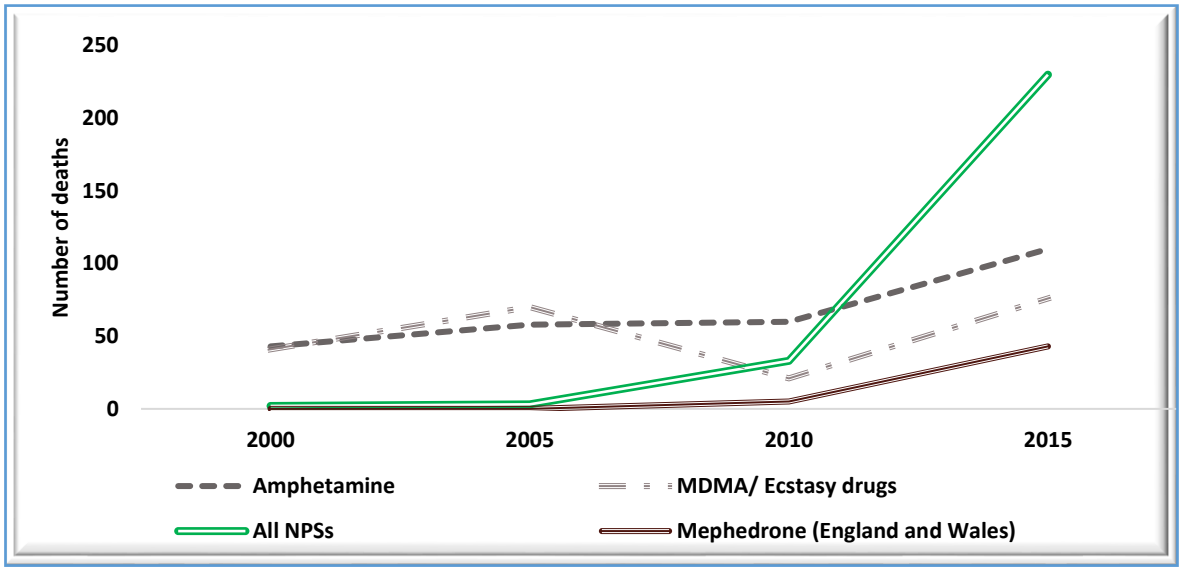


Figure 2-3: Number of deaths from amphetamine, ecstasy, NPS and mephedrone in Scotland, England and Wales

Data was collected from the Office for National Statistics [154, 155] and National Records of Scotland [156].

2.2.7 Legal status of synthetic cathinones

The regulations for the prohibition of NPS are not similar between countries [18]. Globally, the legal response has been to implement several approaches in order to control NPS, including 'the individual listing system' and 'supplementary regulatory frameworks'. The individual listing system has some flexibility in controlling NPS, and by now has been used in most countries. This approach introduces generic regulation to extend to isomers of substances; for example, to include ethers and esters of substances. Several countries, for example Denmark, use temporary or emergency bans to restrict NPS for a period of time until final decisions are made regarding legislation. Some other countries, for example, Sweden, Norway and Poland, use the rapid procedure to ban NPS permanently [157]. Currently, most countries, such as the UK, USA and Canada, use the generic, analogue systems or blanket ban systems, where the legislation covers a wide range or defined group or analogue instead of naming the specific drug. The above applied procedures contribute to limiting abuse of NPS (slowing the pace) in the recent years [22, 153].

2.2.8 Prevalence and history of synthetic cathinones

The prevalence of SC across countries has been reviewed in [27, 92, 158-164]. The data obtained for NPS prevalence were uncertain since abusers often take the drug(s) yet do not recognise which drug(s) have been taken. This is why the trend data shows fluctuation; some surveys showing growth while others were decreasing or even stable trends [165].

The prevalence of NPS in the UK was discussed by the Home Office in 2016, concluding that the prevalence of SC was low compared to traditional drugs. 2.7% of people in England and Wales between the ages of 16 and 59 had taken NPS, with only 0.7% taking the drugs in 2015. The prevalence of NPS was lower in Scotland (1.6%) and Northern Ireland (2.2%). Prevalence of mephedrone (1.3%) was similar to ecstasy in 2010, and in the recent years, the prevalence of mephedrone dropped after being controlled, reaching 0.3% in England, Wales and Scotland in 2016 and 0.6% in Northern Ireland [153]. In Europe, the lifetime prevalence of NPS is 8% with 3% confirming use in 2013 alone, between ages of 15 and 24 (from the Eurobarometer survey) [165]. In Germany, in 2015, 0.9% of individuals between 18 and 64 confirmed use [165].

Historically, the first active components of SC were made in the 1920s for medical purposes, to treat depression and obesity [27, 33, 40, 162, 166]. The active component of cathinone was synthesised in 1929 [42], methcathinone in 1928 [33, 166], buphedrone in 1928 [166], mephedrone in 1929 [33], MDPV in 1969 and pyrovalerone in 1964 [139]. In 1940, methcathinone and cathinone were used for the treatment of depression in Russia. However, between 1970 and 1980, the substances were misused in many countries, including the USA, Russia, the Republic of South Africa and finally in Japan in 1995. Recently, bupropion has been used for cathinone and methcathinone withdrawal treatment [167, 168].

Methcathinone has been misused since the 1970s and became widespread in Europe, USA and Australia in the 1990s. Mephedrone was primarily used for the treatment of depression and for appetite suppression in the USA between the 1930s and the 1950s. In the 1960s, MDPV was initially synthesised for the treatment of chronic fatigue [27, 29, 169]. Pyrovalerone was developed in 1969, and later

used illegally in 1975 [170], Methylone was used as an antidepressant and for the treatment of Parkinson's disease in 1996 [14, 171]. After the year 2000, methylone and the first generation of SC compounds appeared for recreational users. From 2000 to 2009, the pioneer SC in Europe was the mephedrone followed by methylone, MDPV, butylone, ethylone, buphedrone and flephedrone, and after 2009, pentedrone and α-PVP were also included. The second generation of SC were later seen (from 2015) as the group of α-pyrrolidinopentiophenone [33, 38, 58, 60, 114, 172].

The most commonly seized SC in 2015 were α-PVP, 3-MMC, ethylone, and pentedrone [29, 31]. Three SC compounds (methylone, mephedrone and MDPV) made up 98% of all SC used in the USA in 2014, even when these drugs were controlled [20, 173]. In 2005, the European Monitoring Centre on Drugs and Drug Addiction (EMCDDA) included methylone as the first SC illegally used. After two years, mephedrone had been distributed around the world, in countries such as Israel, Australia and the UK [79]. Later, mephedrone increased in Europe and the USA [174]. Mephedrone and methylone were the two most misused SC in Europe [175]. In 2009, up to 41% of the people who attended dance music clubs (the sample size was 2295 participants) in the UK had used mephedrone [176]. MDPV was misused by more than 6% of 259 drivers in Finland between August 2009 and August 2010 [177].

Meanwhile, almost 150 SC have been documented through distribution into the market [15, 178]. More than 376 SC have been discovered in the National Forensic Laboratory Information System (NFLIS) data [1]. Examples of new SC, recently reported in 2016 and 2017 were hexedrone, 4-Cl-α-PPP, 4-Br-α-PVP, 4-bromoethcathinone [179], 4-methylpentedrone, N-ethylnorpentylone [180], N-ethylhexedrone, propylone, 4-Cl-EAPP, 6-Methoxy-bk-MDMA, α-PiHP, 4-F-α-PHP, 4-Cl-α-PHP [181], α-PBT and some other related drugs were reported by [182], as well as thiothinone reported by [183].

2.3 Amphetamine-type stimulants (ATS)

2.3.1 General view

In the last decade, the abuse of ATS compounds, such as amphetamine, methamphetamine and MDMA has become a global concern, but especially in East Asia and the Middle East. According to a recent UNODC report in 2017, the abuse of ATS has globally increased and they are now the second most abused drug in the world after cannabis [22]. In 2015, global seizures of methamphetamine increased 21% (132 tonnes) from 2010. Similarly, amphetamine seizures increased 8% (52 tonnes) and ecstasy decreased 35% (6 tonnes). There were nearly 200 tonnes seized and over 37 million users of ATS compounds in 2015. In 2010, there were 100 tonnes of ATS seized; these figures indicate the sharp rise of ATS between 2010 and 2015 [165]. The quantities of amphetamine, methamphetamine and MDMA seizures in 2015 in the UK were roughly 4.5, 0.1 and 3.5 tonnes, respectively [31].

Amphetamine was first manufactured in 1887 and from 1935 has been used for the treatment of hypertension, obesity, attention-deficit/hyperactivity disorder (ADHD) and narcolepsy. Because of potential addiction, it is controlled in almost all countries. Amphetamine is a form of a phenethylamine derivative compound that has two enantiomers: dextrorotatory or levorotatory, or a mixture of both, each one having different plasma half-lives. The dextrorotatory isomer is three to four times more potent than the levorotatory isomer [184, 185]. Methamphetamine is the methyl derivative of amphetamine and is a strong potent stimulant in the CNS. It was synthesised in 1919 for the treatment of ADHD and obesity [186]. Methamphetamine has commonly been sold illegally as a racemic mixture of dextrorotatory and levorotatory with strong and rapid stimulant effects [187].

2.3.2 Chemical structure of amphetamine-type stimulants

ATS substances can be classified based on the structural characteristics and the substitution patterns on the ring into three sub-groups. The first group has no substituents on the benzene ring, such as amphetamine and methamphetamine. The second group has methylenedioxyphenol-substitution on the aromatic ring,

such as MDMA and MDEA. The third group has other chemical substitution patterns with alkyloxy groups, such as PMA and PMMA [188]. The chemical structures of ATS discussed in this thesis are illustrated in **Figure 2-4**. These compounds were selected based on apparent availability in the market and trade. Substances were also chosen from each group of ATS in order to cover many ATS structures.

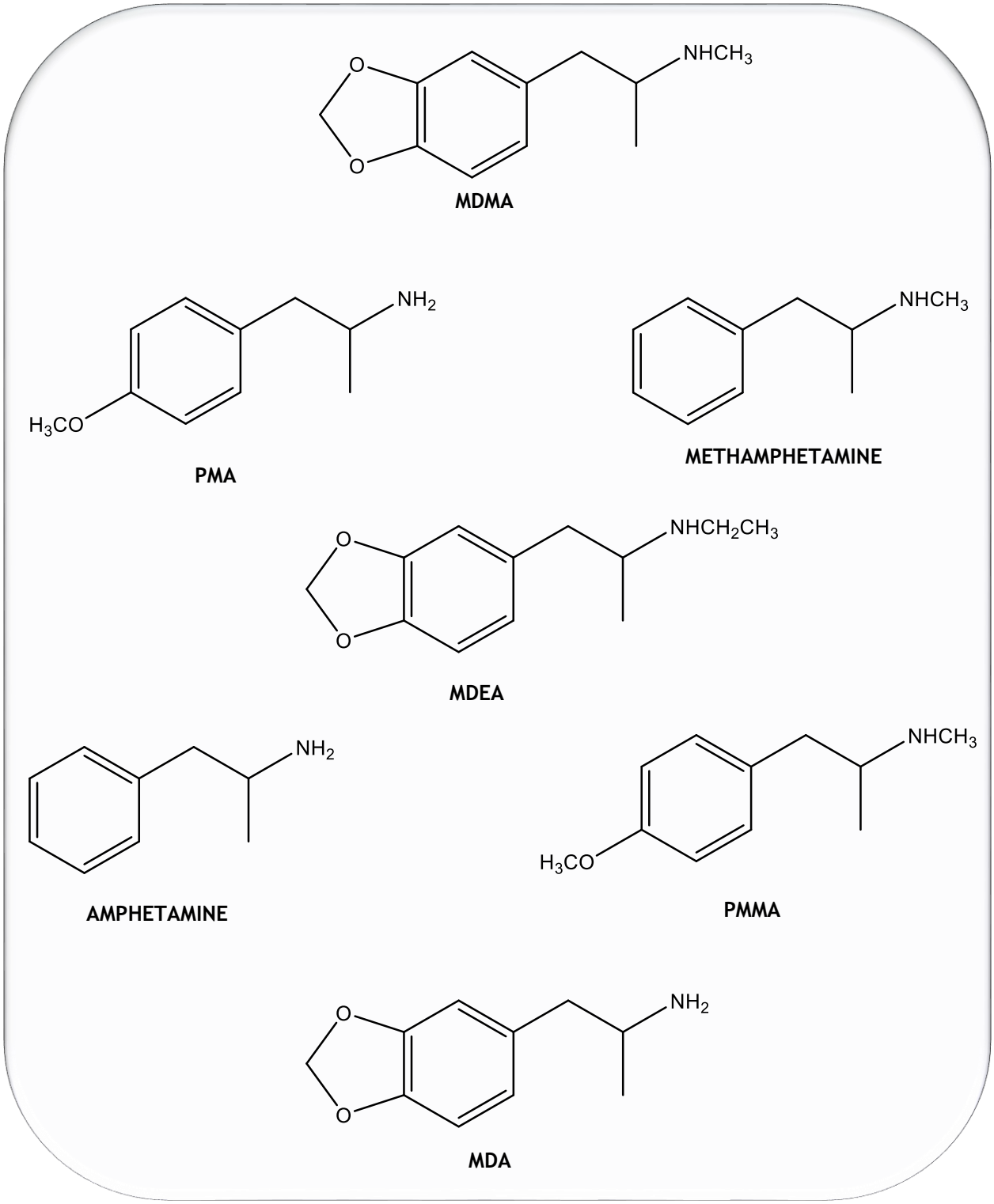


Figure 2-4: The chemical structures of ATS included in this thesis

2.3.3 Pharmacology of amphetamine-type stimulants

Stimulant drugs of the CNS, such as ATS and amphetamine-like stimulants (ALS), effect the cerebral cortex and produce effects similar to adrenaline. For instance, amphetamine crosses the blood-brain barrier into the monoamine neurotransmitter system and acts similar to dopamine. ATS substances such as amphetamine and methamphetamine can react, bind and act in some processes. The substances act as substrates of a monoamine transporter protein with low-affinity in the dopamine and noradrenaline systems, resulting in high concentrations of neurotransmitters. Conversely, MDMA acts more heavily on the serotonin system and results in more potent effects than amphetamine substances [188-190].

The symptoms related to ATS substances are euphoria, elevated blood pressure and heart rate, a weakness in memory, increased body temperature and respiration. Hallucinations, tremors, violent behaviour, agitation, memory loss, psychosis and paranoid delusions can occur with chronic abuse [191].

ATS compounds are absorbed rapidly in the abdominal system. They are lipophilic substances that pass the blood brain barrier and concentrate in the brain, lungs, cerebrospinal fluid and kidneys. The primary active pathway of amphetamine includes 4-hydroxyamphetamine and norephedrine. In urine at pH 7, 30–40% of the amphetamine is excreted unaffected 24 hours after the oral administration, and nearly 50% is excreted as inactive metabolites, including the metabolites of hippuric acid and 4-hydroxyphenylacetone. The concentration of amphetamine in plasma is at a peak level between one and three hours after taken orally, and full absorption occurs after four to six hours. Methamphetamine is 40% excreted in urine unchanged along with 5% amphetamine (a metabolite of methamphetamine) after 24 hours (orally). The half-life in human plasma is 10 hours and the peak occurs after three hours. The active metabolite of methamphetamine is 4-hydroxymethamphetamine [186, 187, 192-194].

2.3.4 Administration of amphetamine-type stimulants

ATS substances are produced in a variety of forms, such as tablets, powder, capsules or white crystals that can be administrated orally, smoked, injected or snorted [187].

2.3.5 The concentration of ATS in abuse samples

The cutoff concentration of amphetamine, methamphetamine, MDMA, MDA and MDEA for a drug of abuse test in urine samples is 500 ng mL^{-1} for screening and 250 ng mL^{-1} for a confirmatory test; this is based on the Substance Abuse and Mental Health Services Administration (SAMHSA) [195]. The amphetamine and methamphetamine cutoff in the EU and the USA are 300 ng mL^{-1} and 500 ng mL^{-1}, respectively. According to the World Anti-Doping Agency (WADA), the detection of ATS substances initially measures qualitatively the ATS groups rather than on a quantitative basis; this is because the trace amount of ATS compounds with metabolites are essential for detection. Therefore, the development of an instrument for the wide determination of ATS substances with the ability to detect small concentrations of ATS even after several days will enable more stringent regulations on the abuse of ATS drugs [187, 196].

2.3.6 Legal Status of amphetamine-type stimulants

ATS substances are heavily regulated and controlled in most countries, restricting access, such that the substances are only used for medical treatment purposes [197].

2.3.7 Prevalence of amphetamine-type stimulants

In 2015, ATS prevalence rates (excluding MDMA) were 0.5 and 0.6% in Europe and North America, respectively [198]. The lifetime prevalence of ATS for young populations (15-34) varied from 0.1% to 12.4%, with an average of 5.5% in European countries. In the USA, the prevalence of methamphetamine use in people aged 15-64 increased from 0.5% to 0.8% between 2012 and 2015 [165].

MDMA prevalence in the UK (England and Wales) decreased from 4% to 3.2% from 2000 to 2015. In Europe (amongst 15-64 year olds) MDMA was misused by 9.3 million males and 4.7 million females in a lifetime, and amphetamines were misused by 8.4 million males and 4.2 million females [199]. The estimation of amphetamine prevalence for young Europeans aged 15–34 was 1.3% (1.7 million) in 2012 [200]. The most commonly misused drugs after cannabinoids were MDMA,

amphetamine, cocaine, methamphetamine and LSD in European countries by students 15-16 years old, with a lifetime prevalence of 5%, excluding cannabis [22].

2.4 Sample preparation methods and matrices

Sample preparation techniques are used in the first stage, before the samples are analysed using chromatographic mass spectrometric methods. These techniques eliminate some components of the sample, such as lipids and proteins, and maintain the target analyte for detection even in small concentrations. Biological matrices frequently contaminate the instruments, particularly when samples are directly inserted into the instruments. Therefore, the requirements for eliminating contamination using sample preparation methods play an essential role in any laboratory [56, 201].

Sample preparation techniques can generally be classified into two categories: exhaustive or non-exhaustive techniques. **Figure 2-5** illustrates the classification of sample preparation techniques [202].

Exhaustive extraction techniques are employed for the extraction of the entire analyte from the matrix by applying a large quantity of organic solvent in liquid or sorbent. This procedure is used to confirm that the analytes are fully extracted from, especially when development parameters are applied. Subsequently, the outcome products easily reach the desired recovery. The SPE and LLE are both good examples of the exhaustive technique. Non-exhaustive techniques depend on reaching the equilibrium between the target analytes and the stationary phase [202].

A review of extraction techniques in the toxicological analysis of drugs was reviewed by Maurer [203].

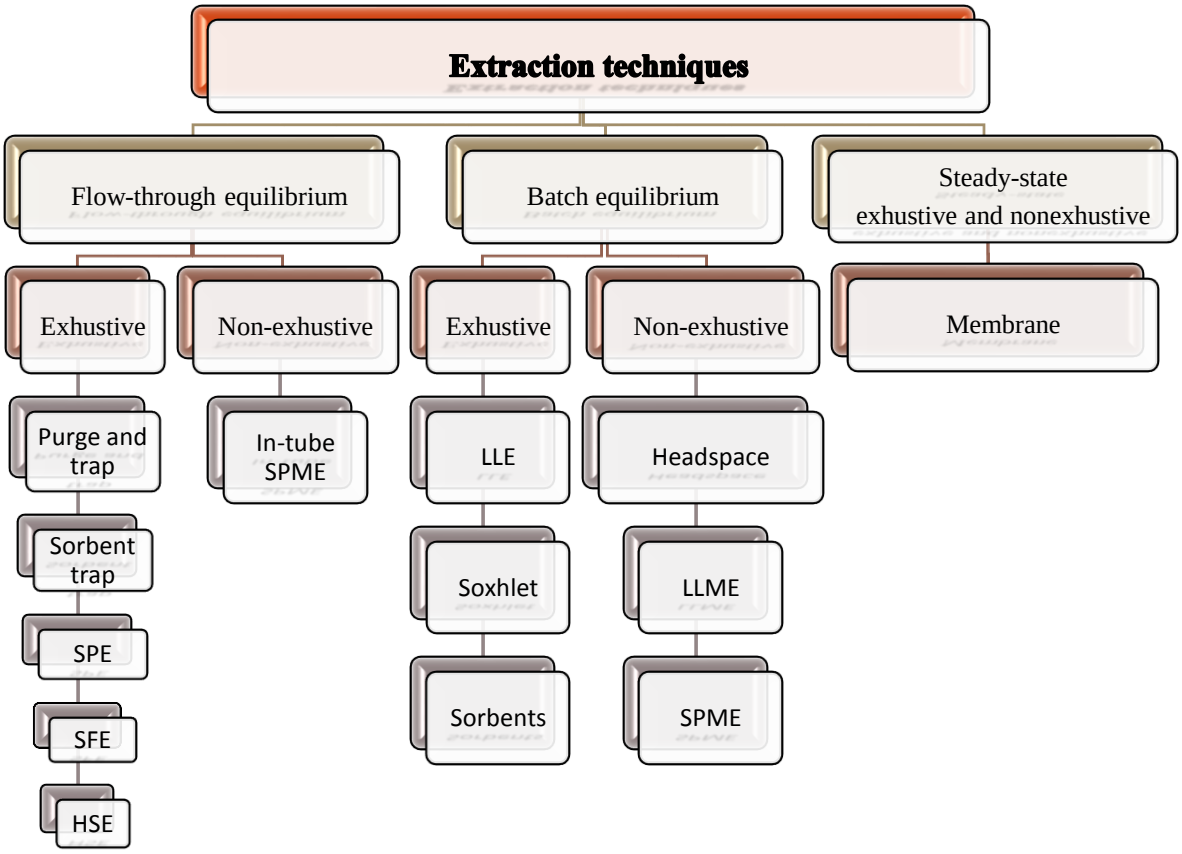


Figure 2-5: General classification of extraction techniques [202]

2.4.1 Liquid-liquid extraction (LLE) technique applied to SC

Liquid-liquid extraction (LLE) has been extensively used for the analysis of forensic biological samples, and it was the first sample preparation method historically used in analytical chemistry [204]. It potentially offers high recovery and reproducibility, and covers a large range of drugs in a single procedure for forensic toxicology matrices. LLE is a batch extraction technique, which involves the direct addition of a large quantity of organic solvent to the matrix. Target analytes are distributed among the sample matrix and the organic solvent phases, and because the organic solvents have different miscibility, density and solubility, a large amount of the analyte is extracted into the organic solvent phase or the aqueous phase. Additional steps, including evaporation with sometimes derivatisation should be performed before the samples are inserted into the analytical

instrumentation for analysis. Even though LLE is a straightforward technique for preparing the sample and has been employed in a wide range of matrices, it is labour intensive, time-consuming and harmful to the environment and general health. The limitations also include emulsion formation, a large sample volume and the use of toxic organic solvents. LLE can result in inadequate cleaning and increases the interference of components [202, 205].

LLE has been widely applied for the extraction of cathinone derivatives coupled with GC or LC-MS-MS. The selected test procedures applied to SC using LLE techniques are illustrated in **Table 2-4**.

Li et al. [206] used a 0.5 mL plasma sample and 5 mL of organic solvent methyl-tert-butyl-ether (MTBE) for the extraction of 11 cathinone derivatives. The top of the organic layer was transferred for evaporation and analysis. Ammann et al. [207] utilised whole blood samples mixed with 1 mL of 1-chlrobutane and 10% isopropanol (v/v), and the sample was analysed using LC-MS-MS for the detection and quantification of 25 components of cathinones.

2.4.2 Solid-phase extraction (SPE) technique applied to SC

The solid-phase extraction technique was developed with the objective of eliminating at least one of the limitations of the LLE technique. In SPE, the organic solvent is substituted for a solid phase and the matrix travels through a sorbent bed. The analytes in the sample settled completely on the solid sorbent. By using solvents and distilled water for washing, the components that are not required are selectivity discarded from the solid sorbent. Conversely, the analytes of interest are desorbed by an eluting solution. The eluent resulting from desorption of the analytes is then concentrated by evaporation. The SPE technique, unlike LLE, can be utilised both offline or fully automated online. It consumes less organic solvent and incorporates a clean-up step. However, it has a number of limitations, including the fact that it is a multi-step technique, it is time-consuming and the volume of the sample must be large to meet the limit of detection [208].

Table 2-4: Selected procedures applied to SC using LLE techniques

Name of SC Drugs	Matrix	Sample preparation methods	Detection	Validation parameters	Instrumentation	Ref.
Mephedrone, MDMA, 3-FMC, MDPV and some other compounds	Urine	LLE	EI, full scan	LOD	GC–MS	[209, 210]
Mephedrone	Hair	LLE	EI, SIM	Linearity, accuracy, precision, selectivity, LOD, LOQ	GC–MS	[211]
Mephedrone, methedrone	Blood	LLE, TFA	EI	Linearity, accuracy, precision	GC–MS	[143]
MDPV	Urine	LLE, HFBA	EI, SIM	Linearity, accuracy, precision, LOQ, LOD	GC–MS	[212]
Cathinone, methcathinone, ethcathinone, mephedrone, flephedrone, methylone, methedrone, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine and mephedrone.	Blood	LLE	ESI, SRM	Linearity, accuracy, precision, selectivity, recovery, LOD, LOQ, stability, matrix effect	LC-MS-MS-QQQ	[213]
Mephedrone	Hair	LLE	ESI, MRM	Linearity, accuracy, precision, selectivity, LOD, LOQ	LC-MS-MS-QQQ	[214]
Mephedrone and methedrone	Hair	LLE	ESI, MRM	Linearity, accuracy, precision	LC-MS-MS-QQQ	[143]
Methcathinone and mephedrone	Blood and serum	LLE	ESI, MRM	Linearity, accuracy, precision, selectivity, recovery, LOQ, LOD	HPLC-DAD	[215]
11 cathinone derivatives	Equine plasma	LLE	ESI, MRM	Linearity, accuracy, precision, recovery, LOD, LOQ, selectivity	LC-MS-MS	[206]
25 designer cathinones	Blood	LLE	ESI, MRM	Linearity, accuracy, precision, LOD, LOQ	LC-MS-MS	[207]

Abbreviations: MDMA (methylenedioxy-methamphetamine), TFA (trifluoroacetic anhydride), HFBA (heptafluorobutyric anhydride), 3-FMC (3-fluoromethcathinone), MDPV ((methylenedioxy-pyrovalerone), LLE (liquid-liquid extraction), EI (electron ionisation), SIM (selected ion monitoring), ESI (electrospray ionisation), SRM (selected reaction monitoring), MRM (multiple reaction monitoring), LOD (limit of detection), LOQ (limit of quantification), GC-MS (gas chromatography-mass spectrometry), LC-MS-MS-QQQ (liquid chromatography-mass spectrometry-triple quadrupoles), HPLC-DAD (high performance liquid chromatography-diode array detection).

The SPE technique is the method most used for the extraction of compounds in simple or complex matrices. It provides the desired recovery using clean products, without harming the instruments. There are innumerable articles published using the technique on biological samples. Reviews of SPE in biological samples and trace elements were completed by Ibrahim et al. [216] and Buszewski et al. [217]. Modern trends in solid-phase extraction (review) were recently published to explore its application to new sorbent media [218]. A review on its application to imprinted polymers and the binding assay was discussed by Caro et al. [219]. There are five devices for SPE, based on applications in food, environment, forensic toxicology and biomedical samples: multi-well plates, cartridges, pipette tips, disks and sorbent mixed with sample [218].

Multi-well SPE plates have been extensively illustrated for clinical applications [220] by monitoring several types of xenobiotics [221]. It can be used for the extraction of drugs in human urine and plasma. The main advantage of the multi-well device is its ability to deal with large samples (96, 384 or 1536 wells in one plate [220, 222]) in a short period of time with less labour and less elution solvents [218]. A 96-well plate was used to analyse cis-3-(4-((4-chlorophenyl) sulfonyl)-4-(2,5difluorophenyl) cyclohexyl) propanoic acid in a plasma sample using HPLC-MS-MS with a recovery between 82 and 89% [223]. A 384 well plate device was used for the extraction of methotrexate and 7-hydroxymethotrexate in urine and plasma using liquid chromatography-mass spectrometry (LC-MS)-MS. The results showed a high recovery of >95% [224]. Both examples above used the octadecyl modified silica extraction material. Another device for the extraction of various drugs in matrices is **the cartridge SPE**. It was used for the extraction of glyphosate, glufosinate and bialaphos in serum and urine using UPLC–MS-MS and provided a recovery between 63 and 74% using zirconia-coated silica as the material of extraction [225]. An additional study used the cartridge device for the extraction of the theophylline drug in serum using HPLC-UV. The recovery was between 79 and 84% using molecularly imprinted polymers as the material of extraction [226].

SPE pipette tip has recently become a desirable technique because it can provide, to some degree, the requirement for green analytical methods. It is a miniaturisation device used for the extraction of substances in biological samples. Pipette tip SPE successfully extracted methamphetamine and amphetamine in

human whole blood, in conjunction with GC–MS. It showed excellent linearity, and the LOD for both drugs was 0.2 ng mL^{-1}. Relative standard deviation (RSD) and bias were both less than 14% for all drugs tested [227]. The technique was also used for the determination of mequitazine in human plasma by GC–MS. The results showed that the device monotip C_{18} tips bonded with monolithic silica gel was robust for the analysis of the medical drug with an LOD equal to 0.06 ng mL^{-1} and 90% recovery [228].

The disk SPE efficiently extracted amphetamine, methamphetamine, MDA and MDMA from urine using gas chromatography-mass spectrometry (GC–MS). The recoveries were between 70 and 105% with LOD less than 4 ng mL^{-1} for all drugs tested [229]. It was also used for the extraction drugs of abuse in urine for screening purposes in a toxicology laboratory. The RSD was less than 5%, and the recovery was 75–100% for all drugs tested [230]. The application of **SPE sorbent mixed** with the sample was reviewed by Augusto et al. [231]. The evaluation of three different SPE, hydrophilic balance, mixed-mode and molecularly imprinted polymer sorbents was undertaken for the extraction of five amphetamines using LC-MS-MS by Iria González et al. [232]. Molecularly imprinted polymer sorbent was the preferred choice, as it provided clean extracts with great precision and accuracy and less matrix effects.

SPE and cathinones, Castro et al. [233] developed an SPE and LC-MS-MS procedure for the determination of eight cathinones in oral fluid. The extraction device used was the Strata X cartridges. 0.5 mL of oral fluid and 2 mL of borate buffer pH 9 were added. The target analytes were eluted using dichloromethane. 0.1% (v/v) HCl was added before the contents were evaporated using nitrogen and then reconstituted by adding 0.1% (v/v) formic acid. The samples were then introduced into the LC-MS-MS system.

Mayer et al. [234] developed a selective and rapid HPLC–diode array detection method for the confirmation of mephedrone, flephedrone and 4-MEC in human urine. Samples were prepared by SPE using as internal standard procaine hydrochloride. The specimens were eluted twice using 400 µL of 0.5 M ammonia–acetonitrile. The recovery of SPE was between 71 and 82%, while LOD and LLOQ were 40 and 100 ng mL^{-1} respectively. Other papers on the subject of SC detection using SPE techniques are summarised in **Table 2-5**.

Table 2-5: Selected toxicological applications applied to synthetic cathinones using SPE

Name of Drugs	Matrix	Sample Preparation	Detection	Validation parameters	Instrumentation Name	Reference
Methedrone, methylone, mephedrone, MDPV, fluoromethcathinone	Oral fluid	SPE	MRM	Selectivity, LOD, LOQ, precision, accuracy, recovery	LC-MS-MS	[233]
Mephedrone, 4-MEC and flephedrone	Urine	SPE	Diode array detection	Stability, recovery, matrix effect, linearity, accuracy, precision, LOD, LOQ	HPLC–DAD	[234]
MDPV, MPHP, PPP, MPPP, MOPPP, MDPPP, MPBP, α-PVP	Urine	EHCC, SPE, TMS	EI and full scan	LOD	GC–MS	[127, 235]
Methylone and its metabolites	Rat and human plasma	Protein precipitation and SPE	MRM	Precision, stability, accuracy, LOQ, LOD, ion suppression	LC-TMS	[236]
Some cathinones and more than 150 drugs of abuse and poisonous compounds	Human whole blood	Online-SPE	Full scan-ESI	Screening methods, LOD, recovery	LC-TOF-MS	[237]
32 cathinone derivatives	Serum	Supelco Visiprep-DL Disposable Liner SPE	MRM	LOD, LOQ, precision, accuracy	LC-QQQ-MS-MS	[238]
28 synthetic cathinones	Urine	Solid phase cation exchange extraction (SOLA SCX)	EI	Stability, recovery, matrix effect, linearity, accuracy, precision, LOQ, LOD	LC-HRMS	[129]
MDPV, Mephedrone, BZP and TFMPP	Whole blood, serum, urine	CSDAU206 Clean Screen SPE	ESI	Stability, LOQ, LOD	LC-TMS	[239]
30 synthetic cathinones	Urine	Solid phase cation exchange extraction (SOLA SCX)	ESI	Stability, matrix effect, linearity, accuracy, precision, LOD, LOQ, ionization suppression/enhancement	LC–HRMS	[240]
10 synthetic cathinones	Oral fluid	SPE	ESI-MRM	Accuracy, precision, linearity, selectivity, matrix effect, recovery	UHPLC –MS-MS	[241]
Methcathinone, mephedrone and 4-MEC	Autopsy, blood	SPE with 0.1 M carbonate buffer (pH 9.3)	ESI	Matrix effect, LOD, LOQ, precision, accuracy	LC-MS-MS	[130]

Abbreviations: MDPV (methylenedioxy-pyrovalerone), 4-MEC (4-methylethcathinone), MPHP (4-methyl-α-pyrrolidinohexiophenon), PPP (α-pyrrolidinophenone), MPPP (methyl-α-pyrrolidinopropiophenone), MOPPP (methoxy-α-pyrrolidinopropiophenone), MDPPP (methylenedioxy-α-pyrrolidinopropiophenone), MPBP (methyl-α-pyrrolidinobutyrophenone), α-PVP (α-Pyrrolidinopentiophenone), BZP (benzylpiperazine), TFMPP (trifluoromethylphenylpiperazine), SPE (solid-phase extraction), EHCC (extrahepatic cholangiocarcinoma), TMS (trimethylsilyl), EI (electron ionisation), ESI (electrospray ionisation), MRM (multiple reaction monitoring), LOD (limit of detection), LOQ (limit of quantification), GC-MS (gas chromatography-mass spectrometry), LC-MS-MS (liquid chromatography-mass spectrometry), HPLC-DAD (high performance liquid chromatography-diode array detection), LC TMS (liquid chromatography-tandem-mass spectrometry), LC–HRMS (liquid chromatography-high resolution mass spectrometry).

2.4.3 Other exhaustive techniques applied to SC

There are many other exhaustive extraction techniques that have been applied for SC, including matrix solid-phase dispersion (MSPD), protein precipitation, dilute-and-shoot technique, microwave assisted extraction (MAE), ultrasonic assisted extraction (UAE), the toxic lab system, perchloric acid, enzymatic hydrolysis, methanol, enzymatic digestion and the ultra-filtration technique.

Matrix solid-phase dispersion technique was developed for the extraction of cathine, psychoactive phenylpropylamino alkaloids, norephedrine and cathinone from khat by HPLC with diode array detection [242]. Protein precipitation was used for the extraction of mephedrone from post-mortem samples by GC–MS and HPLC [141]. Other papers using this method applied to SC are summarised in **Table 2-6**. Dilute-and-shoot (DS) or direct injection method has been successfully applied for the detection several components in toxicology samples using LC-MS (DS-LC-MS-MS). The applications of DS applied to analytical toxicology matrices were reviewed by Deventer et al. [243]. This method has several disadvantages, including yielding bad responses and detection with high noise in the background, it harms the injector port and column in GC. However, it is easy and rapid for screening and reduces the effect of the matrix [243, 244]. Papers published using this method applied to SC are summarised in **Table 2-6.**

Microwave-assisted extraction (MAE) and ultrasonic assisted extraction (UAE) are the techniques used for the extraction the target analytes by heating the solvents in contact with a matrix by emitting microwave energy in order to partition the target analytes from the matrix into the solvent [245]. The applications of MAE and UAE for the identification of drugs of abuse were published by [244, 246] and [247] specifically for cathininones, see **Table 2-6.** Toxi-lab system (or thin-layer chromatography) is a device for the extraction of toxicological drugs for screening only. The evaluation and performance of this technique were evaluated by [248]. The papers that used this technique for the analysis of illegal drugs were reported by [249, 250]. The-toxi lab was used to evaluate the effects of pesticide on khat leaves using GC–MS [251]. It was also similarly applied to methcathinone [167].

Perchloric acid can be used in deproteinisation to remove any protein remaining

in the sample (whole blood or body fluids). It additionally enables any small molecules in the target analytes to be stabilised. The deproteinisation procedure was used for the quantification of SC in oral fluid [252] and in other matrices [76, 253, 254]. This method was thought to improve the recoveries of SC [76]. Acidic or enzymatic hydrolysis with ethylation or acetylation were performed coupled with SPE extraction and GC−MS in biological samples for the extraction of SC [255, 256]. The evaluation of methanol as an extraction solvent for SC was demonstrated by [206, 257].

Enzymatic digestion was employed in human hair samples by placing the hair in a glass tube containing enzyme proteinase K and Cleland's reagent for the digestion of hair samples. The extraction was carried out using LLE, and full validation was successfully achieved for the detection of mephedrone with two metabolites: 4-methylephedrine and 4-methylnorephedrine, using LC-MS-MS [214].

Ultra-filtration technique (UF) was developed [213] for the detection of more than 10 cathinones by LC–ESI-MS-MS in a whole blood sample. Methanol was added to the blood sample for extraction, and the supernatant was then ultra-filtrated by UF filter cup. Before centrifugation, 10 µL of formic acid was added and diluted twice by distilled water. There was no significant loss of SC after the filtration was applied (the drugs were stable even though the filtration procedure was employed) [258].

Table 2-6: Selected applications that were applied to SC using protein precipitation, dilution techniques and ultrasonic-assisted extraction

Name of Drugs	Matrix	Sample Preparation	Detection	Validation parameters	Instrumentation	Ref.
Mephedrone	Autopsy samples	Protein precipitation	Diode array	Not mention	HPLC–UV and GC–MS	[141]
BZP, TFMPP, MeOPP, MDBP, 4-MTA, mephedrone, MDMA, 3-FMC, 3-BMC, MDPV	Urine	Protein precipitation	HR-ESI-MS	LOD	LC–HR-MS	[210]
Mephedrone, methylone, butylone, methedrone, BZP, TFMPP and MDPV	Urine	Dilution	ESI, SRM	Rapid screening, LOD, LOQ, recovery, precision, matrix effect, linearity, accuracy	LC-MS-MS QQQ	[259]
Screening of synthetic cathinones and some other related NPS	Urine	Dilution	Reversed-phase method	Linearity, LOD, LOQ, matrix effect	LC-MS-MS	[260]
Butylone, ethylone, ethcathinone, mephedrone, methylone, MDPV, methedrone, cathinone and some other related NPS	Pills and powders	Ultrasonic-assisted extraction with 3 mL of acetone	ESI	Not mention	LC-QTOF-MS	[247]
Cathinone, methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylone, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine	Whole blood	Protein precipitation, addition of methanol and ultrafiltration method	SRM	Recovery, LOD, LOQ, stability	LC–ESI-MS-MS	[213]

Abbreviations: BZP (benzylpiperazine), TFMPP (3-trifluoromethylphenylpiperazine), MeOPP (para-methoxyphenylpiperazine), MDBP (methylenedioxybenzylpiperazine), 4-MTA (4-methylthioamphetamine), 3-FMC (3-fluoromethcathinone), 3-BMC (4-bromomethcathinone), HR-ESI-MS (high resolution- electrospray ionisation), SRM (selected reaction monitoring).

2.4.4 Other exhaustive sample preparation applied to forensic toxicology matrices.

Other exhaustive extraction methods applied for the detection of substances in forensic toxicology matrices are supercritical fluid extraction (SFE), supercritical fluid chromatography (SFC), pressurised-liquid extraction (PLE), accelerated solvent extraction (ASE), solid-supported liquid-liquid extraction (SLLE), solid phase dynamic extraction (SPDE) and soxhlet extraction.

The general applications of supercritical fluid extraction (SFE) coupled with chromatographic analysis in the forensic field were published by [261, 262], and specifically for drugs of abuse by [263]. SFE is a safe and rapid method but it is expensive. It yields better precision compared to conventional techniques [264]. SFE and pressurised-liquid extraction (PLE) were applied for the extraction of amphetamines by [265-267]. PLE achieved a short extraction time with minimum solvent. The extraction products using the filtration technique were high compared with SFE and microwave-assisted extraction (MAE). Solid-supported liquid-liquid extraction (SLLE) was used for the extraction of benzodiazepines in [260, 268]. The application of solid-phase dynamic extraction (SPDE) to drugs of abuse was published in [246, 260, 269]. Accelerated solvent extraction (ASE) was applied to extract cocaine and benzoylecgonine [270]. Soxhlet extraction apparatus were generally used for herbal, soil and solid samples. It was used for the extraction of herbal drugs of abuse by Meyer [271], and it was used for the extraction of khat by Glick and Kuehne [272]. The automated soxhlet extraction system was applied for extraction from hair specimens for the detection of 20 NPS coupled with HPLC–ESI-MS-MS, as well as benzodiazepines and metabolites in [273, 274].

More than 40 therapeutic drugs were extracted using dispersive solid-phase extraction (DSPE) and GC-ion trap MS in whole blood and plasma. The DSPE was developed and validated for eight drugs. Acetonitrile, magnesium sulfate and sodium chloride were initially added to blood, and the contents were mixed, shaken and centrifuged for separation. The organic layer was then cleaned from the residual of water by D-SPE using bulk sorbents with magnesium sulfate. The

method in this article used the QuEChERS approach, which was developed for the determination of pesticides in food. The D-SPE coupled with the PSA sorbent obtained more acceptable results than aminopropyl and styrene-divinylbenzene sorbent. The recovery was more than 80% and RSD was 10% for most drugs tested. LOD was less than 20 ng mL^{-1} [275].

Six fluoroquinolones in serum specimens were extracted using a molecular imprinting matrix dispersion technique combined with a chromatographic separation instrument. The method was developed using the sorbent and ethylene glycol dimethacrylate as a crosslinker for the elimination of interferences in the serum. The recovery was above 70% for all compounds tested with an RSD of 6.6% [276]. The molecularly imprinted matrix of dispersant sorbent was also developed for the extraction of fluoroquinolones collected from swine tissues and chicken eggs. The molecular imprinting matrix sorbent material was compared with other sorbents, such as Florisil, C18, silica and sand. The recovery was greater when the molecular imprinting matrix sorbent was used and no interferences were observed [277].

2.5 General review of microextraction techniques

Microextraction is a common approach of non-exhaustive extraction technique that employs small quantity of sorbent or liquid in the extraction phase for the extraction of the analytes from the matrix. Sample preparation using non-exhaustive extraction techniques has been used to decrease the volume size of the solvents, chemicals and samples while ensuring that the sampling is introduced conveniently with minimum time and cost. Additionally, this technique can be operated in automated system coupled with hyphenated chromatographic spectrometry instruments [278]. Many review papers have been published focus on microextraction techniques. See **Table 2-7** for the papers published in the last decade.

Table 2-7: Review papers have been published on microextraction techniques within (2007-2018).		
Title	**Year**	**Ref.**
Review: automation of solvent microextraction techniques	2007	[279]
Review: automated, on-line membrane extraction	2007	[280]
Application of solid-phase microextraction in analytical toxicology	2007	[281]
Sorbent and liquid-phase microextraction techniques with gas chromatographic analysis: a review	2008	[282]
A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation	2009	[283]
Review of solvent microextraction techniques- general methods	2010	[284]
Liquid-phase microextraction approaches combined with atomic detection: A critical review	2010	[285]
Review of environmental fate and toxicities of ionic liquids	2010	[286]
Recent developments and applications of microextraction techniques in drug analysis	2010	[287]
Solid-phase microextraction in bioanalysis: New devices and directions	2010	[288]
Protocol for solid-phase microextraction method development	2010	[289]
Recent developments in matrix solid-phase dispersion extraction	2010	[290]
Recent developments in solid-phase microextraction	2010	[291]
Recent advances in applications of single-drop microextraction: a review	2011	[292]
Liquid-phase and dispersive liquid–liquid microextraction techniques with derivatization: recent applications in bioanalysis	2011	[293]
Dispersive liquid-liquid microextraction	2011	[294]
Recent advances in SPME techniques in biomedical analysis	2011	[295]
Review: comprehensive sampling and sample preparation for biological and medical applications	2012	[296]
Review: recent advances in coupling single-drop and dispersive liquid–liquid microextraction	2012	[297]
Single-drop microextraction as a powerful pretreatment tool for capillary electrophoresis: a review	2012	[298]
Trends in liquid-phase microextraction, and its application to environmental and biological samples	2012	[299]
Solid-Phase Microextraction in Perspective	2012	[300]
Single-drop microextraction for bioanalysis: present and future	2013	[301]
Review: automation of solvent microextraction techniques	2013	[302]
Advances in solvent-microextraction techniques: a review	2013	[303]
Perspective: Hollow fibre liquid-phase microextraction – principles, performance, applicability, and future directions	2013	[304]
Recent developments and future trends in solid phase microextraction techniques towards green analytical chemistry	2013	[305]
Review of liquid-phase microextraction techniques based on ionic liquids	2014	[306]
Review of ionic liquid-based microextraction techniques for trace-element analysis	2014	[307]
Review: recent developments of liquid-phase microextraction techniques	2014	[308]
Review of derivatisation approaches using solvent microextraction techniques	2014	[309]
Applications of liquid-phase microextraction techniques in natural product analysis: a review	2014	[310]
Application of solid-phase extraction for trace elements in environmental and biological samples: a review	2014	[216]
Review of solvent microextraction techniques theory and practice text	2015	[311]
Sample preparation with solid phase microextraction and exhaustive extraction approaches: Comparison for challenging cases	2015	[312]
Application of molecularly-imprinted polymers in solid-phase microextraction techniques	2015	[313]
Recent Developments and Applications of Solid Phase Microextraction (SPME) in Food and Environmental Analysis—A review	2015	[314]
New developments in microextraction techniques in bioanalysis. a review	2016	[315]
Modern trends in solid-phase extraction: new sorbent media	2016	[280]
Strengths and weaknesses of in-tube solid-phase microextraction: A scoping review	2016	[316]
A review on procedures for the preparation of coatings for solid phase microextraction	2016	[317]
Review of microextraction techniques for forensic drug analysis in saliva	2017	[318]
Liquid-phase microextraction of biomarkers: a review on current methods	2017	[319]
Ten years of dispersive liquid–liquid microextraction and derived techniques	2017	[320]
Microextraction and its application to forensic toxicology analysis	2017	[321]
Review of geometries and coating materials in solid phase microextraction: Opportunities, limitations, and future perspectives	2017	[322]
Advances in Solid Phase Microextraction and Perspective on Future Directions	2018	[323]

2.5.1 Liquid-phase microextraction (LPME)

Liquid-phase microextraction (LPME) was industrialised in 1989 to minimise the volume of organic solvent and matrix to the microlitre scale [324] and to reduce the disadvantages of LLE [287, 325]. LLE is time and solvent consuming, forms emulsions and no automation can be achieved. The exposure to large amounts of toxic solvents can cause the following; increased waste, decreased safety, more negative impacts on health and the environment. For solving or reducing the above effects, liquid microextraction (miniaturisation) methods have been introduced [325, 326]. Liquid-microextraction or liquid-phase microextraction techniques (LPME) can be classified [327] as follows (**Figure 2-6**):

Liquid microextraction techniques	Solvent-liquid microextraction	Single-drop microextraction-two phase	- Directly suspended drop [212] - Direct immersion [213] - Continuous flow [214] - Drop-to-drop [215]
		Single-drop microextraction-three phase	- Simultaneous solvent microextraction with back-extraction [216] - Head space [217] - Liquid liquid lquid microextraction [173]
		Dispersive liquid liquid microextraction [188, 218-220]	
		Solvent bar microextraction [349]	
	Membrane assisted microextraction	Film membrane or Tube membrane	- Membrane bag [221] - Hollow fiber [222]
		Steady state exhaustive technique	- Flat sheet membrane [223]

Figure 2-6: The classification of liquid-microextraction techniques

2.5.1.1 Single-drop microextraction technique (SDME)

In SDME, a single drop of the organic solvent (sized 1-10 µL) is placed on the tip of a syringe and then introduced in the matrix for extraction. The syringe withdraws the extraction contents to directly inject into the instrument for analysis. The hand-operated system, formation of air bubbles and losing the single drop of solvent from the needle have kept this technique from being widely used in

laboratories [325]. SDME is used for the extraction of the analyte through either two (organic solvent and donor phases) or three phases (here, the back extraction in the acceptor phase is included). SDME use a non-exhaustive technique for extraction. SDME use as either a static approach (direct immersion) or a dynamic approach (headspace). After the extraction processes, the micro drop of acceptor phase (extraction phase) is suspended by the syringe, and then analysed using quantitative instrumentation methods such as GC−MS or HPLC [284, 324, 325]. Although SDME is simple, cheap, rapid and in micro-scale, the method is not suitable for the complex matrices, unionised substances and any substance not sensitive to pH [284, 324, 325].

There was no extensive study in the literature that showed the strength of the method for the analysis. For example, no study was published when used for quantification purposes in forensic toxicology samples. Most applications of the method were covered with water, oil, wine and juice samples. The reasons for the limitations in using the method on complex matrices resulted from: instability of solvent, drop dislodgment, the formation of air bubbles, extra care required during extraction, the filtration necessary and the time required for stirring. These factors showed that the negative impacts outweighed the positive for its suitability to be used in a routine procedure [311, 325, 326].

2.5.1.2 Single-drop-liquid-liquid-liquid microextraction (SD-LLLME)

LLLME was designed for ionisable substances that must have three phases: sample, organic and acceptor. The three-phase LLLME has two modules. The first module occurs in the organic phase that can be placed as a single drop onto a top of the sample phase to form a solvent membrane or into a porous hollow fibre membrane. The second module occurs in the acceptor phase, and it can either be placed into the hollow fibre membrane via microsyringe, or it can be withdrawn from the single drop after it is formed into the solvent membrane [187]. The main drawback of three-phase SDME is that the target analytes are extracted twice in the procedure (the first one in the organic phase, and the second in the acceptor phase) and as a result, the extraction efficiency can be low. However, this may not always be the case; for example, the efficiency factor (EF) increased from 500 to 730 when the three phases microextraction system in SD-LLLME was used for

weakly basic compounds (amphetamine and methamphetamine) in urine samples combined with HPLC–UV detection [187]. The urine sample in the donor phase is adjusted to a strong basic state using NaOH for ionisation and protonation of the basic amines. The single drop of solvent was then transferred into the sample solution using stirring assistance and a hot plate to complete and promote the extraction. The basic amines were protonated in the back-extraction phase (acidic acceptor phase) and yielded charged species that had a weak affinity for the organic solvent phase. The total products in this procedure produced a high EF because the volume ratio of analytes in the donor and acidic acceptor phases were large [284, 328]. Another study was performed using three-phase SD-LLLME with a CE instrument in human urine for chiral analysis of amphetamines. The weakly basic amine drugs were treated by adding NaOH in the donor phase. A single drop of the acidic acceptor phase was covered by an octanol layer to separate the different pH donor and acceptor phases. These processes were used to concentrate the basic analytes into the acceptor phase drop. The (+)-(18-crown-6)-tetracarboxylic acid was selected for the separation of enantiomers amines, and the result was an EF increasing 1000-fold with a 0.5 ng mL^{-1} LOD for amphetamine [196].

2.5.1.3 Headspace-single-drop-liquid-liquid microextraction (HS-SDLLM)

HS-SDLLM or headspace in a single-drop microextraction technique (HS-SDME) technique is similar to headspace solid-phase microextraction (HS-SPME) but the fibre in SPME is replaced with a single micro-organic solvent drop. This provides a significant advantage for this method. After the optimisation of the method, it can be automated with an autosampler system like SPME [196]. In addition, the headspace mode in SDME is used as an alternative to DI-SDME because the solvent in DI-SDME may dislodge from the tip of the syringe during the direct immersion. HS-SDME was developed for the extraction of analytes in biological samples to avoid contamination. The method was developed for the extraction of acetone, hexanal, heptanal, anisaldehyde isomers, amitriptyline, nortriptyline, isomers of 12 non-hydrolysed amino acids, short-chain fatty acids and the lung cancer biomarkers in urine and blood plasma using GC–MS [329-334].

2.5.1.4 Drop-to-drop technique

Drop-to-drop technique is one form of SDME technique exclusively used for the analysis of limited volume matrices; for instance, if the volume of the urine sample is in microscale. Less than 30 µL of blood, serum and urine were successfully extracted using this method, providing excellent LOD. 30 µL of patient plasma was used for the extraction of ß-blocker drugs by adding 5% NaCl (pH 11) (w/v) and drop to drop of toluene (1.8 µL) on the tip of a 10 µL syringe. The acceptor phase was suspended and deposited into another vial for analysis of ß-blocker using matrix-assisted laser desorption/ionisation MS (MALDI-MS). The extraction efficiency of the method was low compared to traditional exhaustive methods, such as SPE technique, because the recoveries after using the technique were ten-fold less than the unextracted recoveries in similar matrices and drugs [335]. The extraction of nicotinic acid in urine specimens used a similar technique, procedure and instrument [336]. The conclusion illustrated the expediency and purification of the method for extraction from microlitre volumes of known drugs in complex matrices [311].

2.5.1.5 Directly suspended drop

The directly suspended drop with melting liquid technique was developed for the extraction of chlorpyrifos (the pesticide chlorpyrifos is very potent and causes neurotoxicity) and its metabolites in urine samples [337]. Due to the popularity of using the pesticide, the study provided an efficient, simple, sensitive and cheap technique for medical laboratories to examine this pesticide. 5 mL of urine was filtered and mixed with 10 µL of 2-dodecanol at 800 rpm and 70°C for 40 minutes. The vial was then placed in a freezer until the drop became solid (10 minutes was sufficient). The solid drop (acceptor phase) was transferred and centrifuged for melting and separation purposes using a conical vial (the conical vial was used for the separation of the acceptor phase from the remaining water content). The 7 µL of extracted content (bottom layer) was taken and analysed by GC–MS.

2.5.1.6 Continuous flow droplets

Continuous flow droplets was a low-cost technique developed by integrating the

microfluidic device with the waveguide mid-IR optical detector for the extraction of cocaine from human saliva specimens [338]. The flow droplets of perchloroethylene and saliva were continuously passed through the waveguide channels of the microfluidic extractor method for cocaine analysis. The flow rate of droplets was 5 and 20 μL min^{-1} for perchloroethylene and saliva, respectively. The validity of the method was not apparent as the cocaine was only tested when it was spiked in saliva in high concentrations (500 μg mL^{-1}).

2.5.1.7 Dispersive liquid-liquid microextraction (DLLME)

DLLME was used to avoid the above-mentioned drawbacks specifically when solvent microextraction methods are applied in complex matrices. DLLME was invented by Assadi et al. in 2006 [339]. It is cheap, fast and delivers high extraction efficiency with great recovery. However, the technique is difficult to install and automate [303, 340, 341]. The mixture of the extraction and solvent contents is quickly injected by high turbulence to form small droplets. As a result, a cloudy extracting system is formed when the surface area increases. After centrifugation, the solvent appears in the bottom of the tube for collection. For instance, DLLME followed by HPLC-UV detection were used for the extraction and examination of cannabidiol, Δ9-tetrahydrocannabinol and cannabinol in urine specimens. The method was effectively applied for the analysis of three male positive urine samples, and it offered great recoveries [342]. A similar extraction method combined with the solidification of a floating organic drop was applied for the detection of amphetamine and methamphetamine in human urine using HPLC-UV [343].

2.5.1.8 Solvent-bar microextraction (SBME)

SBME is demonstrated in **Figure 2-7**. Most papers show similar experimental design for the development of solvent-bar extraction procedures in biological matrices. The main differences between studies were in the experimental processing design or the type of solvent used (differences in pH, extraction time, stirring rate, ionic strength, the temperature in the extraction step and matrix volume). These parameters played a significant role in increasing the extraction efficiency, and the selectivity and sensitivity of the method. These parameters are not only used

for SBME, but are also fundamental for developing sample preparation methods, especially for non-exhaustive techniques, such as LPME and SPME.

SBME was optimised for the determination of tramadol in plasma and urine using GC–MS. The experimental parameters optimised were: extraction time, solvent type, pH, the volume of contents, ionic strength and stirring rate. They were optimised using a box–Behnken and a plackett–Burman system. The hollow-fibre membrane (1.5 cm segments) was cleaned and one end in the membrane was sealed. 4 µL of n-nonanol solvent was suspended in the microsyringe. The needle was inserted into the hollow fibre through the open hole to introduce the organic solvent for 20 seconds, and then the end was sealed. Next, the solvent bar (SB) was immersed in the matrix (12 mL) for extraction. The SB was the removed from the fibre and withdrawn into a microsyringe for analysis. LOD was 0.02 µg $mL^{-1,}$ and the RSD was 4.5%. The data was reported using plackett–Burman screening design, ANOVA and Pareto charts. The pH, stirring rate and extraction time were the most significant parameters that increased the extraction efficiency of a target analyte. No significant effect on the extraction efficiency was observed when the ionic strength and temperature of the extraction step were modified [344].

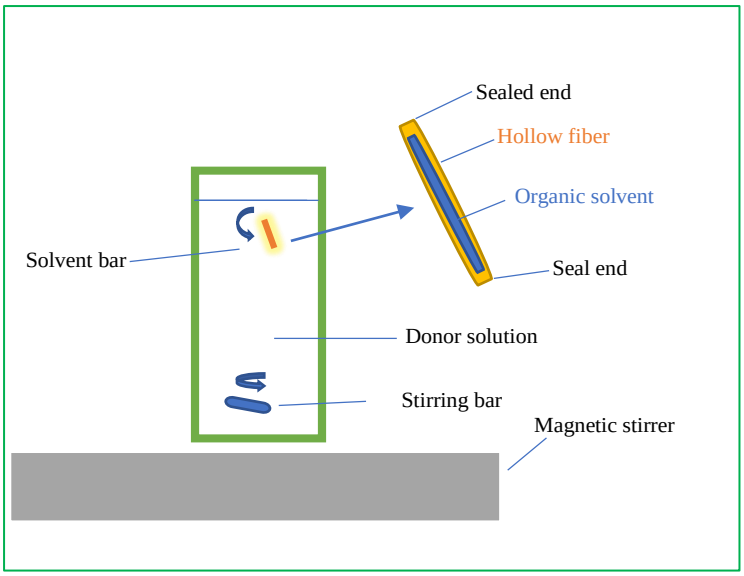


Figure 2-7: The experimental design of SBME

2.5.1.9 Hollow-fibre membrane liquid-phase microextraction (HF-LPME)

HF-LPME is derived from membrane assisted-microextraction techniques for the extraction of target analytes in different matrices. Some of the disadvantages of solvent microextraction techniques were avoided using hollow fibre LPME (HF-LPME). It was invented by Pedersen et al. in 1999, is convenient and cheap, and prevents organic solvents from being lost [345]. The method has two models: the hollow fibre film membrane and the hollow fibre in tube membrane [310, 346]. It was reviewed in terms of its application in [287, 299, 345, 347]. The extraction efficiency, recovery, extraction speed, enrichment factor, application and selectivity of HF-LPME coupled with the instruments of capillary electrophoresis, capillary gas chromatography, HPLC were reviewed by Rasmussen and Pedersen, where it was found that the HF-LPME can provide an excellent clean technique with high extraction efficiency for pre-concentration target analytes [348].

Most articles on the HF-LPME method used urine and plasma matrices. There were few studies showing HF-LPME in whole blood. Polypropylene (PP) fibre was the most commonly used fibre, followed by polyvinylidene fluoride (PVDF) and polyethersulfone fibres, respectively. Cocaine and its metabolites in urine were extracted using the two fibres, and the PP fibre provided more reproducible results than the PVDF fibre [349]. Another study showed irreversible results, due to the specification of the fibres used [350]. Using HF-LPME in complex matrices required hard work in development to obtain the desired results. For example, the extraction of ATS and barbiturates in hair and liver samples used three-phase HF-LPME in [327, 351].

2.5.1.10 Electro membrane extraction (EME)

EME is also another form of LPME. It is a recent technique that has been used in the electrical field. It initially appeared in 2006. The review and development of this technique were discussed in [352-354]. According to Huang et al. [354] there are more than 100 papers published on EME.

2.5.2 The applications of LPME used for the extraction of SC

LPME was reviewed in terms of its application in [287, 299, 347]. The detection of cathinone and five other drugs of abuse was investigated by Jamt et al. [355]. The study used electro membrane extraction and LPME methods by UPLC-MS-MS with MRM in forensic blood samples. The samples were collected from three forensic autopsy cases and authors used a supported-liquid membrane containing 1-ethyl-2-nitrobenzene. The supported-liquid membrane provided an effective barrier by segregating the macromolecules and acidic substance in the sample. When the electric field was applied, only the cationic compounds were efficiently removed across the membrane. The procedure produced clean extracts using LC-MS for detection. When 15 V was applied across the SLM with an extraction time of 5 minutes, 10-30% of recovery was achieved. The results obtained were in conformance with the results of the analysis conducted using conventional sample preparation methods, such as LLE. The LOD was between 40 and 2610 pg mL^{-1}, and the linearity was between 10 and 250 ng mL^{-1}.

2.5.3 Solid-phase microextraction (SPME)

SPME was used for the first time in 1989 by Pawliszyn and colleagues. It was used to reduce the amount of solvents and samples on the microlitre scale when analysing water pollutants [356]. The method is used as a sample preparation method for known and unknown drugs in matrices for screening and confirmation determinations. SPME is easy to employ, allowed for rapid screening and minimum contact with the toxic solvents. It can be both manual and automated, is sensitive, efficient and reduce the time required for the extraction and its cost [287]. It is highly efficient for screening purposes due to its speed and ease of use. The problems of sample loss, contamination and dilution are avoided using this technique. The main functional differences between SPE and SPME are that SPE relies on exhaustive extraction technique whereas SPME relies on the equilibrium reaction between the analytes and the stationary phase [357]. There are two modes commonly performed: Headspace (HS-SPME) and Direct Immersion (DI-SPME). HS-SPME is appropriate for the analysis of highly volatile compounds, simple and complex liquid matrices. DI-SPME is suitable to compounds with high polarity, low-to-medium volatility compounds, gas or simple liquid matrices, and

it can be immersed in any liquid sample [287, 356-358]. SPME is classified into two main categories [295, 359, 360]; see the **Figure 2-8**.

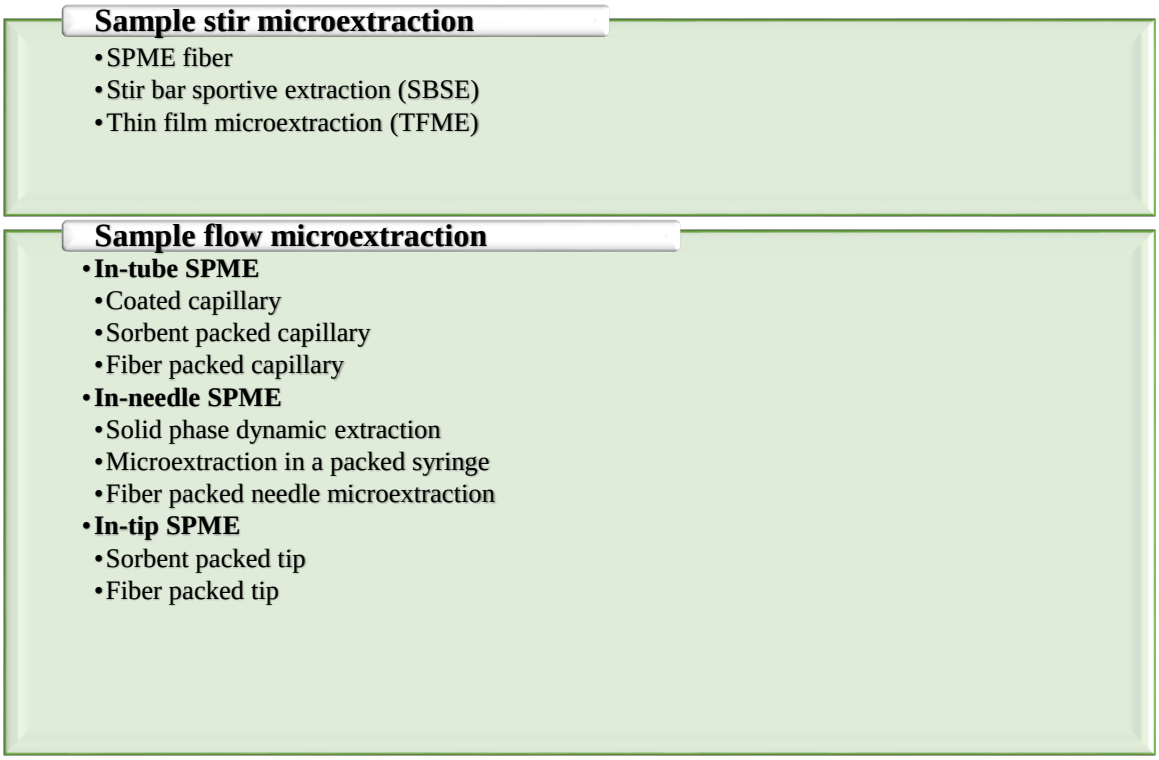


Figure 2-8: The classification of SPME

2.5.3.1 The SPME fibre

The SPME fibre has been successfully employed for the extraction of forensic toxicology samples. It is a basic syringe device consisting of three main components: micro-tubing for holding the fibre, the needle and the septum.

The history, fundamentals and development of SPME fibre were stated in three books by Pawliszyn et al. [202, 361] and Moldoveanu et al. [362]. Fibre coatings can be applied using fused silica with an appropriate stationary phase coupled with a device, such as polydimythyl silocoxane (PDMS), carboxen (CAR) /PDMS, PDMS/ divinylbenzene (DVB), polyacrylate (PA), carbowax (CW), CW/ templated resin (TPR), immunoaffinity fibre, alkyl-diol-silica (ADS) fibre, sol-gel fibre, molecularly imprinted polymer (MIP)-coated fibre, silica particle coated fibre and multi-carbon-tape fibre (thickness between 7–100 µm) [288, 295, 359]. The optimisation of the method and selecting the right coating are crucial. Fibre

selection is based on the physical and chemical properties of the coating and analytes, including volatility, polarity, the thickness of fibre [202, 288]. The key drawbacks are that the fibre gets broken-up easily and it is expensive. Fused silica fibre is used in both SPE and SPME techniques. The fibre in SPME is enclosed in a stainless-steel casing in order to avert mechanical damage when the septum is being pierced.

In HS-SPME, the SPME needle penetrates the septum cap, causing the SPME fibre to be exposed to the space above the sample. The sample vial is sealed, agitated and incubated to increase the movement of analytes in the space above the matrix until dynamic equilibrium is achieved. This process leads to the absorption of the gaseous analytes onto the fibre phase. Once optimum conditions are applied, and equilibrium reaction is reached, the fibre containing the absorbed analytes is withdrawn enclosed in its shielded steel casing. The needle can then be removed from the sample vial and enters the GC injection port for desorption of the analyte. The analytes are then released thermally from the fibre in the injector port and remain concentrated on the top of the GC column.

Direct Immersion (DI) SPME is similar to HS-SPME but differs in the way the fibre is exposed. In DI SPME, the SPME fibre is immersed in the liquid sample matrix [202, 363-365]. There are a number of factors influencing the sensitivity and efficiency of SPME, and a number of operational parameters must be developed in order to maximise affinity between the coating fibre and the analyte. These parameters include the type and thickness of the coating fibre, pH, ionic strength, agitation of sample, salts, temperature, extraction and desorption time and speed. It is essential that these parameters are optimised to attain the required LODs and recoveries for the detection of the target analytes [202].

2.5.3.2 Stir-bar sorption extraction (SBSE)

SBSE is a form of SPME. The method was reviewed in [282, 305, 366-373]. The most significant indicator showing that SBSE has advantages over other sorbent microextraction methods is the sheer volume of papers published, with over 1000 scientific papers published in the last decade alone. Hundreds of papers were published on the advantages and applications. Good examples of SBSE-PDMS

applications are the analysis of target substances in the areas of forensics, pharmaceuticals, food, natural products and biomedicine. The complex matrices can strongly affect the efficiency of SBSE, which could yield low recovery and increases interferences. Therefore, the optimisation of the method is crucial, and validation should be performed to confirm the target analyte behaviour and to avoid any possibility of interference. In some cases, the standard addition can be added to compensate the matrix effect. SBSE-PDMS was found to be a remarkable sorption-bar microextraction for polar solutes, volatile to semi-volatile, non-polar and medium polar compounds in different matrices. The direct immersion SBSE linked with the in-situ derivatisation was applied for the detection of forensic toxicology and biomedical samples [374].

SBSE was developed for the extraction of steroid sex hormones from the urine of pregnant women using HPLC- diode array detection [375]. The novelty of the work was the use of the polymethacrylic acid stearyl ester-ethylene dimethacrylate as the sorbent in SBSE. The development parameters were performed before validation. The LOD and LLOQ were (0.062–0.38) and (0.20–1.20) ng mL^{-1}, respectively. This new technique can efficiently extract polar analytes in the monolithic material copolymerization of methacrylic acid stearyl ester in SBSE with a solvent of a porogen mixed with 1-propanol and 1,4-butanediol, with methanol used for desorption.

The extraction of dimethyl trisulfide (used to treat cyanide poisoning) using SBSE-PDMS and GC−MS in rabbit whole blood was fully validated with an LOD of 0.06 µM, RSD of 10% and bias of 15% [376]. Another similar technique was used for direct immersion fabric phase sorptive media coated and sol-gel poly (ethylene glycol) (sol-gel PEG) for the extraction of benzodiazepines in human serum using HPLC-diode array detection. The recovery was 27% for bromazepam, 63% for lorazepam, 42% for diazepam and 39% for alprazolam [377]. Five fluoroquinolones were extracted by graphene oxide-polyethyleneglycol SBSE and sol-gel techniques using an HPLC-fluorescence detector in chicken muscle and liver. This method achieved great results for polar and less polar substances [378]. SBSE was used for the extraction of three ß2-agonist residues in pork [379], the analysis of pharmaceutical drugs and metabolites in urine [380], pulmonary tuberculosis drugs [381] testosterone and epitestosterone in human urine samples [382], and

glyoxal and methylglyoxal in-situ derivatisation [383].

The SBSE-PDMS coupled with HPLC-FLD (fluorescence detection) was developed for the determination of serotonin reuptake inhibitors (fluoxetine, citalopram and venlafaxine–norfluoxetine, desmethyl, didesmethylcitalopram, o-desmethylvenlafaxine and some active metabolites) in plasma and brain tissue (male and animals) [384]. Matrices were mixed in 1 mL of borate buffer (pH = 11, 0.1 M). The contents were put in 4 mL vial with a stir bar (1100 rpm for 30 minutes at 75°C). The stir bar was removed before the desorption stage, then cleaned with distilled water and tissue. The method was optimised as follows: 300 µL of acetonitrile were used in 15 minutes of desorption. 300 g L^{-1} NaCl was the ionic strength and extraction time was 30 minutes. The method showed excellent sensitivity and selectivity over three matrices. Caffeine and its metabolites were extracted using the alkyl-diol-silica coating and restricted access material in SBSE of rat plasma using HPLC-UV. The procedure used immobilisation material coating and was complicated by many steps over a long period of time; hence, the extraction efficiency showed that the method could be repeated 50 times with minimum loss of the target analytes. LOD was 25 ng mL^{-1} [385].

2.5.3.3 Thin-film microextraction (TFME)

The theory and applications of TFME were reviewed in [386]. TFME was introduced to increase the thickness of the stationary phase and the coated material. According to the theory, the sensitivity of the technique enhances when the area (thickness of the coated device) and the volume of the sample are large. Increasing the thickness of the device coating extended the reaction and equilibrium time. This cannot be achieved using the SPME fibre techniques, due to the small space (diameter) of the SPME needle [387].

2.5.3.4 In-tube SPME

In-tube SPME was reviewed regarding its development and application by Kataoka and Hiroyuki [388]. It was used online for organic compounds in liquid samples that were typically connected with the autosampler of HPLC or LC-MS. The extraction and concentration of the sample was monitored through the stationary

phase of the capillary column using an exposed tubular fused-silica by repetitively drawing and ejecting the sample matrix. It is a cheap, automatable, solvent-free and fast technique applied to medicine, forensic, food and environment matrices.

2.5.3.5 In-tip SPME

SPME tip or SPME pipette tip was used for the first time in 2011 for drug analysis by Xie et al. [389, 390]. It was developed and validated with derivatisation for the examination of vitamin D_3 using HPLC–MS-MS methods in human serum. The tips were coupled with the handling automation system and delivered a new sample preparation method for routine drug analysis. The fibre in tips can be coated in fibres that were usually applied in traditional SPME fibre, such as PDMS. The disposable tips were used for eliminating the carryover effect and precondition steps that are normally seen in the traditional SPME fibre technique. The technique provided accuracy and precision with a simple, convenient, fast and high throughput method.

2.5.3.6 General application of SPME

The application of SPME has been reviewed in biological samples and forensic toxicology fields in several papers [281, 295, 317, 359, 391-393]. The recent development of microextraction techniques and application in drug analysis has been reviewed in [295, 359, 391-393].

In general, SPME has been widely used as the extraction method in various matrices without issue, except for its application in whole blood. Whole blood contains large macromolecules, metabolites, parent products, proteins, platelets, phospholipids and cellular components. These components co-elute and interfere with known or unknown drugs, producing matrix effects. In forensic cases, the preferred post-mortem specimens sample is whole blood collected from the femoral veins as the sample remains unchanged for long periods after death [394]. There are limited studies that show the proper coating available to isolate the target analytes in whole blood. Hence, the use of polyacrylonitrile coating fibre in direct immersion with the C18 phase for the analysis of benzodiazepines in whole blood has been tested using liquid chromatography-tandem mass

spectrometry [395].

Recently, in 2018, SPME was used in the transmission mode device made from polyether ether ketone mesh for the determination of drugs of abuse in urine and oral fluid via direct analysis in real time tandem mass spectrometry. The method could be used in workplace or roadside for rapid screening [396]. A new method of unknown organic iodine was developed in microvolume using in-tube SPME and nanospray high-resolution mass spectrometry (HR-MS). The new scheme was a combination of in-tube and nanospray HR-MS, and was confirmed using iodine in urine. The unknown compound $C_{12}H_{23}O_{11}I$ was identified in human milk, and the method also can be used for the detection of unknown compounds by interpreting fragmentation ions [397].

2.5.4 The applications of SPME on the extraction of SC

The selected application of SPME for the extraction of SC is illustrated in **Table 2-8** and for whole blood [140, 143, 207, 213, 215, 237, 239, 355, 398-402], plasma [206, 236, 403], serum [140, 215, 238], hair [143, 211, 214, 404-408], oral fluid [241, 252] and urine [108, 122, 140, 210, 409-416]. Various instruments were used for the detection of SC, in GC−MS or GC−MS-MS [108, 122, 143, 146, 404, 416-419], LC-MS or LC MSMS [122, 125, 143, 206, 214, 233, 403, 415, 420, 421], nuclear magnetic resonance (NMR) [44, 45, 419, 422], HPLC [423] and Fourier transform infrared spectroscopy (FTIR) [422].

LaPointe et al. [413] examined three cathinones and three of metabolites in urine using direct analysis in real time mass spectrometry (DART-MS). The work was performed for the development and validation of SPME as a rapid screening method with no internal standards added as well as no corrections, extraction, derivatisation and sample preparation used. The fibres of SPME were developed, coated with 200 µm of C18, 200 µm of PDMS/DVB and a strong cation exchange resin. PDMS/DVB provided higher peak area responses for the metabolites of cathinones than C18 and vice versa for cathinones. The downside of this study was that the validation of the method was not performed.

Saito et al. [398] evaluated four types of fibres to analyse whole blood in a fatal poisoning case for the detection of *a*-PVP, MDPV and PV using GC–MS. The favoured extraction fibre was 65 µm PDMS/DVB; the fibres were used under similar criteria and conditions. The development parameters were evaluated based on pH, temperature and the extraction time. Method validation was performed, LOD and LOQ were (0.5 -1) and (1-10) ng mL^{-1}, respectively, and precision and bias were 9.5 and 9.8%, respectively. The recovery was 5%. Although the quantification method was achieved, no other identical samples were tested to ensure similar results were obtained.

2.5.5 Other microextraction techniques

Ultrasound-assisted emulsification microextraction is a sensitive, selective, effective and reliable method [424]. It was applied to pesticide, pollution and water samples [425]. Carbamazepine and amphetamines were detected using GC-FID in biological samples [244, 426]. Micro pulverised extraction technique was used for the detection drugs of abuse in hair or nails in [427-429]. However, there was no paper on the detection of SC by applying the above two methods.

2.5.6 Conclusion

In this chapter, SC and ATS are discussed in terms of their history, prevalence, chemical structures, metabolites, pharmacology, toxicology, administration, fatal cases and legal status. The chapter also involves review for the most preparation methods used in forensic toxicology laboratories. The review includes more than 46 techniques, most of them are related to the one from the following: solid-phase extraction, liquid-liquid extraction and microextraction techniques such as solid-phase microextraction and liquid-phase microextraction. General applications, advantages, and disadvantages of each were briefly discussed with linking in somewhat their uses in forensic toxicology. Sample preparation methods applied to SC are also reviewed and discussed. In the next chapters, some of these are developed and applied to the selected substances.

Table 2-8: SPME applied to SC

Name of Drugs	Matrix	Type of coating	Extraction mode	Instrument name	Ref.
3,4-dimethylmethcathinone (3,4-DMMC), 3,4-DMMC metabolites, 4-EMC, ß-hydroxy metabolite of pentedrone, 2-methylmethcathinone, mephedrone metabolite (ß-hydroxy)	Urine	C18, PDMS/DVB and a strong cation exchange resin	Automated sit between the DART source and the mass spectrometer inlet	DART-MS	[413]
Butylone, diethylpropion, flephedrone, mephedrone, methedrone, MDPV, methylone, and naphyrone	Oral fluid	PDMS/PA	HS-SPME and DI-SPME	GC–MS	[430]
MDPV and α-PVP	Blood	PDMS/DVB	HS-SPME	GC–MS	[398]
Some synthetic cathinones and some other NPS for screening	Hair	Not mention	Not mention	LC- TMS	[408]
4-FMC, α-PVP, MDPV and 11 NPS	Liquids, powders and herbs	PDMS	DH-HS-SPME	GC–MS	[431]

Abbreviations: MDPV (methylenedioxypyrovalerone), α-PVP (α-pyrrolidinopentiophenone), PDMS/DVB (polydimethylsiloxane/divinylbenzene), PA (polyacrylate), DART-MS (direct analysis in real time mass spectrometry), HS-SPME (head space-solid phase microextraction), DI-SPME (direct immersion-solid phase microextraction)

3. Investigation of derivatisation agents for determination of synthetic cathinones using gas chromatography–mass spectrometry

3.1 Introduction

Immunoassay methods traditionally used for screening purposes in forensic toxicology specimens have not proven effective for the detection of SC compounds. ATS or SC immunoassays such as Randox DOA−V assay or (methcathinone/mephedrone) kit [432, 433] often do not detect a wide range of ATS or SC, due to how fast design drug stimulants with variety of chemical structures that appears in the recreational illegal market. Some SC produce false-negatives, false-positives or even cross reactivity in immunoassays with high variability between manufacturer kits [433-435]; for example, de Castro et al. stated that SC have obtained cross reactivity with ATS compounds, such as amphetamine and MDMA [233]. A variety of structural SC substances presents the need for chromatographic mass spectrometry, not only for screening but also for confirmation determination.

The GC−MS is desirable because no cutoffs have been mandated by the government for screening and confirmation of SC in all matrices, and due to the popularity of GC−MS in forensic toxicology, it was used in this study.

3.1.1 Gas chromatography – mass spectrometry (GC−MS)

Chromatography was invented by Mikhail Tsvet in 1905 during research on chromatographic adsorption analysis. Martin and Synge suggested the use of partition chromatography between gas and liquid in 1941. The first GC detector, capillary column and mass spectrometry were invented by James and Martin, M.J.E. Golay and Gohlke et. al in 1952, 1957 and 1959, respectively [436-440].

The general principle of GC is very simple: a volatilised substance is injected into the injector port for transforming the phase from liquid to gas by increasing the temperature. The volatile compound travels from the injector port into the column carried by a mobile phase (carrier gas). The column contains a stationary

phase (silica particles- these are not analysed) that interacts with the analytes during mobilisation. The different analytes reach the detector at different times based on the physical and chemical properties of those phases and analytes. These compounds all detected as peaks in the chromatogram.

The fragmentation of analytes occurs in a mass detector after the ionisation of molecules by one of the following methods: electron ionisation (EI), chemical ionisation, electrospray ionisation, fast−atom bombardment, atmospheric−pressure chemical ionisation or matrix−assisted laser desorption ionisation [441, 442]. The technique most often used in forensic toxicology laboratories is EI, and accordingly, it was used in this project. In EI, the analyte passes through the interface line into the ionisation chamber, where a stream of high energy electrons from a heated filament in the ion source (typically 70 eV) bombards the molecules of the analyte. The molecules are then positively charged and lose an electron (see **Equation 3−1**). In the repeller, lenses are focused on the positively charged molecules before the mass detector. Finally, an analysis is conducted by the mass detector and associated software [442, 443].

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

Equation 3−1: The EI of a molecular ion (parent ion) [443]

Each volatile and non−polar compounds can be detected by GC−MS without the need for derivatisation, whereas detection of polar compounds can be improved using the derivatisation agents. The interpretation of fragmentation ions of a compound is crucial in forensic laboratories. Scan mode can be used to produce a total ion chromatogram, which is mostly used for the detection of unknow compounds or to study interferences. Alternatively, selective ion monitoring (SIM) mode can be used in routine work for the detection of target analytes. Interpretation of fragmentation patterns of known or unknown compounds depends upon the scope of the analysis; for example, the study of fragmentation patterns of unknown compounds should match a library (such as National Institute of Standards (NIST)) and should present sufficient knowledge of the mass spectrum.

3.1.2 Derivatisation reagent, SC and GC−MS analysis

The derivatisation reagent is a compound that is used to chemically amend an analyte to yield a new substance which is suitable for GC−MS analysis. GC methods are designed for the detection of volatile and compounds with low-to-medium polarity compounds, and derivatisation reagents may be used to improve the volatility and decrease the polarity of the mixtures. Derivatisation for the analysis of xenobiotics and drugs of abuse in matrices using GC have been comprehensively discussed and reviewed [444-447].

In forensic toxicology, the most derivatised functional classes of molecules are hydroxyl−groups, aminoalkenes and carboxylic acids. In general, alkylation, acylation and silylation reactions are the most commonly used techniques in GC−MS, but the reagent techniques most widely used in forensic toxicology are acylation and silylation. The acylation reaction is dominant and effective for ATS and SC, using acetic acid anhydrides, acid halides or fluorinated anhydrides such as PFPA, TFA and HFBA. Active hydrogen presents in cathinones (−NH), is submitted to the acylation reaction where the cathinones are converted into amides. Hence, tertiary amines lack the active hydrogen necessary to complete the reaction because of the presentation of the benzene ring instead of a hydrogen substance [448]. The reactions included in this study are summarised in **Figure 3-1**.

The GC−MS analysis of SC mostly involves the usage of derivatising agents to improve suitability (by modifying the chemical structure of the SC), efficiency (by improving the peak resolution and reducing interference or co−elution) and detectability (by increasing the sensitivity and producing multi fragmentation patterns or more mass ions in the detector) [449]. To choose suitable derivatisation reagents for GC−MS analysis, the following criteria should be considered as guidelines [449]:

a) More than 95% of complete derivatives should be produced by reagent.
b) The new derivative products that result from the reaction should not rearrange or alter the structure of the compound during formation of the derivative.

c) The sample should not be lost during the reaction.

d) The derivative compounds should not interact with the column in GC.

e) The derivatives should be stable over time.

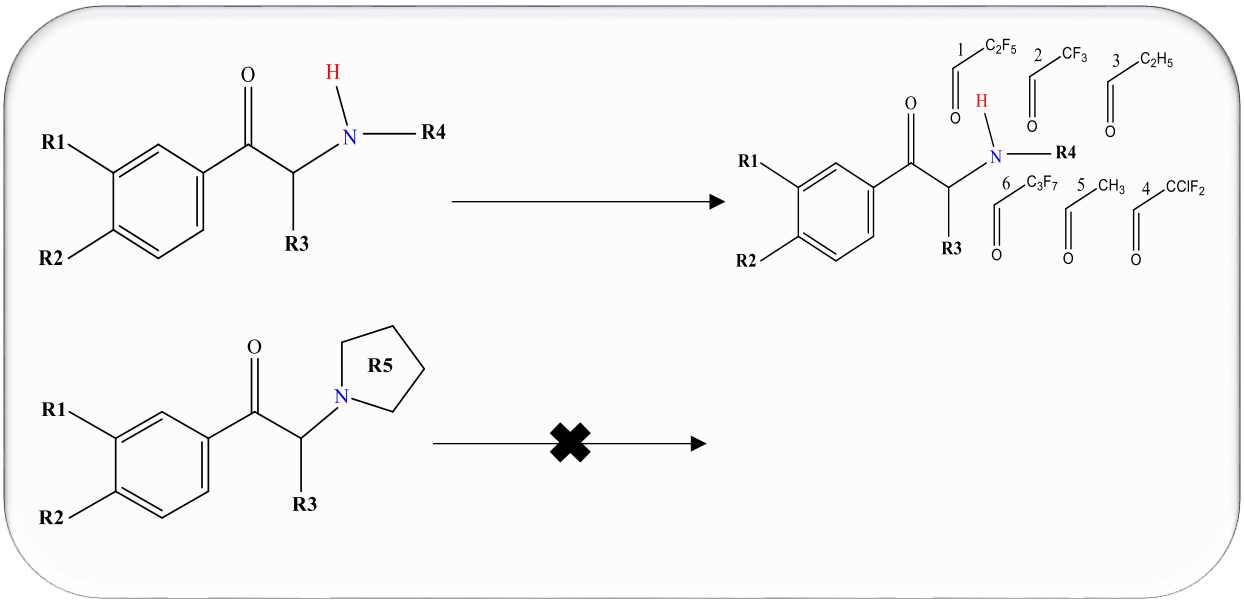


Figure 3-1: Acylation of primary, secondary and tertiary SC using PFPA (1), TFAA (2), PA (3), CLF_2AA (4), AA (5), HFBA (6); the active H is illustrated in red.

Acylation was preferred for this work because it is a common technique applied to SC and GC–MS, as **Table 3-1** shows, documenting the up-to-date published GC-MS analysis methods and derivatisation reagents applied to cathinones. These reagents were extensively used to enhance the sensitivity and specificity of the electron capture detector (ECD), provide more fragmentation ions in the mass spectrum by altering the original compounds, improve the resolution of the chromatogram and reduce the artifact peaks by dropping the polarity of analytes. Acylation reagents are similarly beneficial for thermolabile drugs when the analyte of interest has inadequate detection ions, which commonly occurs to various SC. However, the use of extra reagent may damage the column or cause detector contamination. The acylation reagents pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFA), chlorodifluoroacetic anhydride (CLF_2AA), heptafluorobutyric anhydride (HFBA), acetic anhydride (AA) and propionic anhydride (PA) were selected for this evaluation study. See **Figure 3-2** for the chemical structures of the reagents. This is the first study that includes CLF_2AA derivative for the detection of SC. It is also the first evaluation study that presents the fragmentation patterns of the selected cathinones using the CLF_2AA reagent.

Table 3-1: The recent published techniques including the analysis of derivative cathinones using GC-MS

SC	Derivatisation	Year	Ref.
Three methcathinones	PFPA and HFBA	2006	[48]
MBDB, Methylone and methcath	PFPA	2007	[404]
Butylone and ethylone	TFAA	2009	[125]
Mephedrone	PFPA	2010	[146]
Methedrone	TFAA	2010	[143]
Mephedrone	BSTFA	2011	[141]
Methylone	HFBA	2012	[450]
GHB and Mephedrone	PFPA	2012	[451]
3-bromomethcathinone and 3-FMC	Acetic anhydride-pyridine mixture	2012	[210]
DMMC	TFAA	2013	[452]
Ethylone	PFPA	2014	[453]
4-MEC	Acetic anhydride-pyridine mixture	2015	[454]
Mephedrone	MSTFA	2017	[455]
26 stimulants drugs	Hexyl chloroformate	2018	[456]

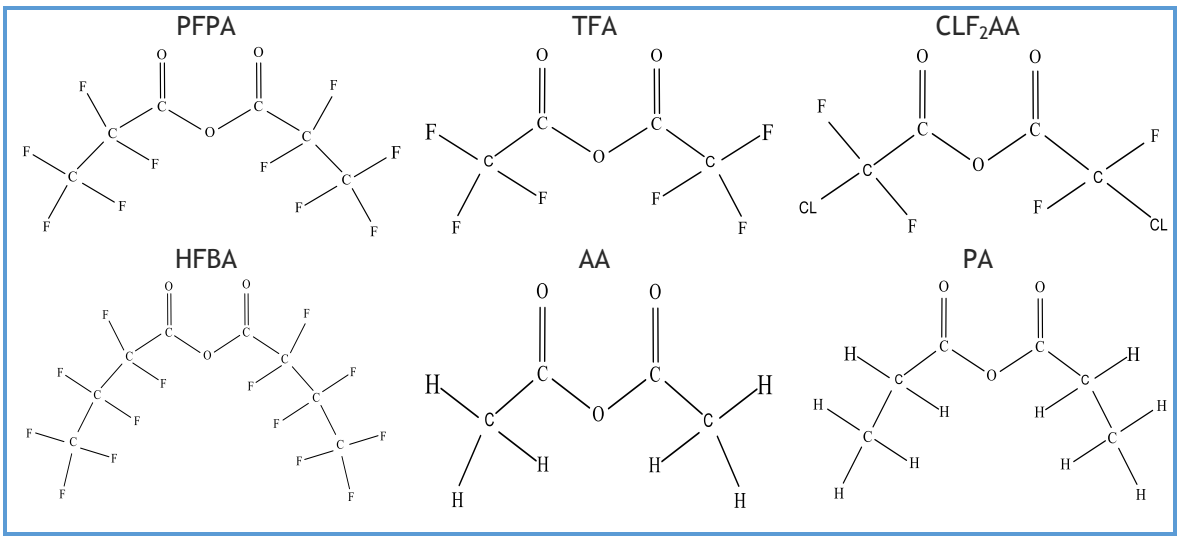


Figure 3-2: Chemical structure of selected derivatisation reagents

3.1.3 Problems and aims

The GC–MS conditions were optimised until excellent responses and separations were achieved in the chromatogram. During the development work, however, it was noted that MDPV–PFPA (tertiary amine) had one mass spectra fragment with a base peak of m/z 126, while the remaining ions were relatively small (less than

5%). Cathinones are widely known for having poor detection characteristics (sensitivity) with little fragmentation of mass spectra (selectivity), meaning that there are very few qualifier ions. Some SC have positional isomers, such as butylone and ethylone that have identical fragmentation patterns with only minor differences in spectra intensity. MDPV and pyrovalerone are not derivatised, therefore the SC are reliant on a limited number of mass spectra ions. Internal standards (ISD) of cathinones overlap with high abundance ions of cathinones, such as mephedrone and mephedrone-d_3. Thermal degradation of SC in the injector port is another concern when the temperature is very high.

From the viewpoint of the above problems, an investigation should be undertaken to avoid the legal implications of false interpretation, particularly in the screening tests for SC. The sensitivity and selectivity could be improved by adding derivatisation techniques and acidified methanol. The derivatisation agents are required to produce more fragmentation patterns and increase the resolution of peaks. After an extensive literature review, there was a distinct lack of publications on this significant issue. Therefore, the detailed aims of the project are as follows:

1. Comparison of six derivatising agents: trifluoroacetic anhydride (TFA), acetic anhydride (AA), chlorodifluoroacetic anhydride (CLF_2AA), heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA) and propionic anhydride (PA) for the determination of nine SC in GC–MS: mephedrone, flephedrone, pentedrone, methylone, ethylone, methedrone, MDPV, butylone and pyrovalerone. This is after optimisation of the conditions and investigate the thermal lability of SC in the injector port, the assessment of which included:
   - Reaction time and temperatures during the incubation and evaporation stages.
   - The maximum values of peak areas.
   - Quality of fragmentation patterns in each reagent.
   - The fragmentation patterns in terms of relative intensity ions.
   - Interference study.
   - Number of fragmentation ions in each cathinone derivative.
   - Quality of the reagents, using recovery, linearity, LOD, accuracy and precision applied to SC.
   - Complete three–way ANOVA for data treatment analysis [457].

- Study underivatised tertiary amines of cathinones, such as MDPV and pyrovalerone.
- Study ISD vs derivatisation agents using best−fit regression approach.

2. Study the effects of adding acidified methanol on the sensitivity of the method.
3. Study the recovery of two extraction methods: SPE and LLE.
4. Determination of five cathinones (mephedrone, flephedrone, methylone, methedrone, butylone) in whole blood, SPE−PFPA.

The GC−MS method was optimised to provide the best separation, selectivity and sensitivity in the chromatogram and detector. The SC for this project were selected as those being frequently abused in the UK [458]. Additionally, compounds from each class of SC (secondary and tertiary amines) were included to cover a wide range of cathinones. All compounds stated above are previously illustrated in **Table 2-2.**

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Chemicals and reagents

Nine SC reference standards at 1 mg mL^{-1} (butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone), five internal standards (ISD) at 100 µg mL^{-1} (butylone−d_3, mephedrone−d_3, ethylone−d_5, methylone−d_3 and MDPV−d_8) as their hydrochloride salts and seven derivatisation reagents (propionic anhydride (PA) ≥ 99%, chloro di−fluoro acetic anhydride (CLF_2AA) ≥ 98%, pentafluoro−propionic anhydride (PFPA) ≥ 99%, heptafluoro−butyric anhydride (HFBA) ≥ 99%, trifluoro−acetic anhydride (TFA) ≥ 99%, acetic anhydride (AA) ≥ 99% and butyric anhydride (BA) ≥ 98%) were purchased from Sigma−Aldrich, Gillingham, UK.

Ethyl acetate (EtOAc), sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride (NaCl), sodium dihydrogen orthophosphate monohydrate (NaH_2PO_4), disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), methanol (MeOH), isopropanol (IPA), dichloromethane (DCM), ammonium hydroxide

(NH_4OH), hydrochloric acid (HCl) and acetic acid were supplied by VWR International, East Grinstead, UK. Human whole blood was obtained from the Scottish National Blood Transfusion Service collected at Gartnaval Hospital, Glasgow. Sodium phosphate and phosphate buffer were purchased from Fisher Scientific, Loughborough, UK. Solid-phase extraction (SPE) 200 mg cartridges (part number ZSDAU20) were supplied by Chromatography Direct, Runcorn, UK. The deionised water was produced by ultrapure water purification (Water deionizer–Merck Direct QR 3UV).

3.2.1.2 General laboratory equipment

Table 3-2: General laboratory equipment used in the thesis	
Equipment	Specification
Balance	Sartorius TE 64-0CE
	Mettler Toledo XPE105 Deltarange
Vortex mixer	VWR Analog Vortex Mixer Fisher Scientific Topmix FB15024
Centrifuge	VWR Microstar 17
	Sigma 4-16
Nitrogen evaporator	Thermo Scientific Reacti-Therm III #TS18826 Evaporation Unit
Ultrasonic bath	Grant XUBA3
	Grant XUB5
pH meter	pH Electrode SJ223 662-1395 + Hanna Instrument pH210 Microprocessor
Microbiological safety cabinet	MSC12 BS5726-Jouan Part 1-1992
Thermometer	Fisher Scientific Traceable Calibration

3.2.1.3 Saline solution

The solution was prepared by dissolving 9.5 g of NaCl in 500 mL of deionised water (d.H_2O) (w/v). The contents were then transferred to a 1 litre volumetric flask and made up to the mark using d. H_2O.

3.2.1.4 Blank blood preparation

Blank human whole blood was prepared by adding 500 mL of packed red blood cell to a volumetric cylinder with a 1% saline solution defrosted in a ratio of 1:1 (v/v). The blood saline solution was then mixed carefully, transferred to a glass bottle, and stored in the refrigerator at 4°C until use.

3.2.1.5 Preparation of phosphate buffer (pH 6)

Phosphate buffer at 0.1 M at a pH of six was prepared by dissolving 1.7 g of Na_2HPO_4 and 12.14 g of NaH_2PO_4 in 800 mL of d.H_2O in a beaker. The contents were then transferred to a 1 L volumetric flask and d.H_2O was added to the mark. The volumetric flask was then inverted several times to mix the contents. The pH was adjusted to six using 0.1 M dibasic sodium phosphate to increase the pH or 0.1 M monobasic sodium phosphate to decrease the pH. The solution was then stored at 4°C for use within three months.

3.2.1.6 Preparation of 0.1 M acetic acid

5.75 mL of glacial acetic acid (99.6% acetic acid; stock solution is 17.4 M) was transferred into 800 mL of water in a volumetric flask in a fume hood. This was gently mixed and then topped up to 1 L mark with d.H_2O. The solution was then kept at room temperature (RT) to be used within six months.

3.2.1.7 Preparation of DCM: IPA: NH_4OH (78:20:2) (v/v/v)

2 mL of 28% NH_4OH solution was transferred to 20 mL of IPA in a 100 mL volumetric flask and then mixed. DCM was then added up to the 100 mL mark and mixed again. This solution was freshly prepared each day and then kept at RT until use. The preparation was carried out in a fume hood.

3.2.1.8 Preparation of derivatised SC

Acylation reagents are difficult to prepare, because interferences may occur during the reactions between the reagents and other products. Unreacted

reagents must be removed before GC analysis. Acylation derivatives are odorous, sensitive, hazardous and moist [448], therefore solvents were introduced to the mixture to reduce negative impacts.

The preparation of PFPA and EtOAc (2: 1), TFA and EtOAc (2: 1, v/v), CLF_2AA and EtOAc (2: 1, v/v), HFBA and EtOAc (3: 2, v/v), AA and EtOAc (3: 2, v/v), PA and pyridine (2: 1, v/v) and BA and pyridine (2: 1, v/v) was carried out in a fume hood. 4 mL of PFPA, TFA and CLF_2AA with 2 mL of EtOAc were aliquoted to 7 mL glass tubes for each derivative. 3 mL of HFBA and AA were separately transferred to 7 mL glass tubes and mixed with 2 mL of EtOAc for each agent. 4 mL of PA and BA were added to 7 mL glass tubes and mixed with 2 mL of pyridine in each reagent. All tubes were then capped and mixed for a few seconds. These reagents were again prepared by the same procedure after consumed.

3.2.1.9 Preparation of acidified methanol (1:9) (v/v)

1 mL of concentrated HCl was mixed with 9 mL of MeOH in a 10 mL volumetric flask and then transferred to an amber glass bottle. The solution was stored at RT until use.

3.2.1.10 Preparation of stock standards

Stock standard solutions were prepared for each substance individually, by dilution of butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone to achieve a concentration of 100 µg mL^{-1}. These were prepared by transferring 1 mL of each reference substance (1 mg mL^{-1}) into a 10 mL volumetric flask using MeOH to fill the flask up the mark (1:10, v/v dilution). The flasks for each drug were then shaken several times before each stock solution was transferred to single amber glass bottles and stored at −20°C.

3.2.1.11 Preparation of standard solutions

An individual solution for each drug was prepared to achieve concentration of 10 µg mL^{-1} in methanol. These were prepared by adding 1 mL from each stock solution

(butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone) at a concentration of 100 µg mL^{-1} to a 10 mL volumetric flask, the volume was made up to the mark.

3.2.1.12 Preparation of working solution (mixture)

Comparison of derivatisation study

Preparation of the mixture working solution was made by adding 1 mL from each stock standard (100 µg mL^{-1}) to a 10 mL flask of MeOH in order to achieve 10 µg mL^{-1}. The mixture of nine drugs (butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone) was transferred to an amber glass bottle and stored at −20°C until use. This working solution was used for the comparison study of derivatisation. A mixture of five SC (methylone, mephedrone, flephedrone, methedrone and butylone) was also prepared and used for the determination of whole blood samples.

For determination of five SC in whole blood sample study

Working Solution One was prepared by diluting stock solutions (100 µg mL^{-1}) at a 1:10 ratio in methanol for preparation of five SC (mixture) at 10 µg mL^{-1} (methylone, mephedrone, flephedrone, methedrone and butylone). Working Solution Two (1 µg mL^{-1}) of the five mixture drugs was prepared by diluting Working Solution One (10 µg mL^{-1}) by a ratio of 1:10 (v/v) in methanol.

3.2.1.13 Preparation of mixture working solution for ISD

A mixture of butylone−d_3, mephedrone−d_3, ethylone−d_5, methylone−d_3 and MDPV−d_8 was produced by transferring 1 mL of each drug (100 µg mL^{-1}) to a 50 mL flask of MeOH in order to reach 2 µg mL^{-1}. The mixture of ISD was transferred to an amber glass bottle and stored at −20°C until use. Similarly, a mixture of two ISD (mephedrone−d_3 and methylone−d_3) was prepared for the determination of the selected drugs in whole blood.

3.2.2 Methods

3.2.2.1 Optimisation study of six derivatisation reagents

The general procedure was as follows:

SC were derivatised using the following procedure. 50 µL of nine SC (mixture of 10 µg mL^{-1} from working solution stated in **section 3.2.1.12**) and 50 µL of five ISD (mixture of 2 µg mL^{-1} from working solution stated in **section 3.2.1.13**) were added to a 7 mL vial. The sample was evaporated under a stream of nitrogen at RT. The sample was then placed under the fume hood for the preparation of the derivatisation reagents. One reagent was added to each sample (glass tube), which consisted of 50 µL of PFPA and EtOAc (2:1, v/v); 50 µL of TFA and EtOAc (2:1, v/v), 50 µL of CLF_2AA and EtOAc (2:1, v/v), 65 µL of HFBA and EtOAc (3:2, v/v), 50 µL of AA and EtOAc (3:2, v/v) or 50 µL of PA and pyridine (2:1, v/v). Each sample was covered and mixed rapidly for 5–15 seconds and then incubated for various durations (5–10–15–20–25–30–35 or 40 minutes) and temperatures (RT, 40°C, 55°C or 70°C). The samples were evaporated again at different temperatures (RT, 40°C or 50°C) under a stream of nitrogen using a hot block. 50 µL of EtOAc was then added for reconstitution of the sample. The content of the EtOAc was transferred to a GC vial for GC–MS analysis. The syringe of GC was rinsed in EtOAc three times before the analysis. 1 µL of each sample was injected at 225°C and GC–MS was run under the conditions outlined in **section 3.2.2.12**.

In detail:

Triplicate samples were prepared and repeated eight times on eight different days at final concentrations of 0.50 µg mL^{-1} for the mixture of SC and 0.10 µg mL^{-1} for the mixture of ISD. A total of 72 derivatised tubes included 18 tubes in each specific time at 10, 20, 30, and 40 minutes at RT on day one, 40°C on day two, 55°C on day three and 70°C on day four. There were 72 tubes in each day = 3 (triplicate) × 6 (derivatisation reagents) × 4 (times) × 1 (temperature).

The samples were then set similarly to previous days but the times were modified to 5, 15, 25 and 35 minutes, carried out from day five until day eight. The samples

were then transferred for evaporation under nitrogen gas at RT for all reagents on days one through four. On days five through eight PFPA, TFA and HFBA were placed at RT, AA and CLF_2AA were set to 40°C and PA was set at 50°C.

On day nine, the 72 tubes included 18 tubes for each temperature under a stream of nitrogen examined using turboVap® for solvent evaporation in the following procedure. Triplicated specimens were set at 50°C under the hot block, and the incubation was 20 minutes at RT, 40, 55 and 70°C. 72 tubes = 3 (triplicate) × 6 (derivatisation reagents) × 4 (temperatures) × 1 (time).

An additional method was carried out on day ten to observe the effect of pyridine as a solvent when mixed with BA and PA using the following technique. 200 µL of the SC mixture (10 µg mL^{-1}) was added to all tubes and then the samples were left at RT for evaporation. The triplicates of 18 samples of BA and PA were capped and mixed for 15 seconds and then incubated at 90°C for 30 minutes. The samples were kept for evaporation again at RT, 40 and 50°C. 18 tubes = 3 (triplicate) × 2 (derivatisation reagents) × 3 (temperatures) × 1 (time).

54 tubes were set similarly to the above on a separate day for the assessment of temperature on the reaction, considering RT, 55°C and 70°C in 30 minutes (18 samples for each temperature). Both evaporation steps were completed at RT. 54 tubes = 3 (triplicate samples) × 6 (derivatisation reagents) × 3 (temperatures). This procedure was to confirm if the obtained results on one day are within an acceptable error range of the previous results using similar parameters**. Figure 3-3** shows an example of data analysis and **Figure 3-4** illustrates an example of derivatisation laboratory work.

Test	Sample	Compound name	tR	Target ion	Unite	Q. value *	Date	n value	Derv.	Temp. (°C)	Time	EVA. (°C)	Response	Conc. (500) **	Mean	Median	SD	RSD	Mean conc.	ng/ml	RSD of conc.	Accuracy
1	1	Flephedrone	10.23	123	ng/ml	95	11-10-2015	3	PFPA	RT	10 MIN	25	1530670	477	1697264	1755969	146356	8.3%	499	20	3.9%	-0.2%
2	1	Mephedrone	11.82	204	ng/ml	77	11-10-2015	3	PFPA	RT	10 MIN	25	748555	429	808182	824527	53366	6.5%	438	8	1.8%	-12.4%
3	1	Pentedrone	12.02	232	ng/ml	88	11-10-2015	3	PFPA	RT	10 MIN	25	1367910	394	1518876	1575956	132029	8.4%	413	17	4.0%	-17.4%
4	1	Methedrone	13.60	135	ng/ml	99	11-10-2015	3	PFPA	RT	10 MIN	25	4063497	455	4373964	4441701	282751	6.4%	476	18	3.9%	-4.9%
5	1	Methylone	14.62	204	ng/ml	93	11-10-2015	3	PFPA	RT	10 MIN	25	628778	436	663477	665104	33915	5.1%	447	11	2.4%	-10.6%
6	1	Butylone	15.16	218	ng/ml	92	11-10-2015	3	PFPA	RT	10 MIN	25	709915	430	777529	787954	63051	8.0%	441	11	2.4%	-11.8%
7	1	Ethylone	15.30	190	ng/ml	80	11-10-2015	3	PFPA	RT	10 MIN	25	297417	428	342158	343571	44051	12.8%	438	12	2.7%	-12.3%
8	1	Pyrovalerone	15.68	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	1525590	401	1495499	1525590	184537	12.1%	433	37	8.5%	-13.4%
9	1	MDPV	18.24	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	1201168	436	1107582	1201168	213038	17.7%	439	5	1.0%	-12.3%
10	2	Flephedrone	10.22	123	ng/ml	94	11-10-2015	3	PFPA	RT	10 MIN	25	1755969	509								
11	2	Mephedrone	11.83	204	ng/ml	74	11-10-2015	3	PFPA	RT	10 MIN	25	824527	440								
12	2	Pentedrone	12.03	232	ng/ml	89	11-10-2015	3	PFPA	RT	10 MIN	25	1575956	422								
13	2	Methedrone	13.56	135	ng/ml	99	11-10-2015	3	PFPA	RT	10 MIN	25	4441701	482								
14	2	Methylone	14.61	204	ng/ml	89	11-10-2015	3	PFPA	RT	10 MIN	25	665104	447								
15	2	Butylone	15.16	218	ng/ml	76	11-10-2015	3	PFPA	RT	10 MIN	25	787954	442								
16	2	Ethylone	15.30	190	ng/ml	82	11-10-2015	3	PFPA	RT	10 MIN	25	343571	436								
17	2	Pyrovalerone	15.68	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	1297765	473								
18	2	MDPV	18.23	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	863771	436								
19	3	Flephedrone	10.21	123	ng/ml	94	11-10-2015	3	PFPA	RT	10 MIN	25	1805153	512								
20	3	Mephedrone	11.82	204	ng/ml	74	11-10-2015	3	PFPA	RT	10 MIN	25	851465	445								
21	3	Pentedrone	12.02	232	ng/ml	89	11-10-2015	3	PFPA	RT	10 MIN	25	1612761	423								
22	3	Methedrone	13.59	135	ng/ml	98	11-10-2015	3	PFPA	RT	10 MIN	25	4616694	490								
23	3	Methylone	14.62	204	ng/ml	82	11-10-2015	3	PFPA	RT	10 MIN	25	696549	458								
24	3	Butylone	15.16	218	ng/ml	91	11-10-2015	3	PFPA	RT	10 MIN	25	834718	452								
25	3	Ethylone	15.30	190	ng/ml	86	11-10-2015	3	PFPA	RT	10 MIN	25	385485	451								
26	3	Pyrovalerone	15.69	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	1663141	425								
27	3	MDPV	18.24	126	ng/ml	100	11-10-2015	3	PFPA	RT	10 MIN	25	1257808	444								

6480 720

Figure 3-3: An example of data treatment analysis in excel sheet on day one using three samples applied to nine SC in PFPA

The incubation time and temperature were 10 min at RT, hot block for the evaporation was set at RT. *Q. value is the relative ion intensities % (Q. value interprets how the quantification ions in ratio % related to the qualification ions). **The concentrations above were calculated using ChemStation software data analysis (the true value was 500 ng mL^{-1}). The mean, median, standard deviation (SD) and RSD above are related to the responses of triplicate samples for each drug. The mean of concentration, SD, RSD and accuracy are related to the concentration results (ng mL^{-1}). All the 720 samples including 6480 tests were calculated in similar way.

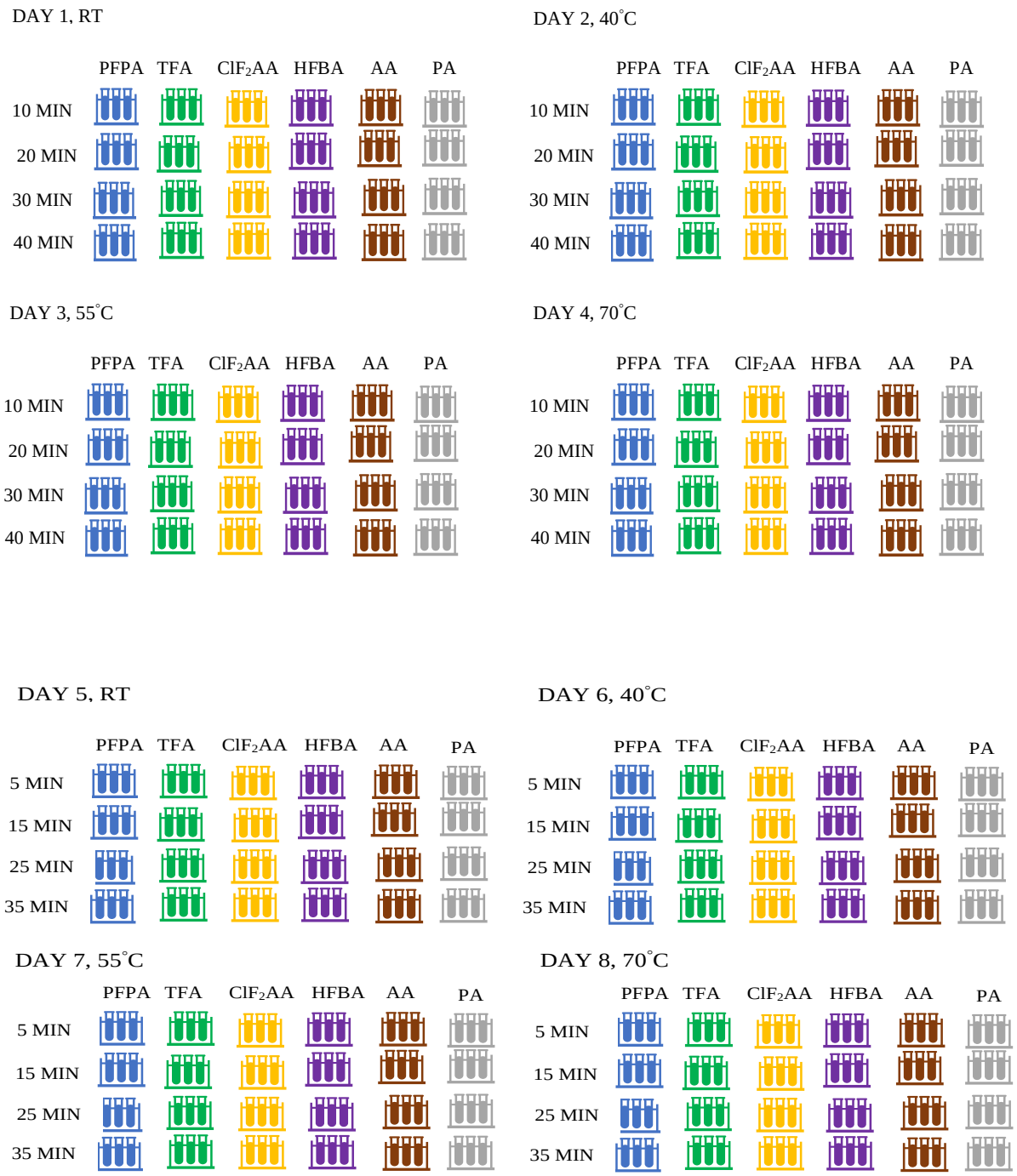


Figure 3-4: Diagram of the laboratory work from day one to day eight at varied temperatures and times

3.2.2.2 Optimised procedure

The final method is illustrated in **Table 3-3**. This procedure was determined after the optimisation of incubation times and temperatures, as well as the temperatures of hot block for the derivative of SC. The excel sheet produced

contained 6480 tests: (9 days × 72 samples × 9 drugs) + (1 day × 54 samples × 9 drugs) + (1 day × 18 samples × 9 drugs). The concentration, average, median, SD, RSD and error of accuracy (± %) of each triplicate sample were calculated (see the **Equation 3−4** for the RSD calculation and **Equation 3−5** for the accuracy calculation). The average of the highest responses in each target mass ion for each derivative and compound was used for conclusion the optimised method.

The optimum procedure was subsequently applied to study the validation parameters, including precision, accuracy, linearity and recovery. Additionally, this procedure was applied to the samples used for the determination of the five PFPA SC in whole blood and urine.

Table 3-3: The optimised procedure of derivative cathinones

Derivatisation reagents	Incubation time	Temperature of incubation	Temperature of hot block
PFPA and TFA	20 min	RT−PFPA and 40°C−TFA	RT
CLF_2AA and HFBA	25 min for CLF_2AA and 20 min for HFBA	55°C	40°C
AA and PA	25 min	70°C	50°C

3.2.2.3 Linearity study

Linearity study for comparison of derivatisation reagents:

Triplicate unextracted samples were prepared at seven concentrations (2, 1, 0.75, 0.50, 0.25, 0.10, and 0.05 µg mL^{-1}) and then spiked with the mixture of nine SC. These concentration points are commonly used in forensic toxicology laboratories for the detection of ATS substances. The work was accomplished in two days; the first day was for PFPA, TFA and CLF_2AA and the second day was for HFBA, AA and PA derivatives. 63 samples were analysed per day. (7 (concentration points) × 3 (triplicate) × 3 (derivatisation agents) per day).

Linearity study for determination of 5 SC in whole blood sample:

Duplicated samples were prepared and repeated at nine concentration points (5, 2, 1, 0.500, 0.250, 0.100, 0.050, 0.025, 0.010 μg mL^{-1}). This procedure was performed for the extraction of SC (flephedrone, mephedrone, methedrone, methylone and butylone) using SPE and whole blood, and it was also repeated for the examination of unextracted samples. The purpose of plotting unextracted and extracted SPE calibration curves is to study the recovery over these points. The calibration points were prepared for derivatisation and determination of whole blood studies as illustrated in **Table 3-4** and **Table 3-5** , respectively.

Table 3-4: The volume of nine SC solutions added of unextracted samples for the linearity study of derivatisation

Conc. of calibration points (μg mL^{-1})	Mixture of nine SC at (10 μg mL^{-1}), the volume added (μL)	Mixture of nine SC at (1 μg mL^{-1}), the volume added (μL)
2	200	–
1	100	–
0.750	75	–
0.500	50	–
0.250	–	250
0.100	–	100
0.050	–	50

Table 3-5: The volume of five SC solutions spiked to 1 mL of whole blood for the linearity study

Conc. of calibration points (μg mL^{-1})	Mixture of five SC at 10 μg mL^{-1}, volume added (μL)	Mixture of five SC at 1 μg mL^{-1}, volume added (μL)	Final volume (μL)
5	500	–	1000
2	200	–	1000
1	100	–	1000
0.5	–	500	1000
0.25	–	250	1000
0.1	–	100	1000
0.05	–	50	1000
0.025	–	25	1000
0.010	–	10	1000

Calculation methods:

Each linearity point was calculated using the peak area ratio of the analyte and its ISD, as follows in **Equation 3–2**.

Equation 3–2: Peak area ratio of target analyte

$$= Analyte\ peak\ area\ (target\ ion\ response) \div ISD\ peak\ area\ (target\ ion\ response)$$

The calibration curve was generated by plotting the area against the concentration. The equation of linearity and the correlation of coefficient (R^2) were determined.

The ISD applied for each analyte are demonstrated in **Table 3-6** for the comparison study and **Figure 3-5** for the whole blood determination study.

Table 3-6: SC substance with its ISD used for comparison study of derivatisation reagents.

SC/Derv.	PFPA	TFA	CLF_2AA	HFBA	AA	PA
Flephedrone	Mephedrone-d_3	Mephedrone-d_3	Methylone-d_3	Mephedrone-d_3	Mephedrone-d_3	Mephedrone-d_3
Mephedrone	Mephedrone-d_3	Mephedrone-d_3	Methylone-d_3	Mephedrone-d_3	Mephedrone-d_3	Mephedrone-d_3
Pentedrone	Mephedrone-d_3	Mephedrone-d_3	Methylone-d_3	Mephedronevd$_3$	Mephedrone-d_3	Mephedrone-d_3
Methedrone	Mephedrone-d_3	Mephedrone-d_3	Methylone-d_3	Mephedrone-d_3	Mephedrone-d_3	Mephedrone-d_3
Methylone	Methylone-d_3	Methylone-d_3	Methylone-d_3	Methylone-d_3	Methylone-d_3	Methylone-d_3
Butylone	Butylone-d_3	Butylone-d_3	Butylone-d_3	Butylone-d_3	Butylone-d_3	Methylone-d_3
Ethylone	Ethylone-d_5	Ethylone-d_5	Ethylone-d_5	Butylone-d_3	Butylone-d_3	Methylone-d_3
Pyrovalerone	MDPV-d_8	MDPV-d_8	Butylone-d_3	Butylone-d_3	MDPV-d_8	MDPV-d_8
MDPV	MDPV-d_8	MDPV-d_8	Butylone-d_3	Butylone-d_3	MDPV-d_8	MDPV-d_8

Figure 3-5: SC substance with its ISD used to calculate the peak area ratios for whole blood determination study

3.2.2.4 Limit of detection (LOD)

The limit of detection (LOD) was measured by determining the lowest concentration at which the compound could be detected. At least three ions were considered for the assessment of each substance's LOD in each reagent (see **Table 3-9** for the ions used). The signal−to−noise (S/N) ratio must be greater than three to be considered above the LOD. This was achieved using the Agilent instrumentation software (ChemStation software version 6.5 data analysis). The calculation of *S*/*N* ratio is illustrated in **Equation 3−3**.

Equation 3−3: Signal−to−noise *S*/*N* ratio

$$= \frac{\textit{The height of mass fragment ion in the analyte}}{\textit{The noise in the baseline of background}}$$

Seven concentrations (250, 100, 50, 25, 10, 5, 1 ng mL^{-1}) of nine cathinones were spiked in derivative methanolic samples and repeated three times using SIM mode. The preparation of above concentrations was completed in a similar practice to that mentioned in **section 3.2.1.12**. The *S*/*N* ratio in the whole blood sample was determined at a concertation of 10 ng mL^{-1} only for the assessment of LOD for 5 SC.

3.2.2.5 Relative standard deviation and accuracy for the evaluation of derivatisation agents

The RSD (%) values at concentrations of 0.5 µg mL^{-1} for SC and 0.1 µg mL^{-1} for ISD were calculated based on the optimal procedure (see **section 3.2.2.2**) of each derivative and drug using **Equation 3−4,** and the accuracy (bias) values were calculated from **Equation 3−5**.

Equation 3−4: Relative standard deviation (RSD)

$$= \left(\left(\textit{The average of standard deviation } (\textit{SD})\right) \div \left(\textit{The average of target analytes ratio}\right)\right) \times 100$$

Equation 3−5: The accuracy (bias) calculation

$$= \left(\left(\textbf{\textit{The average calculated concentration}} - \textbf{\textit{True values}}\ \left(0.5\frac{\mu g}{mL}\right)\right) \div \left(\textbf{\textit{True values}}\ \left(0.5\frac{\mu g}{mL}\right)\right)\right) \times 100$$

The final calculation results used the above equations for the final determination of RSD and bias results in each derivative and drug. The SDs and the means above were calculated in accordance with triplicate samples obtained from the optimised method only.

3.2.2.6 Recovery study for the examination of derivatisation reagents in whole blood

1 mL of whole blood and 300 µL of the mixture of nine SC at a concentration of 3 µg mL^{-1} were added into a culture tube and vortexed with 1 mL of 0.10 M phosphate buffer (pH=6). All culture tubes were then vortexed for few seconds and centrifuged at a speed of 3000 rpm for 10 minutes. The SPE column was conditioned by adding 3 mL of MeOH and deionized water, respectively followed by 1 mL of 0.10 M phosphate buffer at pH6 to clean the DAU cartridges and eliminate undesirable materials (DAU cartridges are UCT's main cartridge type (copolymeric bonded phase, reverse C8, and benzenesulfonic acid ion exchange phases) for forensic analysis and are the most commonly encountered in publication applicable to acid, basic and neutral drugs). The whole blood specimens were added and permitted to distribute and pass across the columns completely. 3 mL of d.H_2O and 1 mL of 100 mM acetic acid, followed by 3 mL MeOH were added for washing, and the contents were then dried under a full vacuum for five minutes. The specimens were eluted using 3 mL of DCM: IPA: NH_4OH (78:20:2). The samples were then put under a gentle stream of nitrogen for evaporation at RT to dry. The extraction contents were derivatised using the technique mentioned in the **section 3.2.2.2** (optimisation procedure). The triplicate samples for each reagent were extracted without ISD present. The 100 µL mixture of five ISD (10 µg mL^{-1}) was added prior to the evaporation stage.

On the same day, the triplicate of unextracted samples of nine SC were added into 7 mL glass tubes at 3 µg mL^{-1} with 100 µL of the five ISD mixture (10 µg mL^{-1}).

15 samples of the above were extracted (3 × 5 reagents: PFPA, TFA, CLF_2AA, HFBA and AA) and the three samples of unextracted tubes were evaporated at same time. The calculation of recovery % for each compound was used **Equation 3−6**:

Equation 3−6: Recovery (%)

$$= \big((\boldsymbol{Peak\ area\ ratio\ of\ extracted\ standards}) \div (\boldsymbol{Peak\ area\ ratio\ of\ unextracted\ standards})\big) \times \mathbf{100}$$

3.2.2.7 Internal standards (ISD) evaluation

The procedure for ISD evaluation was accomplished on the day of linearity study by adding 50 µL of the mixture ISD drugs at 2 µg mL^{-1} (see section 3.2.2.3 for the procedure used). This was carried out by applying each ISD a lone in each drug using ChemStation Software Version 6.5 and the conclusion results based on the regression square (R^2).

3.2.2.8 Carryover

Carryover was assessed by injecting triplicates of blank blood with the mixture of nine SC at a concentration 10 µg mL^{-1}. 100 µL of 100 µg mL^{-1} was added to three culture tubes of whole blood, and the method was then conducted using SPE followed by PFPA derivative procedures.

3.2.2.9 Acidified methanol study

Duplicate blank blood samples with the mixture of selected drugs were extracted using the SPE procedure at concentrations of 2000, 1000, 500 and 100 ng mL^{-1} using two ISD (mephedrone-d_3 and methylone-d_3 at 100 ng mL^{-1}). 20 µL of acidified MeOH (concentrated HCL: MeOH (9:1) (v/v)) was then added before evaporation. The PFPA derivative was added (50 µL and kept 20 min at RT), followed by evaporation (at RT using nitrogen gas) and reconstitution (by ethyl acetate). The above procedure was repeated with no acidified menthol.

3.2.2.10 Evaluation of two extraction methods (SPE and LLE)

The aim of the study is to determine which extraction method performs a better recovery before proceeding with the long–term stability validation study; thus, two extraction methods were selected for evaluation: SPE and LLE. These techniques effectively remove interferences and contaminants from the specimens, while providing excellent recovery. The recovery calculation methods used for the assessment are already illustrated in **Equation 3–6**.

For long–term stability, a urine sample was selected, and applied as a matrix for evaluation. For that, blank urine samples were collected on the day of the laboratory experiment. Triplicate 1 mL urine samples for each method (SPE and LLE) with triplicate unextracted samples were analysed at a concentration of 1 µg mL^{-1}. The SPE procedure used was similar to that outlined in **section 3.2.2.6**.

For LLE, a 1 mL urine specimen was mixed with 100 µL mixture of 6 SC at 10 µg mL^{-1} in a conical glass tube (the final concentration= 1 µg mL^{-1}). 0.1 mL of 25% NaOH (w/v) and 2 mL of DCM were added to the tube, which was then capped and vortexed for 1 minute. The specimen was thereafter centrifuged at 2500 rpm for 10 minutes. The bottom layer was carefully transferred into a round–bottom glass tube, avoiding gel particles and taking extra care to remove aqueous droplets. 25 µL of acidified methanol (methanol + concentrated HCl, 9:1 v/v) was added to all nine tubes (SPE, LLE and unextracted tubes). This was followed by 50 µL of ISD (mephedrone-d_3 at 10 µg mL^{-1}). The samples were left for evaporation at RT under a gentle stream of nitrogen until completely dry. The procedure was then completed as demonstrated in **section 3.2.2.2** using PFPA for derivatisation.

3.2.2.11 Thermal degradation of SC method

This was completed by injecting 50 µL of nine SC (mixture of 10 µg mL^{-1}). The samples were used for optimisation the GC-MS methods (oven temperature and injector port).

3.2.2.12 The optimum condition methods in GC–MS

GC–MS was operated using a 7890A GC/5975C MSD (triple–axis detector), coupled with a split/splitless inlet and a DB–5MS (5% phenyl/95% methylpolysiloxane; 30 m × 0.25 mm × 0.25 µm film thickness) separation fused–silica capillary column (All Agilent Technologies, Waldbronn, Germany). Helium with a purity of 99.99% was used as the carrier gas at 1.0 mL min^{-1}. Splitless injection at 225°C was employed. The MS transfer line temperature remained at 250°C. The MS functioned in electron impact ionisation mode (70 eV). The ion source continued at 200°C. MS data acquisition began after seven minutes and was set to selected ion monitoring (SIM) mode and scan mode (based on scope). The column temperature was started at 70°C and was then raised 10°C per minute until 280°C with a final hold time of 23 minutes. The mass spectrometer was run in full scan mode (50 – 450 m/z) to study the ion fragmentations and peak interferences. Selected ion monitoring (SIM) mode was obtained to study the validation parameters. All data collection and processing was conducted on the GC/MSD ChemStation Software Version 6.5. See **Table 3-7** summarising the GC–MS parameters.

Table 3-7: Summary of GC–MS parameters

Injector port mode	Splitless
Temp. at injector port	225°C
Column type	DB–5 capillary column (30 m x 0.25 mm I.D., 0.25 µm film thickness)
Carrier gas	Helium
Flow rate	1 mL min^{-1}
Initial temperature	70°C
Ramp 1	10°C per min to finish at 280°C
Run time	23 min
Transfer line temp.	250°C
Solvent delay	7 min
Ionization voltage	70 eV
Mass-to-ion-ratio range	50-450 m/z

3.3 Results and discussion

3.3.1 Study of the fragmentation patterns and interferences with relative ion intensities

GC–MS was primarily conditioned for the separation of nine derivative cathinones until adequate responses and separations were achieved in the chromatogram and

mass with excellent peak shapes. Many GC–MS programs were used in combination with changes to the oven temperature in scan mode from 50 to 450 amu; an example of the optimisation work is summarised in **Table 3-8**. The SIM mode was then selected after determination of the mixture drugs (see **Table 3-9** for the selection ions).

All derivatives drugs (9 (cathinones) × 6 (reagents) = 54 derivatives = 54 tubes) were run separately to determine of the fragmentation patterns, mass spectra and retention time ($_tR$) using nine SC standards. This was completed by adding 50 µL of each stock standard (100 µg mL^{-1}) into a small vial. The content was then evaporated using nitrogen at RT, following the procedure mentioned in **sections 3.2.1.8** and **3.2.2.1** for each reagent.

The fragmentation patterns with relative ion intensities for the SC derivatives are shown in **Table 3-9**. The abundance responses of quantification ions appear in bold, as achieved from the optimum conditions stated in **section 3.2.2.2**. These ion patterns were determined for the calculation of validation parameter assessments in the following sections.

Nine derivative SC were tested to evaluate ion fragmentation; see **Figure 3-6** for the fragmentation and **Table 3-10** for the evaluation. The fragmentation patterns were investigated for each substance in each reagent. The overall points were used for the final evaluation of the fragmentation patterns. The best reagent in each factor was given one point (rank 1) and the second–best reagent was given two points (rank 2), etc.; meaning, the lower number of points, the better the reagents. The factors included for the evaluation were number of ions, number of unique ions and the total relative ion fragmentation percentage. These factors were estimated for each reagent in each individual compound. From **Table 3-11**, the best reagent for the evaluation of fragmentation patterns was AA (8 points) followed by CLF_2AA (9 points), PFPA and PA (10 points), TFA (12 points) and then HFBA (16 points).

The interference study is shown in **Figure 3-7**, where the elution of all peaks was showed a different $_tR$ in chromatogram, which were well separated from one another. Co–elution of peaks was not observed for any substances or reagents,

except in two situations where the ethylone could not be separated efficiently from butylone using AA and PA reagents. In spite of this, ethylone has a unique ion at m/z 178 for both AA and PA, permitting the substances to be discriminated from one another. Ethylone and butylone are very similar in terms of M.W, structure and isomers. The differentiation of fragmentation pattern and chemical structure for butylone and ethylone in GC–EI–MS is discussed in more detail in [2, 459].

Table 3-8: An example of GC–MS conditions used for the optimisation of SC

Conditions	Method number						Final
	1	2	3	4	5	6	
Initial oven temp. (˚C)	100	60	80	75	70	65	70
Ramp 1 (min/temp. (˚C))	10/280	10/260	8/300	11/240	11/220	13/280	10/280
Ramp 2 (min/temp. (˚C))	–	–	–	10/280	10/280	–	–
Run time (min)	18	20	27.5	19	20	16.5	23
The injector– temp. (˚C)	250	250	250	225	225	250	225

Table 3-9: Fragment ions with relative ion intensities in SIM mode
(quantification ions in bold were used in the calculation of peak area ratios, the remaining ions were used for qualification ions (confirmation ions), underlined ions are the unique ions, the ions between the brackets are the target ion of internal standards, the base ions that have 100% were used to calculate the highest peak areas, the italic is the molecular ions.

Target Compounds	PFPA			TFA			CLF_2AA			HFBA			AA			PA		
	(tR)	m/z	Relative ion intensity (%)	(tR)	m/z	Relative ion intensity (%)	(tR)	m/z	Relative ion intensity (%)	(tR)	m/z	Relative ion intensity (%)	(tR)	m/z	Relative ion intensity (%)	(tR)	m/z	Relative ion intensity (%)
Flephedrone	10.22	204	100	10.26	154	100	12.40	170	100	10.63	254	100	12.87	58	100	13.61	58	100
		123	**54**		**123**	**74**		**123**	**51**		**123**	**42**		**100**	**67**		**114**	**63**
		160	32		110	29		95	29		210	27		95	23		95	24
		95	26		95	28		75	11		95	17		123	19		75	12
Mephedrone	11.81	119	100	11.93	119	100	14.00	119	100	12.16	119	100	14.58	58	100	15.25	58	100
		204 (207)	**29** -		91 **154**	25 **21**		**170** 91	**32** 26		**254** (257)	**36** -		**100** (103)	**83** -		**114** (61)	**70** -
		91.1	24		(157)	-		65	11		91	23		91	23		91	23
		160	17		65	2		-	-		210	14		119	21		*233*	*3*
Pentedrone	12.02	**232**	**100**	12.17	**182**	**100**	14.20	**198**	**100**	12.36	**282**	**100**	14.73	**86**	**100**	15.34	**86**	**100**
		190	66		140	68		156	46		240	58		128	61		142	48
		105	41		105	55		105	37		103	30		77	17		77	13
		77	27		77	31		77	29		79	16		105	10		105	11
Methedrone	13.60	**135**	**100**	13.77	**135**	**100**	15.74	**135**	**100**	13.89	**135**	**100**	16.34	58	100	16.93	58	100
		204	12		77.1	10		170	10		254	13		100	77		114	66
		77	11		91	8		77	10		210	7		**135**	**34**		**135**	**28**
		160	8		154	8		-	-		169	7		*235*	6		77	18
Methylone	14.61	149	100	14.77	149	100	16.72	149	100	14.89	149	100	17.29	**58**	**100**	17.85	58	100
		204 (207)	**19** -		**154** (157)	**14** -		**170** 121	**20** 12		**254** (257)	**22** -		100 (61)	65 -		**114** (117)	**57** -
		160	13		121	13		*319*	6		121	11		149	24		149	21
		353	6		*303*	6		-	-		210	11		*249*	22		*263*	9
Butylone	15.15	149	100	15.37	149	100	17.25	149	100	15.41	149	100	17.80	**72**	**100**	18.34	72	100
		218 (221)	**27**		**168** (171)	**19**		**184** (187)	**27**		**268** (271)	**31**		114 (75)	55		**128** -	**48** -
		367	10		121	12		121	12		210	10		149	14		149	16
		160	6		*317*	6		*333*	6		*417*	5		*236*	8		*277*	3
Ethylone	15.30	149	100	15.55	149	100	17.42	149	100	15.51	149	100	17.87	72	100	18.38	72	100
		218 (223)	37 -		168 (173)	28 -		184 (338)	37 -		**268** *417*	**40** 3		**114** -	**60** -		**128** -	**48** -
		190	**18**		**140**	**13**		*333*	12		-	-		149	14		149	16
		121	12		121	12		**156**	**10**		-	-		*178*	7		*178*	2
Pyrovalerone	15.68	**126**	**100**	15.68	**126**	**100**	15.68	**126**	**100**	15.68	**126**	**100**	15.68	**126**	**100**	15.68	**126**	**100**
		149	9		91	7		127	6		127	9		91	7		91	5
MDPV	18.23	**126** (134)	**100** -	18.23	**126** (134)	**100** -	18.23	**126** (134)	**100** -	18.23	**126** (134)	**100** -	18.23	**126** (134)	**100** -	18.23	**126** (134)	**100** -

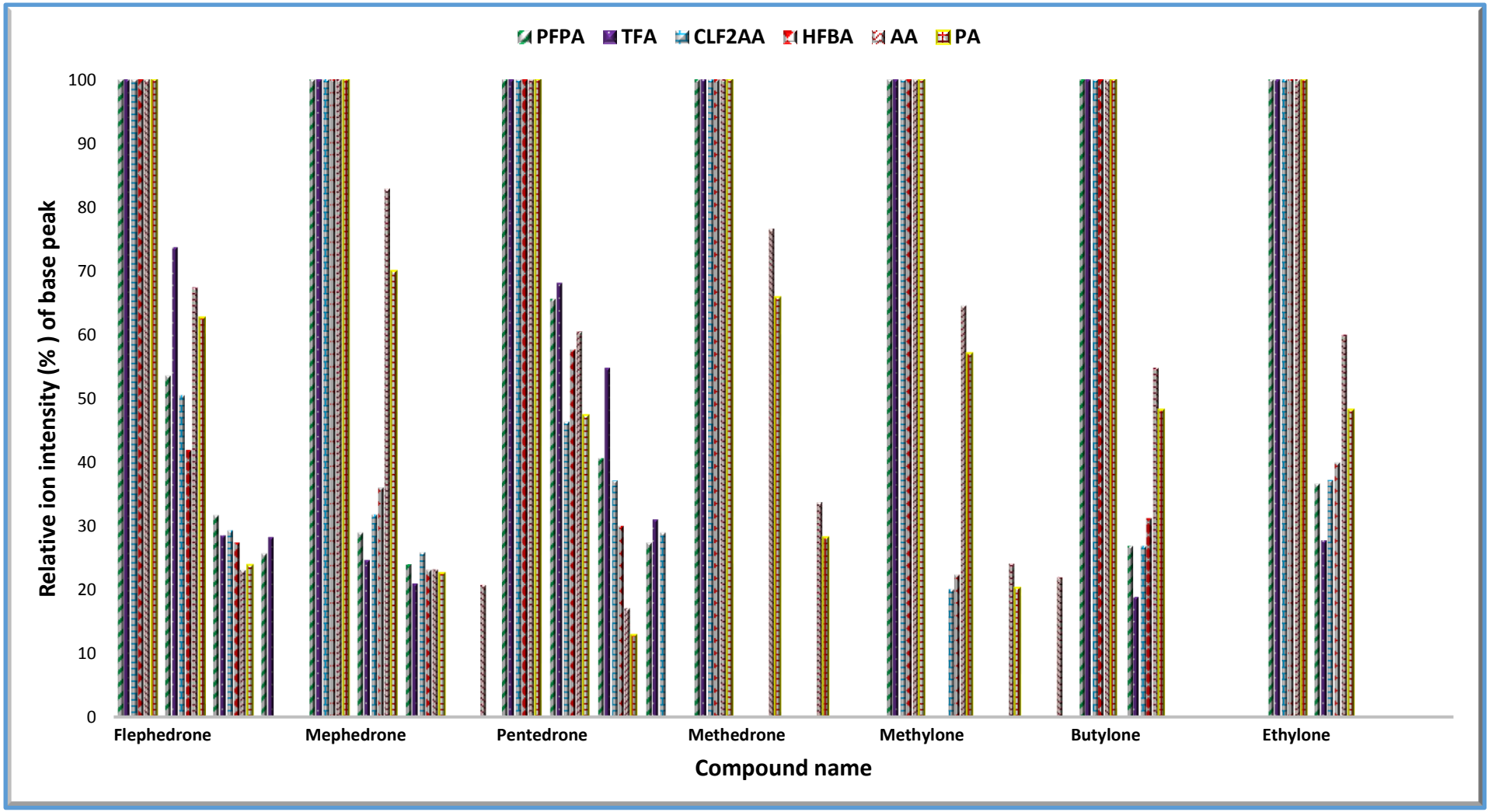


Figure 3-6: Ion fragmentation pattern for each reagent applied to selected SC. Lower than 10% relative fragmentation ions were removed. The fragmentation patterns were using the determined optimum method.

Table 3-10: Evaluation of fragmentation patterns

Reagent & drug names		Flephedrone	Mephedrone	Pentedrone	Methedrone	Methylone	Butylone	Ethylone	Pyrovalerone	MDPV	Total	The reagent rank	Overall points
PFPA	No. of ions	4	4	5	3	3	6	4	1	1	31	1	10
	No. of unique ions	2	1	1	1	0	0	1	0	0	6	6	
	Total relative ion fragmentations %	211	170	234	131	138	143	167	122	113	1428	3	
TFA	No. of ions	5	3	6	2	3	3	4	1	1	28	3	12
	No. of unique ions	3	1	1	1	0	0	1	0	0	7	5	
	Total relative ion fragmentations %	230	147	254	126	132	137	153	120	117	1416	4	
ClF_2AA	No. of ions	4	4	5	3	4	3	4	1	1	29	2	9
	No. of unique ions	3	1	3	1	1	1	1	0	0	11	2	
	Total relative ion fragmentations %	191	169	212	127	138	144	159	114	115	1369	5	
HFBA	No. of ions	4	4	4	2	4	3	2	1	1	25	6	16
	No. of unique ions	2	1	4	2	1	0	0	0	0	10	4	
	Total relative ion fragmentations %	186	173	204	128	144	146	107	121	116	1325	6	
PA	No. of ions	4	3	4	4	4	3	3	1	1	27	5	10
	No. of unique ions	2	1	3	1	1	1	1	0	0	10	4	
	Total relative ion fragmentations %	210	227	188	216	211	177	181	113	111	1632	1	
AA	No. of ions	4	4	4	3	4	3	3	1	1	27	5	8
	No. of unique ions	2	1	4	2	1	1	1	0	0	12	1	
	Total relative ion fragmentations %	199	196	172	212	186	168	166	110	109	1518	2	

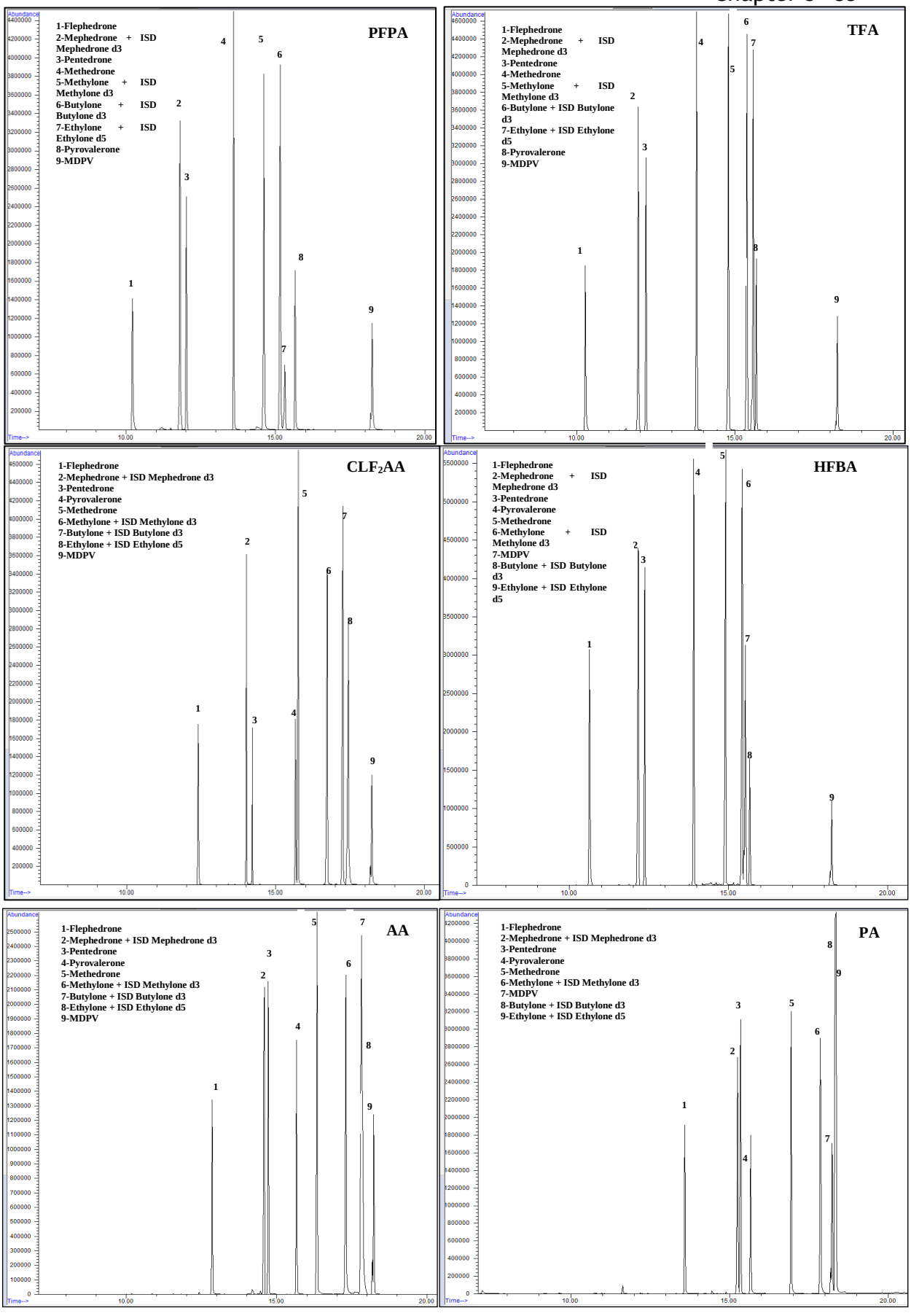


Figure 3-7: Chromatograms for six acetylation derivatives of SC at a concentration of 0.50 μg mL^{-1}

3.3.2 Thermal degradation of SC

The decomposition of cathinones in the injector port of GC has been documented since 1994 by injecting methcathinone [167, 460]. The degradation product was caused by two missing hydrogen atoms, resulting a 2 Da lower mass than the base peak in mass spectra. Kerrigan et al. discussed this issue in terms of the effect of temperature in injector port when underivative cathinones were involved. It was concluded that the lower the temperature in the injector port the more thermally stable the SC compounds [459]. However, reducing the temperature of the injector port may result in an incomplete reaction [444] and a decrease in the volatilisation of analytes [461]. In this project, during the GC method optimisation, the temperature of the injector port was successfully reduced from 250°C to 225°C to prevent the decomposition of SC. Temperatures any lower (for example 200°C or 185°C) produced lower peak area responses. Therefore, 225°C was used as the optimum temperature in the injector port.

3.3.3 The optimum temperature and time reaction

The optimum procedure for each derivative cathinone is shown in **Table 3-11**. The following points were noted:

• All samples applied to SC−PA must follow the optimised procedure; specifically the temperature of hot block in the evaporating stage at 50°C. The GC−MS always provided poor responses or the lowest peak area values when the samples were set at RT in the hot block with the RSD and accuracy typically above 20% error.
• The samples under all derivative reagents occasionally provided bad responses or even above 20% errors, based on accuracy and/or precision calculations.
• The optimised procedures stated in **Table 3-11** were selected because their results provided better responses, accuracy and precision for most derivative drugs tested. It was also selected due to the ANOVA study discuss in the following section.
• Ethylone−AA in most samples provided poor peak area response, though the RSD and accuracy were valid in some samples (under 20% error), this is because the ISD was used in the calculation; see **Figure 3-8**.

• MDPV and pyrovalerone were frequently underivatised under all reagents and conditions.

• In general, according to the maximum peak area responses, the derivative cathinones regularly achieved good responses for a reaction of 20–25 minutes at 70°C for AA and PA; see **Figure 3-9** for the results. While cathinones in AA and PA usually performed well at 70°C, both obtained poor responses at RT during the incubation stage. The effect of temperature on the reagents of CLF_2AA/HFBA and TFA/PFPA are shown in **Figure 3-10** and **Figure 3-11**, respectively.

• Cathinones need high temperature for the completion of the reaction. For most experimental cases, the higher the temperature, the quicker reaction with the better the responses. This may be due to the chemical and physical properties. The boiling point (BP) and molecular weight (M.W) of each reagent and drug are examples that have effects on the reaction. PA, AA, HFBA, CLF_2AA, TFA and PFPA have the following BPs: 167, 140, 120, 97, 72 and 69°C, respectively. When the boiling points of these reagents are high, the temperature in the hot block should also be high for the completion of the reaction, and high peak area values to be observed. The M.W in each SC also has certain effects; mephedrone and flephedrone, for example, are more volatile than other drugs due to a smaller M.W. Therefore, the smaller the M.W, the less temperature required; see **Table 3-12** for the overall explanation.

• The time required for the completion of the reaction was under 15–25 minutes for all reagents and drugs. See **Figure 3-12** for the example results of the PFPA agent.

• Butylone, ethylone, MDPV and pyrovalerone presented better responses and greater peak area values when the samples were left at 50°C in nitrogen gas hot block (after derivatisation) using all reagents.

• Butyric anhydride is the one reagent that was also involved in the evaluation. Unfortunately, despite attempting to increase the temperature during reaction and evaporation, very poor peaks and responses were observed for all nine SC. This might be related to the BP of the reagent, which is high (198°C) and prevents the excess reagent from evaporating even when the hot block temperature was set to 90°C for 20 minutes. As a consequence, this reagent was not involved in the comparison study.

Table 3-11: Optimisation of temperature and incubation time

This is according to the average of the highest peak areas at concentration of 0.50 µg mL^{-1}. The temperature that is presented between brackets is the optimised temperature in the evaporation stage (when the samples were left in hot block after derivatisation).

Drug Name/ derivative	PFPA (RT)	TFA (RT)	CLF_2AA (40˚C)	HFBA (40˚C)	AA (50˚C)	PA (50˚C)
FLEPHEDRONE	20 min RT	20 min 40˚C	20 min 40˚C	20 min 40˚C	25 min 55˚C	25 min 70˚C
MEPHEDRONE	10 min RT	20 min 40˚C	25 min 70˚C	25 min 55˚C	20 min 40˚C	25 min 70˚C
PENTEDRONE	20 min RT	20 min 40˚C	25 min 55˚C	20 min 40˚C	25 min 55˚C	25 min 55˚C
METHEDRONE	20 min 40˚C	20 min 40˚C	25 min 55˚C	25 min 70˚C	25 min 55˚C	25 min 70˚C
METHYLONE	35 min 70˚C	20 min 40˚C	25 min 55˚C	20 min 40˚C	25 min 55˚C	25 min 70˚C
BUTYLONE	20 min 40˚C	20 min 40˚C	25 min 55˚C	20 min 40˚C	25 min 70˚C	25 min 70˚C
ETHYLONE	20 min 40˚C	20 min 40˚C	25 min 55˚C	20 min 40˚C	15 min 70˚C	25 min 70˚C
PYROVALERONE	35 min 70˚C	25 min 70˚C	25 min 70˚C	25 min 55˚C	25 min 70˚C	25 min 70˚C
MDPV	35 min 70˚C	25 min 70˚C	25 min 70˚C	25 min 55˚C	15 min 70˚C	25 min 70˚C
Optimisation	**20 min RT**	**20 min 40˚C**	**25 min 55˚C**	**20 min 55˚C**	**25 min 70˚C**	**25 min 70˚C**

Table 3-12: The correlation between the required temperature, volatility, M.W and BP for the completion of the reaction during the incubation stage.

Drug name	Molar mass g moL^{-1}	Temperature	Volatility	bp.
MDPV	275	70˚C	More volatile	PA, BP. 167˚C
PYROVALERONE	245			AA, BP. 140˚C
ETHYLONE	221	55˚C		HFBA, BP. 120˚C
BUTYLONE	221			CLF_2AA, BP. 97˚C
METHYLONE	207	40˚C		TFA, BP. 72˚C
METHEDRONE	193			
PENTEDRONE	191	RT		PFPA, BP. 69˚C
FLEPHEDRONE	181			
MEPHEDRONE	177			

Abbreviation: bp. (boiling point), M.W (molecular weight)

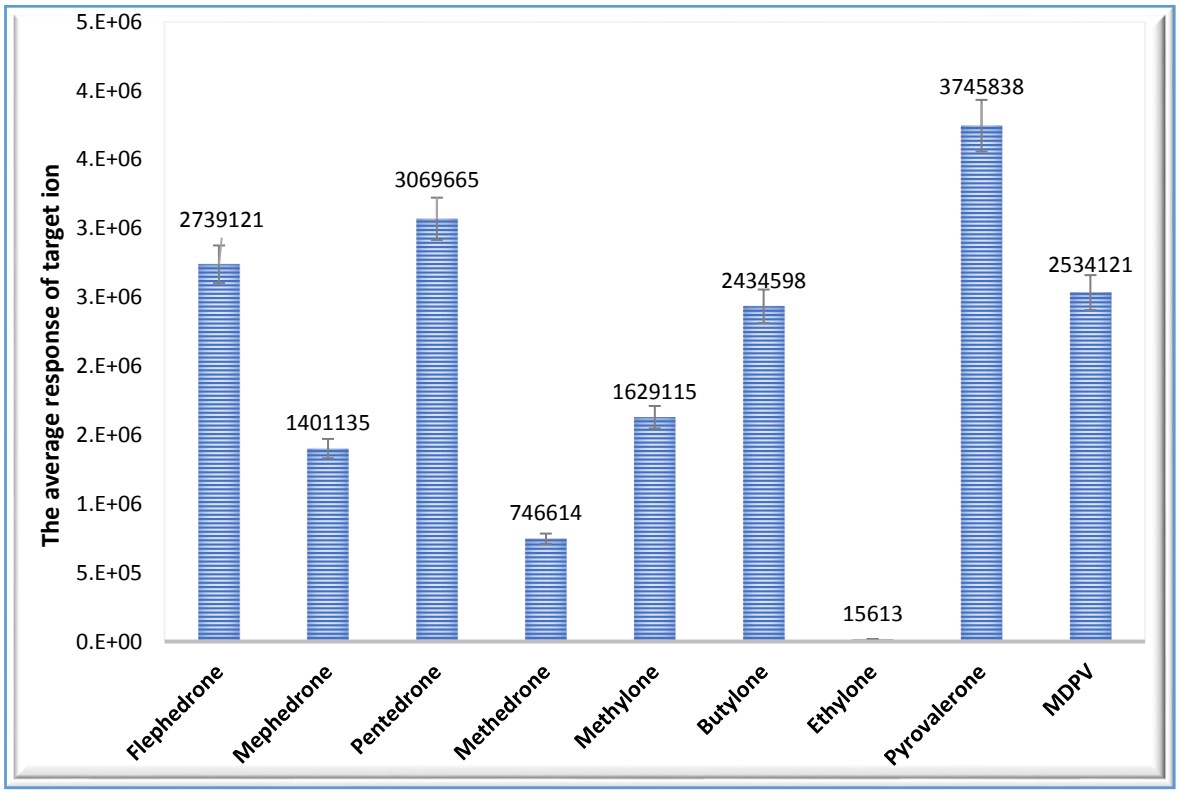


Figure 3-8: Poor response of ethylone derivative by AA at 55˚C in hot block for the completion of the reaction and 50˚C during the evaporation stage, n= 3.

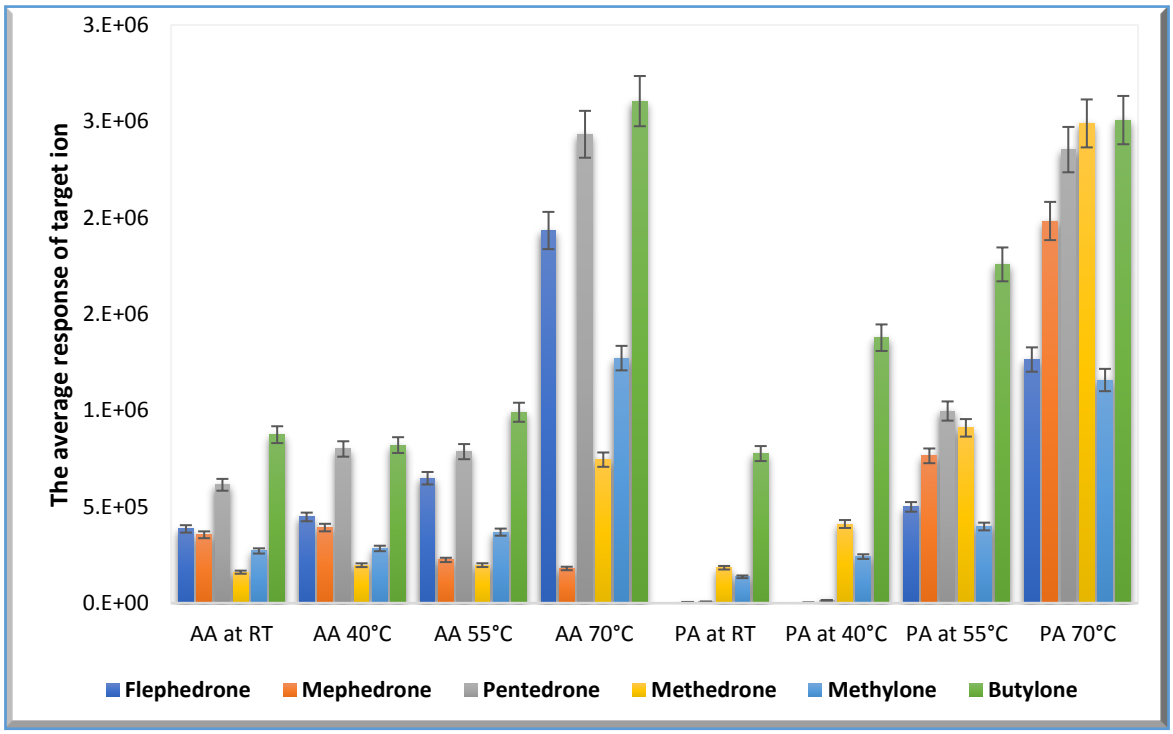


Figure 3-9: The effect of temperature during the reaction of six derivative cathinones using AA and PA reagents after 25 min, where the temperature in the evaporation stage was 50˚C, n= 3.

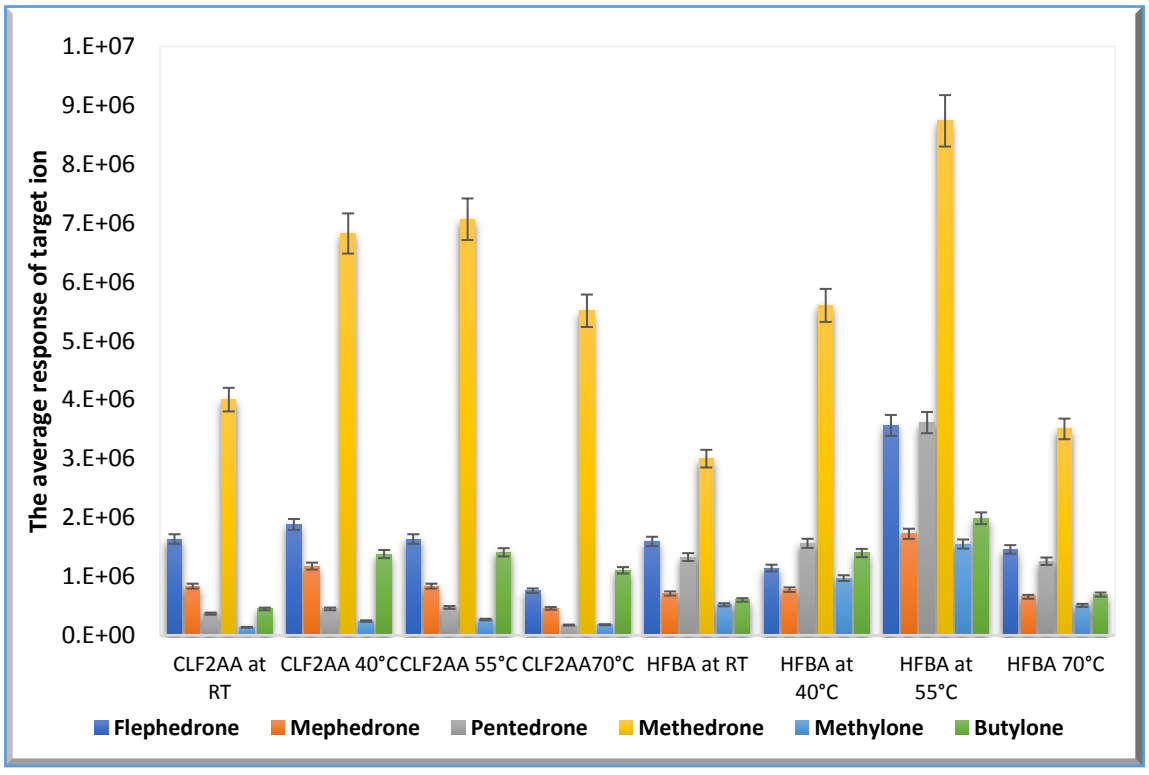


Figure 3-10: The temperature effect on six SC using CLF_2AA and HFBA reagents, the time for the reaction was 20 min and the temperature of hot block during the evaporation stage was 40˚C, n= 3.

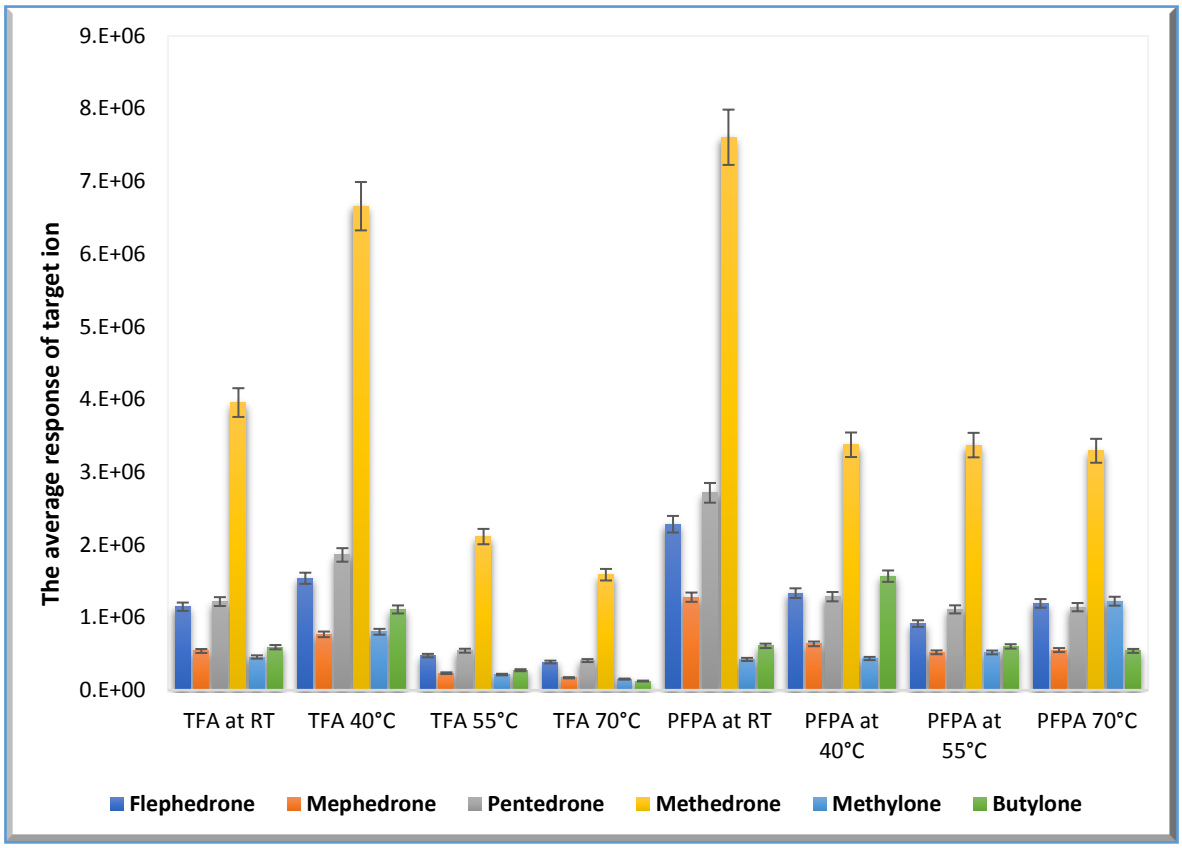


Figure 3-11: The temperature effect on six SC using TFA and PFPA reagents, the time was 25 min and the temperature of hot block during the evaporation stage was RT, n= 3.

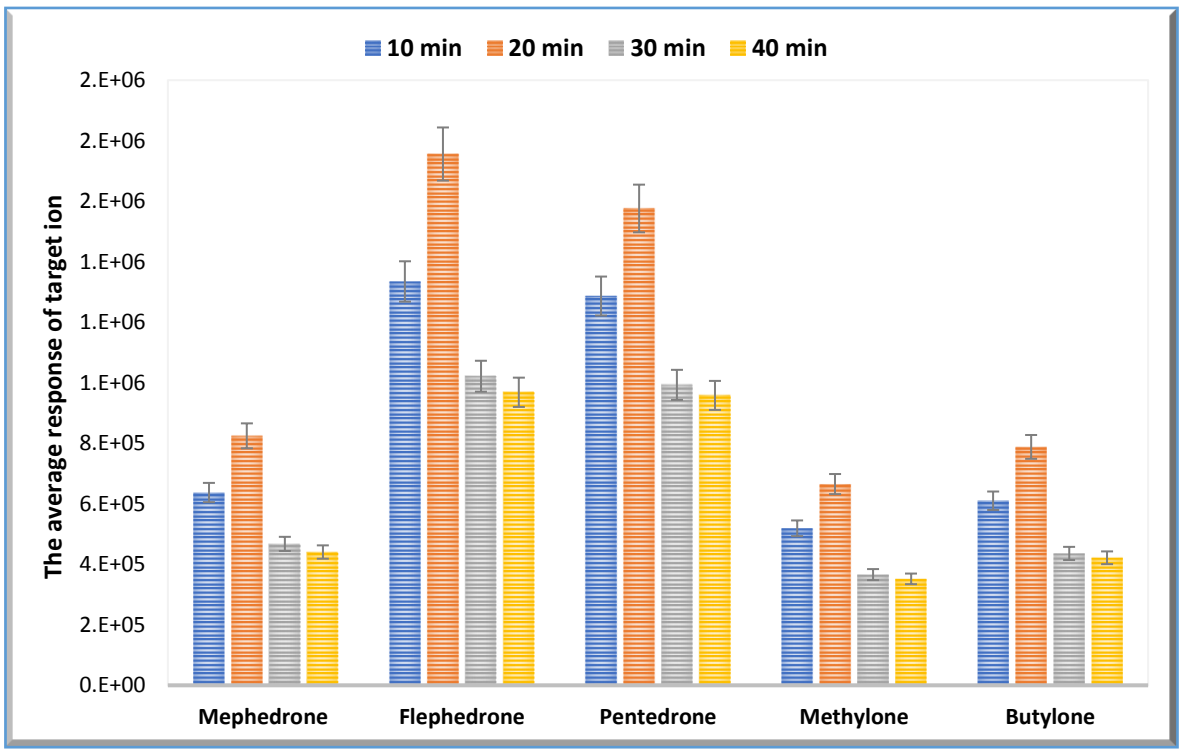


Figure 3-12: Time required for the completion of the reaction applied to five SC in PFPA incubated and evaporated at RT, n= 3.

3.3.4 Three-way ANOVA

The purpose of using three−way ANOVA was to confirm whether the responses were significantly different or not when the procedure of derivative SC was modified over different incubation times and temperatures, as well as hot block temperatures. The R programming language was applied to study a three−way ANOVA by analysing three factors (time and temperature during incubation, and temperature during the evaporation stage) as independent variables. The dependent variables (54 derivative SC = 9 SC × 6 derivatisation reagents) were the average target ion responses for a specific time, temperature, substance and reagent.

The ANOVA was produced from 5184 tests (5184 tests = eight different days × 72 samples per day × nine SC). The tests consisted of the response values of target ions that were obtained from the procedure mentioned in **section 3.2.2.1**.

One of three independent variables must have statistically significant with at least 5% confidence level in order to conclude that there was variance in the result. Once the average response values of SC target ions were higher than 5% (using the *F* factor), the responses within the variables for each derivative drug showed no statistically significant difference. The obtained results in **Table 3-13** show that the derivative sample should be set using a specific time and temperature during the incubation and evaporation periods if there is significant difference. This means the procedure for each reagent should be strictly followed, and if not the response would be variable then resulting in errors or uncertainties. For instance, the optimised procedure should be followed for all samples that were derivatised using PA to provide high responses, and if not the responses values will be beyond the 95% confidence limit and may provide poor responses, accuracy and precision.

The optimum conditions were determined based on the information in **Table 3-11** and **Table 3-13**. For instance, the optimum condition for derivative samples using PFPA was at RT for 20 minutes with RT for the evaporation. The question is, why were these temperatures and times selected as the optimum conditions? The answer, as the ANOVA study states, is that there is no significant difference in the results when the procedure was altered for all derivative drugs in PFPA, with exception of mephedrone and flephedrone.

Table 3-13: Three-way ANOVA for incubation time and temperatures as well as evaporation temperature in hot block.

(Significant difference = probability (<*F*) is always less than 5%), (No significant difference = probability (>*F*) is always higher than 5%), samples above 20% error in RSD were excluded.

Drug Name/ derivative.	PFPA	TFA	CLF_2AA	HFBA	AA	PA
FLEPHEDRONE	SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
MEPHEDRONE	SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	SIG. DIFF.
PENTEDRONE	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
METHEDRONE	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	SIG. DIFF.	SIG. DIFF.
METHYLONE	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
BUTYLONE	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
ETHYLONE	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
PYROVALERONE	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.
MDPV	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	NO SIG. DIFF.	SIG. DIFF.

3.3.5 Study of maximum peak area values

In this study, the greatest base peak values that provide 100% of the relative ion intensities in the background of the mass spectrum ions were used for the assessment of derivatisation reagents. Each reagent in each drug has different fragmentation patterns, therefore base ions were selected instead of target ions for more accurate comparison results. See **Figure 3-13** for the results, where all derivatised drugs have adequate peak areas, excluding AA for ethylone and methedrone as well as CLF_2AA for pentedrone. This is all after using the optimisation procedure conditions. The best reagents were PFPA followed by TFA, HFBA, PA, CLF_2AA and AA, respectively.

3.3.6 Internal standards (ISD)

The reason behind studying ISD was to investigate the application of ISD to cathinones and to answer the following questions:

• Are one or two ISD sufficient to provide the required quantification method when applied to nine derivative cathinones?

• Do the ISD provide the fit–regression with valid linearity results? Are there variances between the results?

Due to the expensive list of available ISD, only five were evaluated. If one or two were successful in providing the fit–regression, then there is no reason to use more, which will reduce costs. Each ISD was investigated alone as it applied to nine derivative SC using the linearity study.

All ISD applied to PFPA and TFA SC worked well and results of R^2 value were greater than 0.990. The ISD that obtained poor regression were avoided in further studies in the next sections and chapters. The ISD results are demonstrated in **Table 3-14**.

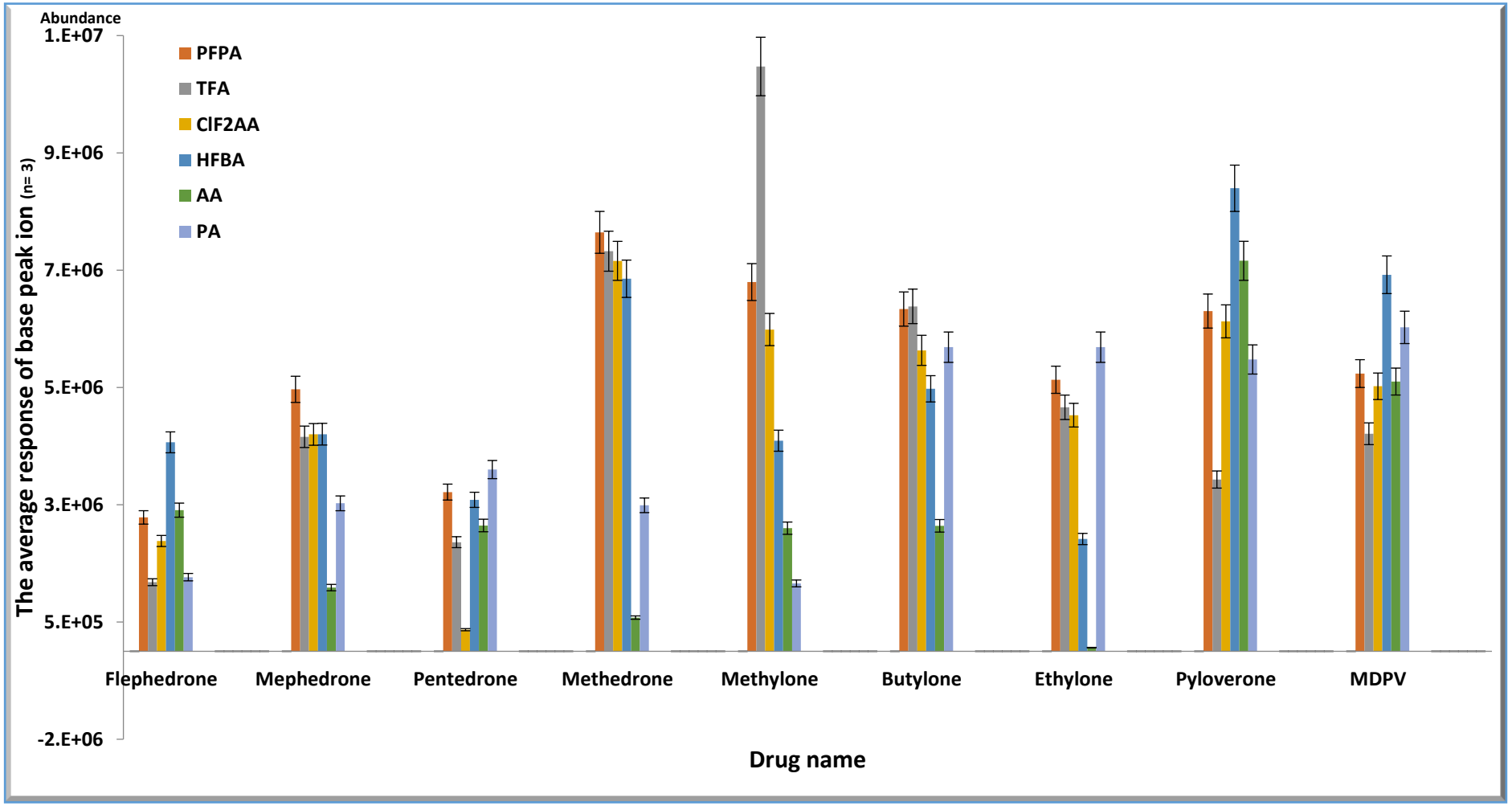


Figure 3-13: The average of the greatest responses of relative ion intensities for base peak area values in nine SC and reagents at a concertation of 0.5 µg mL^{-1}, n= 3.

Table 3-14: Examination of the quality (R^2) of the target ion of selected internal standards for each drug. All drugs listed according to elution in chromatogram ($_tR$).

*ISD in bold were used to study validation parameters. B.R is bad response =<0.900.

Compound with ISD/derivative	PFPA (R^2)	TFA (R^2)	CLF_2AA (R^2)	HFBA (R^2)	AA (R^2)	PA (R^2)
FLEPHEDRONE – MEPHEDRONE-d_3	**0.998**	**0.999**	B.R	**0.998**	**0.997**	**0.999**
FLEPHEDRONE – METHYLONE-d_3	0.996	0.999	**0.999**	B.R	0.990	0.991
FLEPHEDRONE – BUTYLONE-d_3	0.997	1.000	0.999	0.995	0.995	B.R
FLEPHEDRONE – ETHYLONE-d_5	0.995	0.990	0.995	B.R	B.R	B.R
FLEPHEDRONE - MDPV-d_8	0.994	0.998	B.R	0.996	0.991	0.999
MEPHEDRONE – MEPHEDRONE-d_3	**0.999**	**0.999**	B.R	**0.999**	**0.997**	**1.000**
MEPHEDRONE – METHYLONE-d_3	0.997	0.997	**0.997**	B.R	0.942	0.995
MEPHEDRONE – BUTYLONE-d_3	0.997	0.996	1.000	0.994	0.959	B.R
MEPHEDRONE – ETHYLONE-d_5	0.995	0.996	0.998	B.R	B.R	B.R
MEPHEDRONE - MDPV-d_8	0.994	0.989	B.R	0.994	0.941	0.988
PENTEDRONE – MEPHEDRONE-d_3	**0.998**	**0.998**	B.R	**0.998**	**0.997**	**0.997**
PENTEDRONE – METHYLONE-d_3	0.995	0.995	**0.997**	B.R	0.955	0.978
PENTEDRONE – BUTYLONE-d_3	0.995	0.995	0.997	0.997	0.967	B.R
PENTEDRONE – ETHYLONE-d_5	0.994	0.995	0.994	B.R	B.R	B.R
PENTEDRONE - MDPV-d_8	0.994	0.988	B.R	0.998	0.954	0.986
METHEDRONE– MEPHEDRONE-d_3	**1.000**	**0.999**	B.R	**1.000**	**0.999**	0.996
METHEDRONE – METHYLONE-d_3	0.996	1.000	**0.999**	0.999	0.999	**0.999**
METHEDRONE – BUTYLONE-d_3	0.994	0.999	1.000	0.996	0.999	B.R
METHEDRONE – ETHYLONE-d_5	0.998	0.999	0.998	B.R	B.R	B.R
METHEDRONE - MDPV-d_8	0.996	0.996	B.R	B.R	0.999	0.997
METHYLONE – MEPHEDRONE-d_3	0.998	0.999	B.R	0.998	0.999	0.995
METHYLONE – METHYLONE-d_3	**0.999**	**0.999**	**0.998**	**0.999**	**0.998**	**1.000**
METHYLONE – BUTYLONE-d_3	0.999	0.999	1.000	0.998	0.999	B.R
METHYLONE – ETHYLONE-d_5	0.999	0.998	0.999	B.R	B.R	B.R
METHYLONE - MDPV-d_8	0.993	0.998	B.R	B.R	0.997	0.997
BUTYLONE – MEPHEDRONE-d_3	0.997	0.999	B.R	0.996	0.999	0.996
BUTYLONE – METHYLONE-d_3	0.999	1.000	0.995	0.999	0.997	**1.000**
BUTYLONE – BUTYLONE-d_3	**0.999**	**1.000**	**0.999**	**1.000**	**1.000**	B.R
BUTYLONE – ETHYLONE-d_5	0.999	0.999	0.997	B.R	B.R	B.R
BUTYLONE - MDPV-d_8	0.995	0.997	B.R	B.R	0.996	0.996
ETHYLONE – MEPHEDRONE-d_3	0.994	0.999	B.R	B.R	0.995	0.996
ETHYLONE – METHYLONE-d_3	0.998	1.000	0.996	0.942	0.998	**1.000**
ETHYLONE – BUTYLONE-d_3	0.999	1.000	0.999	**0.978**	**0.994**	B.R
ETHYLONE –ETHYLONE-d_5	**0.999**	**0.999**	**0.999**	B.R	B.R	B.R
ETHYLONE - MDPV-d_8	0.998	0.997	B.R	B.R	0.997	0.996
PYROVALERONE – MEPHEDRONE-d_3	0.992	0.994	B.R	0.998	0.995	0.986
PYROVALERONE – METHYLONE-d_3	0.996	0.995	0.997	0.997	0.998	0.998
PYROVALERONE – BUTYLONE-d_3	0.997	0.995	**0.996**	**0.996**	0.994	B.R
PYROVALERONE – ETHYLONE-d_5	0.997	0.994	0.992	B.R	B.R	B.R
PYROVALERONE – MDPV d_8	**0.997**	**0.999**	B.R	B.R	**0.998**	**0.994**
MDPV – MEPHEDRONE-d_3	0.990	0.992	B.R	0.998	0.995	0.982
MDPV – METHYLONE-d_3	0.996	0.993	B.R	0.993	0.995	0.996
MDPV – BUTYLONE-d_3	0.997	0.993	**0.909**	**0.994**	0.988	B.R
MDPV – ETHYLONE-d_5	0.997	0.992	B.R	B.R	B.R	B.R
MDPV - MDPV-d_8	**0.999**	**0.999**	B.R	B.R	**0.995**	**1.000**

3.3.7 RSD and accuracy studies

The RSD and accuracy were obtained for the evaluation of the nine SC within six reagents at concentration of 0.50 µg mL^{-1}. The results in **Table 3-15** were calculated based on the optimised procedure only (see **section 3.2.2.2**).

For both RSD and bias, optimised results were less than 20% for all drugs and reagents. The favoured reagent was determined according to the concentration errors (0.50 µg mL^{-1}) of RSD and accuracy obtained. Hence, the RSD was from lowest to highest was: TFA (2.71%), AA (4.34%), PFPA (4.73%), CLF_2AA (6.0%), HFBA (7.0%) and PA (7.4%).

The bias results from lowest to highest were obtained for TFA (0.68%), PA (−0.71%), PFPA (0.73%), HFBA (1.97%), AA (−2.32%) and CLF_2AA (−13%).

3.3.8 Linearity and limit of detection (LOD) studies

For the linearity study, the regression of correlation coefficients (R^2) was constructed for each reagent and drug using the ChemStation software, based on triplicate samples at seven concentrations (2, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 µg mL^{-1}). All average values of R^2 for all reagents and drugs were above 0.900; see **Table 3-16** for the results.

The best fit regression was greater than or equal to 0.992. The best results (R^2) were PFPA (0.999), HFBA (0.999), TFA (0.998), PA (0.996), CLF_2AA (0.996) and AA (0.996). These were determined based on the average of (R^2) for all derivative drugs, with the exception of ethylone, pyrovalerone and MDPV.

For the LOD study, the SIM mode was calculated using the method from **section 3.2.2.4**. The method was applied in triplicate samples for all anhydrides and SC at seven concentrations from 1 to 250 ng mL^{-1}. The best LOD results were attained using PFPA (2.33 ng mL^{-1}), HFBA (3.83 ng mL^{-1}), TFA (5.0 ng mL^{-1}), PA (7.5 ng mL^{-1}), CLF_2AA (16 ng mL^{-1}) and AA (66 ng mL^{-1}). The conclusions were obtained from the average of the lowest LOD for all substances, excluding ethylone, MDPV and pyrovalerone.

Table 3-15: RSD and accuracy (bias)

The average is the average of the highest peak area value of the base peak ion response at concentration of 0.50 µg mL^{-1} (n=3).

Drug name & reagent		PFPA	TFA	CLF_2AA	HFBA	AA	PA
Flephedrone	Mean	2283223	1178147	1881598	3563229	2407035	1263952
	RSD	4.07%	3.41%	10%	14%	1.13%	5.5%
	Bias	1.81%	−9.8%	−19%	−4.83%	3.67%	1.81%
Mephedrone	Mean	4467040	3657740	3698786	3702338	1086728	2523269
	RSD	1.96%	0.99%	6.4%	2.02%	2.71%	11%
	Bias	4.79%	3.47%	−12%	−0.36%	−9.0%	−12%
Pentedrone	Mean	2714988	1860552	368017	2582720	2145452	3099143
	RSD	1.51%	2.37%	2.20%	4.33%	2.59%	12%
	Bias	10%	4.09%	−9.3%	11%	−9.3%	10%
Methedrone	Mean	7144720	6822530	6657846	6353019	574827	2489097
	RSD	4.49%	2.89%	4.43%	7.7%	2.59%	0.18%
	Bias	5.62%	12%	−7.1%	13%	5.2%	−12%
Methylone	Mean	6296421	9973042	5487420	3591150	2099231	1157643
	RSD	1.46%	1.76%	0.45%	0.98%	1.76%	0.06%
	Bias	−11%	−1.11%	−12%	−2.55%	−6.8%	1.42%
Butylone	Mean	5835783	5881945	5132108	4476375	2139855	5185680
	RSD	1.96%	7.6%	8.2%	9.7%	2.43%	5.6%
	Bias	−3.28%	−13%	−16%	−7.5%	8.5%	−3.28%
Ethylone	Mean	4630147	4161097	4026282	1914781	63541.29	5185680
	RSD	1.14%	1.81%	7.9%	6.8%	3.82%	5.6%
	Bias	2.09%	−12%	−9.0%	14%	−17%	0.44%
Pyrovalerone	Mean	5801857	2929385	5626518	7895943	6658780	4976504
	RSD	12%	1.01%	4.74%	6.8%	10%	12%
	Bias	−13%	15%	−19%	14%	3.43%	13%
MDPV	Mean	4735925	3709708	4519016	6421153	4600039	5523840
	RSD	14%	2.54%	10%	11%	12%	15%
	Bias	9.5%	7.5%	−16%	−19%	0.39%	−5.8%

Table 3-16: Linearity (R^2) and LOD (ng mL^{-1}).

Reagent & drug name		PFPA	TFA	CLF_2AA	HFBA	AA	PA
Flephedrone	LOD	1	5	10	1	50	10
	(R^2)	0.998	0.995	0.992	0.998	0.996	0.997
Mephedrone	LOD	1	5	5	1	25	10
	(R^2)	0.999	0.999	0.995	0.999	0.99	0.981
Pentedrone	LOD	1	5	5	1	1	5
	(R^2)	0.997	0.998	0.997	0.998	0.996	0.997
Methedrone	LOD	1	5	5	5	50	10
	(R^2)	0.999	0.999	0.994	0.999	0.999	1
Methylone	LOD	5	5	25	10	25	5
	(R^2)	0.999	0.999	0.998	1	0.998	0.999
Butylone	LOD	5	5	50	5	250	5
	(R^2)	0.999	0.997	0.998	0.999	0.998	0.999
Ethylone	LOD	5	10	25	100	100	5
	(R^2)	0.998	0.999	0.996	0.935	0.996	0.998
Pyrovalerone	LOD	50	50	50	100	25	5
	(R^2)	0.956	0.912	0.955	0.912	0.998	0.996
MDPV	LOD	50	50	50	100	25	5
	(R^2)	0.944	0.985	0.978	0.905	0.995	0.994

3.3.9 Recovery studies

The evaluation of recovery was investigated to check that the nine substances could be derivatised after extraction from whole blood. A concentration of 3 µg mL^{-1} was extracted from whole blood using the SPE technique. The procedure and calculation method was demonstrated in **section 3.2.2.6**. The results of recovery and RSD are illustrated in **Table 3-17**. The best reagents for the recovery study were AA, TFA, CLF_2AA, PFPA and HFBA.

Table 3-17: The evaluation of recovery in whole blood samples at 3 µg mL^{-1} (n = 3)

Figures between brackets are the rank of the reagent, the best reagent has one point, the second reagent has two points, etc.

Drug names vs. reagents		PFPA	TFA	CLF_2AA	HFBA	AA
Flephedrone	Recovery	69%	69%	100%	59%	81%
	RSD	7.4%	12%	17%	6.2%	2.14%
Mephedrone	Recovery	107%	104%	94%	64%	121%
	RSD	7.1%	1.43%	9.7%	20%	7.6%
Pentedrone	Recovery	70%	112%	92%	43%	68%
	RSD	2.63%	3.48%	17%	20%	5.2%
Methedrone	Recovery	107%	129%	100%	110%	94%
	RSD	9.6%	7.9%	7.5%	19%	10%
Methylone	Recovery	101%	98%	98%	126%	82%
	RSD	0.75%	2.37%	3.59%	16%	2.35%
Butylone	Recovery	145%	51%	37%	53%	75%
	RSD	5.3%	0.84%	56%	1.80%	18%
Ethylone	Recovery	229%	117%	97%	14%	119%
	RSD	32%	1.27%	5.4%	7.3%	15%
Pyrovalerone	Recovery	77%	19%	64%	52%	187%
	RSD	1.90%	11%	23%	15%	15%
MDPV	Recovery	58%	122%	63%	134%	106%
	RSD	1.13%	2.86%	20%	3.76%	3.24%
The average recovery and RSD (%) for all drugs in each reagent (excluding ethylone, MDPV and pyrovalerone)	Recovery	120% (5)	113% (4)	104% (2)	91% (3)	104% (1)
	RSD	6.56% (2)	5.6% (1)	22% (5)	17% (4)	9.1% (3)
Number of drugs between (80–120% recovery) for each reagent		3 (4)	4 (3)	6 (2)	1 (5)	5 (1)
Number of drugs between (0–20% RSD) for each reagent		8 (3)	9 (1)	6 (5)	7 (4)	9 (1)
Overall points for each reagent (the less points, the better the reagent)		14 (3)	9 (2)	14 (3)	16 (5)	6 (1)

3.3.10 Overview for the evaluation of derivatisation reagents

All points from the above sections were collected in order to decide the favoured reagents. This was based on nine parameters, demonstrated in **Table 3-18**. The overall best reagent for the selected SC was PFPA, followed by TFA, AA, CLF_2AA, HFBA and PA. Therefore, PFPA was selected for the following steps of the studies.

The assessment of the reagents above is important for application to forensic sample examination. Each drug has a different fragmentation and response to each reagent, and this study can help decide which drug and reagent provide the results with the best fragmentation patterns. For instance, the best reagent for the detection and quantification of mephedrone is PFPA because:

- It has variety of fragmentation ions.
- It shows the greatest peak area values.
- It is within acceptable error, using the accuracy and precision parameters.
- The sensitivity of the instrument is high, as it offers the lowest LOD compared to other reagents.
- It presents excellent regression under the linearity study.
- It has a unique ion.
- It has more total ions than other reagents.
- The relative ion ratio percentage is high, associated to the base peak of the mass spectra (119 m/z); see **Figure 3-14**.

Table 3-18: The best reagents for selected cathinones and parameters
The best reagent has one point, the second has two, etc.

Parameters & reagents	PFPA	TFA	CLF_2AA	HFBA	PA	AA
No. of ions	1	3	2	6	4	4
No. of unique ions	6	5	2	4	4	1
Total relative ion fragmentation %	3	4	5	6	1	2
The maximum peak area	1	2	5	3	6	4
RSD	3	1	4	5	6	2
Accuracy	3	1	6	4	2	5
Linearity R^2	1	3	5	2	4	6
LOD	1	3	5	2	4	6
Recovery	4	2	3	5	6	1
Total points (the rank)	23 (1)	24 (2)	37 (4)	37 (4)	37 (4)	31 (3)

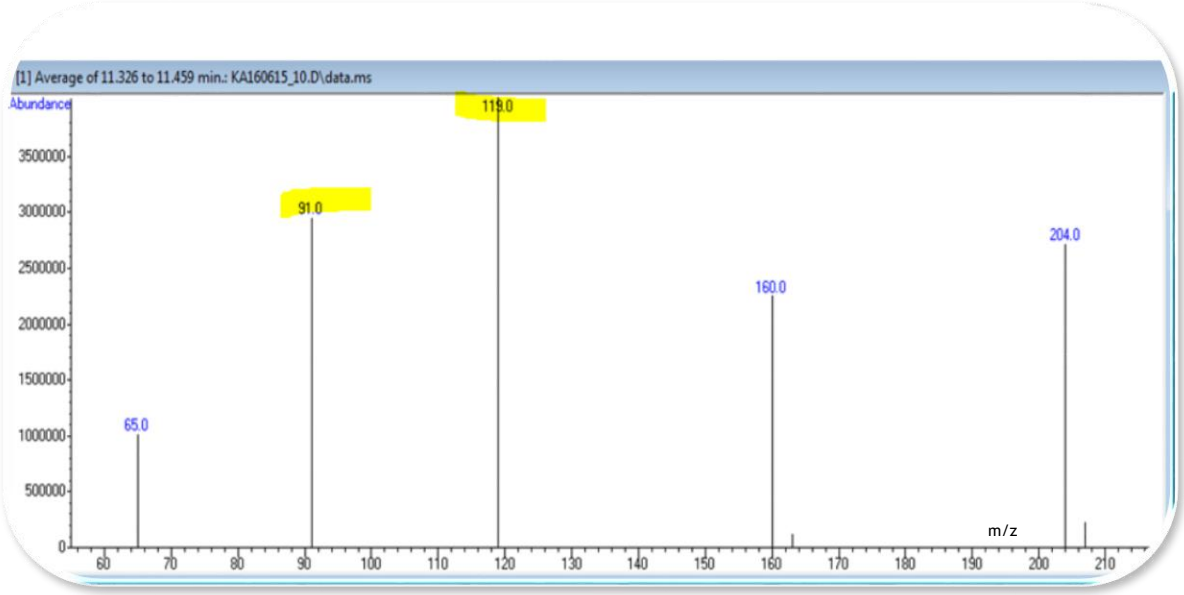


Figure 3-14: Consistent fragmentation with high relative ion intensities using PFPA

3.3.11 Acidified methanol study

The results of the duplicate blank blood samples spiked with the selected SC showed that adding the acidified methanol before evaporation was crucial in order to increase the sensitivity of the instrument. See **Figure 3-15** for the comparison results. This is because the cathinones are basic drugs, where adding the acid will decrease the volatility of the compounds during evaporation under a nitrogen stream.

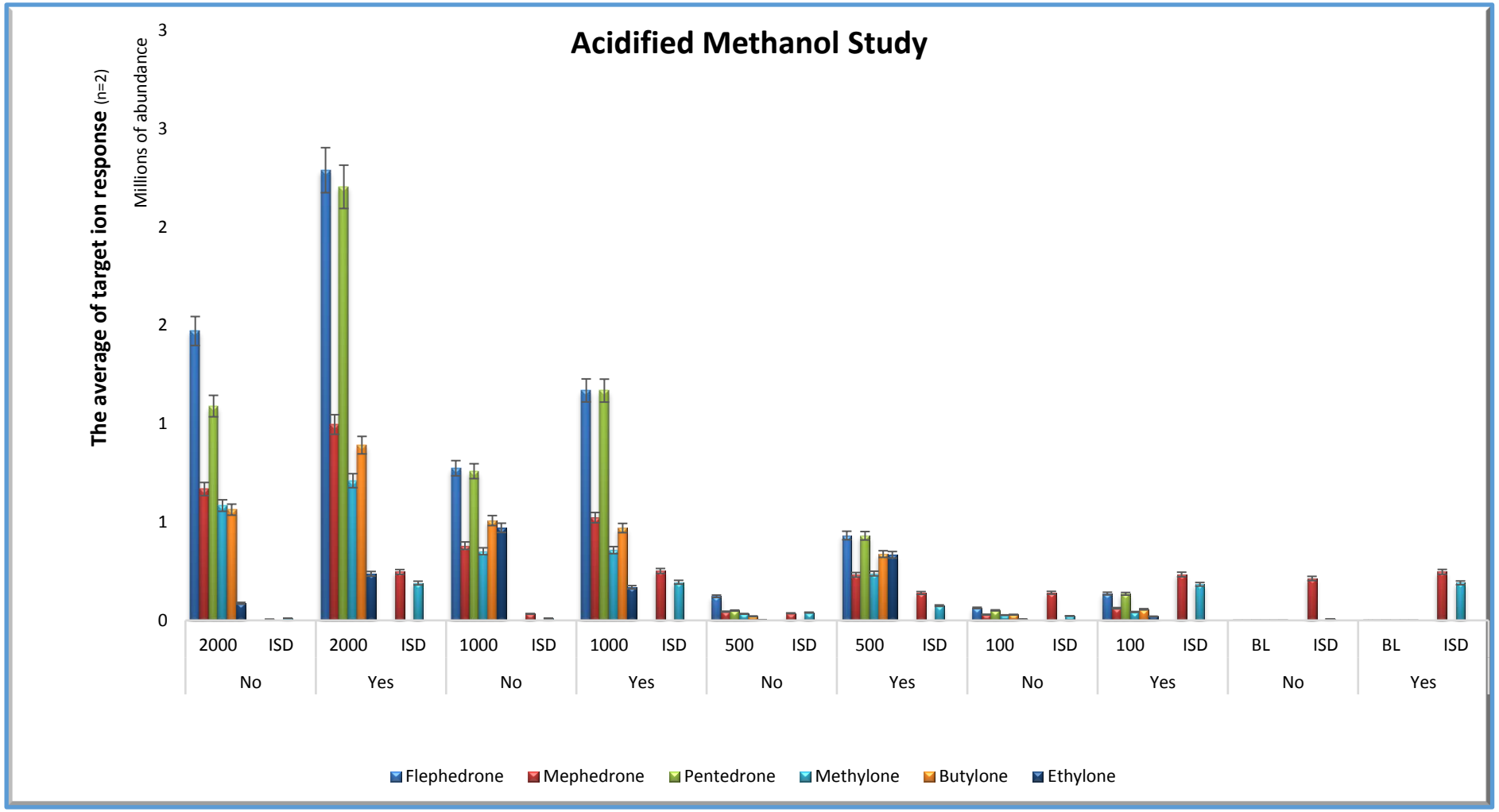


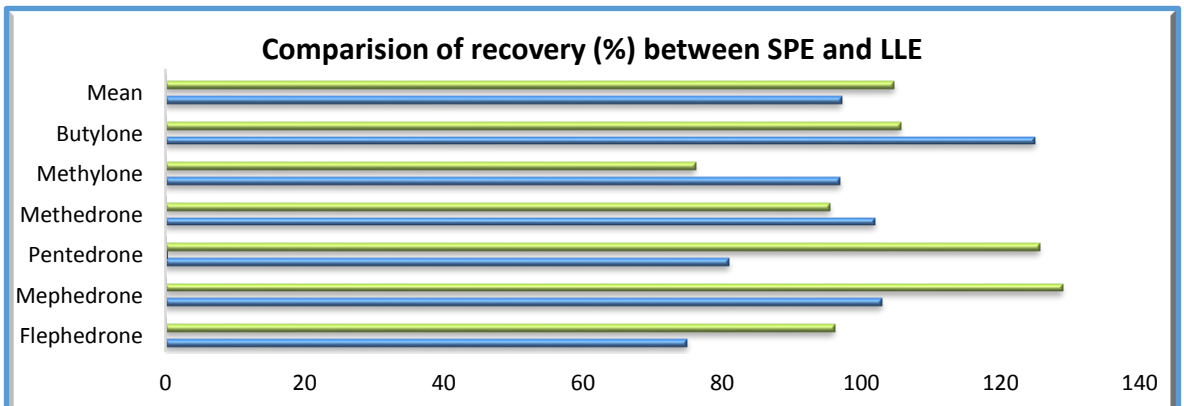
Figure 3-15: Comparison study when acidified MeOH was added to samples or not.

No: no acidified MeOH was added. Yes: the acidified MeOH was added. The concentration points are in ng mL^{-1}. BL is whole blood blank

3.3.12 Evaluation of two extraction methods (SPE and LLE)

This study was carried out to evaluate the recovery results using two sample preparation methods: LLE and SPE. Henceforth, the favoured extraction method is used in the stability work. Two extraction methods were applied to six SC in urine specimens. The results are illustrated in **Figure 3-16**. These results show that both techniques achieved high recovery with no less than 75% in SPE and 76% in LLE for all drugs examined. At least four out of six drugs were between 80–120% in SPE, but LLE had only three drugs in that range. The average of all drugs tested was better in SPE (97%) compared to LLE (105%). Over 100% recoveries were observed for some compounds, which may have originated from the evaporation step, where drugs of unextracted samples were partially lost. In general, and based on the limited repeatability with only triplicate samples, the findings indicate that the both techniques are suitable for application to SC using the procedures stated in the method and material section.

Though LLE provided comparable recovery results, SPE was selected for the upcoming work. SPE is well known for its ability to obtain cleaner products with less interferences in the chromatogram. The advantages and disadvantages of both techniques are discussed in chapter two.



	Flephedrone	Mephedrone	Pentedrone	Methedrone	Methylone	Butylone	Mean
RSD-LLE	1.90%	2.09%	1.49%	4.31%	12%	3.71%	4.18%
LLE %	96	129	126	96	76	106	105
RSD-SPE	5.4%	9.4%	15%	3.51%	4.16%	7.4%	7.6%
SPE %	75	103	81	102	97	125	97

Figure 3-16: Recoveries (%), RSD and average of six SC in triplicate urine samples at 1 µg mL^{-1} by SPE and LLE methods (n= 3),

3.3.13 Linearity, signal−to−noise ratio and carryover examination using whole blood, SPE and PFPA

The method was applied to five SC (flephedrone, mephedrone, methedrone, methylone and butylone) using the SPE−PFPA and whole blood. For the linearity study, all the results were above 0.991 (R^2). **Figure 3-17** displays results for unextracted and extracted drugs. Signal−to−noise (S/N) ratio was inspected at 10 ng mL^{-1}, as demonstrated in **Table 3-19** for each ion of five SC.

No carry over interferes were found after injecting triplicates of whole blood−blank high concentration 10 µg mL^{-1} samples.

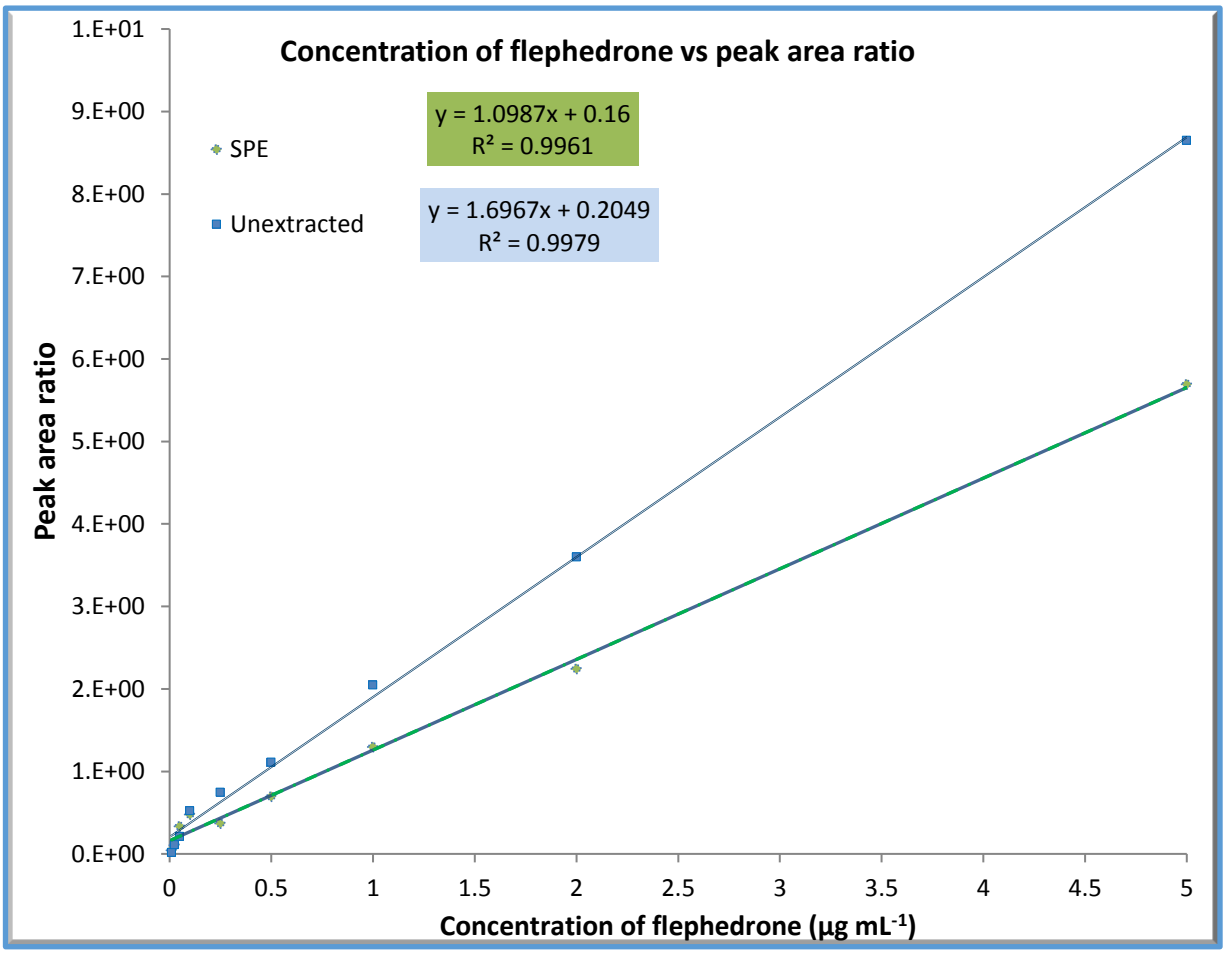


Figure 3-17: The comparison curves between unextracted and extracted SPE−PFPA in whole blood samples for the examination of flephedrone

Note: Similar findings were observed for the remaining five drugs.

Table 3-19: Signal–to–noise (*S/N*) ratio of five SC at 10 ng mL^{-1} in whole blood samples using SPE–PFPA methods.

Mephedrone	Flephedrone	Butylone	Methedrone	Methylone
Ion / (*S/N*) ratio	Ion / (*S/N*) ratio	Ion / (*S/N*) ratio	Ion / (*S/N*) ratio	Ion / (*S/N*) ratio
119/429	123/16	218/11	135/2252	149/3
91/146	95/8	149/103	77/4	218/22
160/34	160/19	160/6	160/87	367/25
65/294	204/24	367/36	204/32	121/7

3.4 Conclusion

Six acylation reagents (PFPA, TFA, CLF_2AA, HFBA, AA and PA) were investigated for nine SC compounds (butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone) using GC–MS. The GC conditions were primarily optimised by altering the temperature of the injector port to reduce the degradation of SC substances. Additionally, the oven temperature was adjusted until the desired separation and detection were achieved. The reagents were optimised for incubation period and temperature as well as the hot block temperature in the evaporation stage. These conditions were briefly discussed using a three-ways ANOVA and peak area values until the optimum procedure was determined. Derivative cathinones generally performed better under higher temperatures for the reagents that have high M.W and BP, though the converse was found for the other cathinones. Five minutes was sufficient for the completion of the reaction; however the maximum peak areas were improved after 20 minutes.

After the method was optimised, various parameters were applied for the comparison study, such as the relative ion fragmentation intensities, number of ions, number of unique ions, the uppermost peak area values, recovery, LOD, linearity, RSD, bias and interferences. The final conclusion was that all acylation anhydrides were suitable for the detection of SC substances. PFPA agent was favoured, based on the criteria and parameters used. PFPA was investigated

further in whole blood using linearity, recovery, signal–to–noise ratio and carryover parameters.

In this chapter, one ISD was found to be sufficient to achieve excellent linearity in all types of anhydrides and SC. Acidified methanol was examined to increase the sensitivity of the GC–MS, and the results showed that adding the acidified methanol before drying the samples significantly improved the detection of the selected drugs. The extraction methods of LLE and SPE were compared using a recovery study. Both techniques had excellent recoveries for most drugs tested, and both could be used for the extraction of SC compounds. However, the application of SPE was adopted, due to its advantages.

Butyric anhydride applied to SC was investigated for the first time. No or very poor responses were observed in the chromatogram during method development. The tertiary amines MDPV and pyrovalerone were mostly not derivatised with the reagents assessed.

Therefore, in the next chapter, the selected PFPA reagent will be fully validated using the optimum conditions of SPE–PFPA and GC–MS in urine samples.

4. Determination of synthetic cathinones and amphetamine−type stimulants in urine using solid-phase extraction and gas chromatography–mass spectrometry

4.1 Introduction

A urine specimen is one of the most commonly used matrices in forensic toxicology laboratories. Collection method of urine specimen is inherently less harmful or at risk for contamination, an adequate volume is usually available, and the concentration of analytes is generally high compared to other matrices. Positive urine samples indicate the presence of the parent drug and/or its metabolites. Parent drug/metabolite ratios occasionally estimate when the drug was taken, due to processing changes in the metabolism. SC are normally taken in high doses and the parent substances can be determined in urine [129, 260, 399, 462].

SC and ATS were previously detected using screening and confirmation methods in urine samples, however published papers that included SC, metabolites and ATS in a single method using GC−MS were non−existent. Therefore, a comprehensive screening and confirmation technique for the detection and quantification of ATS and SC in urine using a single method was demanded. The GC−MS was optimised until a specific and sensitive method was developed for the detection of 29 SC and ATS, including three SC metabolites. 20 out of 29 drugs were valid for confirmation purposes.

New methods should be capable of differentiating between a variety of substances that have similar chemical behaviour, which may be present in the sample. Therefore, it is very important to prove the validity of such methods in order to ensure that it is robust and reliable for screening and confirmation in forensic toxicology. This is significant because the obtained results have substantial authority over individuals encountering the criminal justice system. The validation work should be completed in agreement with international guidelines and recommendations. Even though there are a lot of guidelines available for the purpose, the Scientific Working Group for Forensic Toxicology (SWGTOX) guideline was selected for the validation work, because it was recently designed for validating new methods in forensic toxicology [3].

The aims of this chapter are:

- To optimise the GC–MS for the detection and quantification of 29 frequently tested ATS and SC, including three metabolites, as follows: seven ATS (amphetamine, methamphetamine, PMA, MDA, MDMA, MDEA, PMMA) and 22 SC (cathinone, methcathinone, buphedrine (buphedrone metabolite), flephedrone, 4–methylephedrine (mephedrone metabolite), 4–methyl–N–ethyl–norephedrine (4–MEC metabolite), buphedrone, N–ethylcathinone, mephedrone, pentedrone, methedrone, methylone, butylone, ethylone, pyrovalerone, 4–EMC, 4-MEC, α–PVP, pentylone, MDPPP, naphyrone and MDPV).

- To validate the methods using the selected PFPA reagent and SPE (see chapter 3) for the detection and quantification of the above drugs. The validation parameters were RSD, bias, linearity, carryover, selectivity, interferences, limit of detection (LOD), lower limit of quantitation (LLOQ), recovery and stability. This method was also validated to prove the robustness for studying long–term stability of the analytes and the prevalence of the selected ATS and SC in Saudi samples discussed in chapter 5 and 7.

4.2 Materials and methods

4.2.1 Chemicals and reagents

All 29 standard drugs, 3 ISD (amphetamine–d_{11} at 1 mg mL^{-1}, MDA–d_5 at 1 mg mL^{-1} and butylone–d_3 at 100 µg mL^{-1} with their hydrochloride salts), derivatising reagents, other materials and grade chemicals were obtained from the supplier mentioned in **section 3.2.1.1**. Phosphate buffer and sodium phosphate were acquired from Fisher Scientific, Loughborough, UK. Solid-phase extraction (SPE) 200 mg cartridges (part number ZSDAU20) were purchased from Chromatography Direct, Runcorn, UK.

4.2.2 Drug-free urine (DFU)

Blank urine samples were collected in the forensic toxicology department from at least 10 different healthy volunteers. All collected urine samples were drug-free

from the target analyte drugs. The samples were confirmed drug-free (negative) from the target analytes by running these samples using the methods and procedure outlined in chapter 3 using scan and SIM mode. All urine specimens were kept at 4°C in the refrigerator (RF). The drug-free urine samples were collected under ethical approval. The protocol was reviewed and approved by the MVLS College Ethics Committee, University of Glasgow (200160020), see appendix 1.

4.2.3 Preparation of drug standard (STD) solutions

Purchased standards at a concentration of 1 mg mL^{-1} of all 29 drugs were individually prepared in methanol via 1:10 (v/v) dilution to produce final concentration solutions of 100 µg mL^{-1} of each drug. This was obtained by transferring the purchased ampule to a 10 mL volumetric flask. The ampule was rinsed several times using MeOH to ensure the entire amount was completely transferred. MeOH was then transferred to the flask up to the mark, the flask was inverted and then mixed with the STD. The contents were labeled using amber glass and stored in the freezer (FZ) at −20°C.

4.2.4 Preparation of ISD

The purchased ISD of amphetamine−d_{11} and MDA−d_5 at 1 mg mL^{-1} were diluted to 100 µg mL^{-1} using the preparation method described in the section above. The mixture of these with butylone−d_3 at 100 µg mL^{-1} were prepared to provide a concentration of 10 µg mL^{-1}. This was achieved by transferring the content (1 mL) of each to a 10 mL volumetric flask and then completing the preparation method outlined above in the section of STD preparation.

4.2.5 Preparation of working solution for linearity study

1 mL was taken and transferred from each stock standard solution (100 µg mL^{-1}) to a 50 mL volumetric flask via a 1:50 (v/v) dilution in DFU to a concentration of 2 µg mL^{-1}. This flask was filled up to the mark using DFU. The mixture of the working solution was inverted and gently shaken several times before it was labeled and kept in the FZ at −20°C in an amber glass bottle. The remaining

concentrations (0.05, 0.10, 0.25, 0.50, 0.75 and 1 µg mL^{-1}) were prepared using **Equation 4−1** to determine the volume of the working solution.

Equation 4−1:Dilution equation

$$\left(\frac{C_1}{V_1}\right) \times \left(\frac{C_2}{V_2}\right)$$

where V_1 = volume of working solution (?), C_1 = concentration of working solution (2 µg mL^{-1}), V_2 = final volume of new point of standard (1 mL) and C_2 = final concentration of the new point of standard (for example, 1 µg mL^{-1}).

4.2.6 Linearity method

The assessment of linearity was carried out using five separate calibration curves repeated on five consecutive days using eight calibration points, including DFU (50, 100, 250, 500, 750, 1000, 2000 ng mL^{-1}). 1 mL of the mixture drug in urine for each calibrator point was added to the culture tube and then the sample preparation procedure was executed. The bias of each calibrator point was calculated from each calibration curve which should not exceed ±20%. The average error for 25 accuracy values in each concentration was obtained in order to determine whether the calibrator point was valid or not. Acceptable values should be greater than 0.99 in R^2 to comply with SWGTOX guidelines. The calculation linearity method was previously described in **section 3.2.2.3**.

The SIM ion ratios for each drug were monitored throughout the course of the validation work to confirm constancy. The monitoring SIM ion ratios assisted in interpreting whether a sample was positive or not. The monitoring was carried out using the Q value in the ChemoStation software, which should not less than 80%.

4.2.7 Preparation of working solutions for accuracy and precision study

Three quality controls (QCs) were prepared at concentrations of 250 (QC_1), 750 (QC_2) and 1500 (QC_3) ng mL^{-1}. For that, 125, 375 and 750 µL of each stock standard solution (100 µg mL^{-1}) were added to 50 mL volumetric flasks (1:50 v/v) dilution in DFU for QC_1, QC_2 and QC_3 respectively. The flasks were filled using DFU to the

mark. The mixture in the three flasks were inverted several times and transferred to amber glass bottles stored in the FZ at −20°C.

4.2.8 Accuracy and precision method

Accuracy (bias) of the method was determined using the grand average of the results of each quantity detailed in chapter 3; see **section 3.2.2.5**. The purpose of accuracy determination is to study how close the concentration results are to the expected true concentration of the QCs.

Precision (RSD) should be calculated throughout within runs (intra−day) and between runs (inter−day) to evaluate the validity of the method. Intra−day and inter−day precision were analysed using three quality controls at concentrations of 250 (low−QC_1), 750 (medium−QC_2) and 1500 (high−QC_3) ng mL^{-1}. Urine samples were prepared together on the same days as the linearity study, and were replicated five times each day on five consecutive days (n= 25). Acceptable range criteria for each measurement was ±≤20%. The calculation method for precision was expressed in chapter 3; see **section 3.2.2.5**.

To study precision, intra−day (the largest calculated intra−day precision (%) for each concentration was used to measure intra−day precision acceptability) and inter−day RSD were calculated using the following equations:

Equation 4−2: Intra−day precision calculation (RSD)

$$\boldsymbol{RSD}\ (\%) = \left(\frac{\boldsymbol{SD\ of\ a\ single\ run\ of\ samples}}{\boldsymbol{Mean\ calculated\ value\ of\ a\ single\ run\ of\ samples}}\right) \times \mathbf{100}$$

Equation 4−3: Inter−day precision calculation (RSD)

$$\boldsymbol{RSD}\ (\%) = \left(\frac{\boldsymbol{SD\ of\ Grand\ mean\ for\ each\ concentation}}{\boldsymbol{Grand\ mean\ for\ each\ concentration}}\right) \times \mathbf{100}$$

4.2.9 Preparation of working solutions for LOD and LOQ studies

1 mL was taken and transferred from the working solution (2 µg mL^{-1}) to a 10 mL volumetric flask (1:10, v/v) dilution in DFU to prepare a concentration of 200 ng mL^{-1}. This flask was make using at least three different sources of DFU. The concentration points (50, 25, 10, 5, 1, 0.5 ng mL^{-1}) were prepared using the dilution equation described above.

4.2.10 LOD and LLOQ method

See **section 3.2.2.4** in chapter 3 for LOD and LLOQ method. At least three different sources of DFU specimens were prepared in three replicates for three separate runs spiked at concentrations of 50, 25, 10, 5, 1 and 0.5 ng mL^{-1}. Each replicate was independently evaluated. The LOD approach was designed for the lowest concentration of analyte when the target ion peaks exceed three times the background noise with at least two qualifier ions present at the $_tR$. The LLOQ was measured based on the lowest concentration that provides a repeatable GC–MS response with an acceptable accuracy (±<20%) and an $S/N \geq 10$. The accuracy results of the LLOQ were obtained from the linearity study (n= 25 in each calibrator point).

4.2.11 Sample preparation using the SPE−PFPA (procedure)

The mixture of ISD (50 µL of 10 µg mL^{-1}) and 3 mL of 0.1 M phosphate buffer (pH6) were added to all calibrators, QCs and samples (for the sample results see chapter 7) before being mixed for a few seconds and centrifuged for 10 minutes at 3000 rpm. 2 mL of MeOH, d.H_2O and 0.1 M phosphate buffer were individually added to condition the SPE cartridge. Next, samples were added to the SPE cartridge, followed by 3 mL of d.H_2O, 1 mL of 1M acetic acid and 3 mL of MeOH for washing and extraction, after which the cartridges were dried under full vacuum for a minute. The cartridges were eluted using 2.5 mL of DCM: IPA: NH_4OH (78:20:2) (v/v/v) before 10 µL of acidified MeOH (1:9) (v/v) were added to each tube. The specimens were evaporated at 33°C under a stream of nitrogen until fully dry. The selected PFPA (see chapter 3) was applied as a reagent to derivatise the specimens

by adding 50 µL of PFPA and EtOAc (2:1) (v/v) to all tubes. The tubes were then capped and incubated for 15–20 minutes at 60°C before being evaporated once again under a gentle stream of nitrogen at RT. Finally, the samples were reconstituted using 50 µL of EtOAc prior the contents being transferred to GC–MS vials for analysis.

4.2.12 Recovery method

Four replicate samples of 1 mL DFU, spiked with the 29 mixture drugs were analysed in one day using SPE for extracting the samples at concentrations of 1500, 750 and 250 ng mL^{-1}. The sample preparation procedure using SPE–PFPA mentioned was applied. 29 mixture drugs at the three concentrations were added to each tube for the unextracted samples. The mixture of ISD were added before the evaporation stage for both unextracted and extracted tubes. The calculation method was demonstrated in **section 3.2.2.6**.

4.2.13 Carryover method

Carryover was investigated by rerunning the blank urine sample after the calibrator point of 2 µg mL^{-1} that was prepared in linearity study. This was completed to estimate any possible carryover.

4.2.14 Selectivity and interferences method

Selectivity was evaluated to detect if any other drug can interfere with the target analytes and modify the interpretation of the results. Interferences could either be produced by exogenous substances, by any other analytes implanted in the specimen or by endogenous substances (the matrix itself). The selected independent drugs were analysed in groups, instead of all together.

The method examined the data attained from the 29 SC and ATS substances using SIM mode for a mixture of four groups of specimens.

100 µL of a 10 µg mL^{-1} working solution of each drug were placed in 1 mL DFU samples, and the samples were prepared and completed using the procedure

stated above. The analysed drugs were cocaine, 6–MAM, pregabalin and tetrahydrocannabinol (THC) for culture tube 1; gabapentin, clozapine, olanzapine, midazolam and LSD for culture tube 2; nor- fentanyl, fentanyl, morphine and codeine for culture tube 3; paracetamol, caffeine, oxycodone and hydrocodone for culture tube 4.

The DFU that was applied in the linearity, RSD and accuracy studies were conducted for the assessment of interferences. The drug–free urine samples were collected from at least 10 different donors under the ethical approval stated above. The selectivity was evaluated by looking for new peaks and retention time that interfere the peaks and retention time of the target analytes using scan and SIM modes.

4.2.15 GC–MS conditions

The GC–MS methodology described in **chapter 3.2.2.12** was utilised with the exception that the temperature condition was optimised as follows: the temperature programme of the capillary column began at 70°C then elevated to 200°C at a rate of 11°C/min and was then held for 4 minutes before increasing to 280°C at a rate of 10°C/min and finally held for 1 min with a run time 25 min. All data obtained were used the GC–MS ChemStation Software Version 6.5.

4.3 Results and Discussion

4.3.1 Retention time ($_tR$) and fragmentation ion ratios

Figure 4-1 demonstrates the shape, separation and $_tR$ of the peaks. Even though some peaks had coelution that resulted in bad resolution in chromatogram, the peaks have different $_tR$ with different target ions and ratios with at least two different qualitative ions. This can be simply achieved using an extracted ion chromatogram (EIC) or a total ion chromatogram (TIC) method using a mass detector rather than a GC; see **Table 4-1** for the $_tR$, target and qualification ions. These ions were selected for the evaluation of validation parameters and samples results.

Throughout the validation laboratory work, the five compounds of tertiary amines were derivatised in small peaks under different $_tR$ of underivatised peaks using PFPA, as illustrated in **Figure 4-1**, however the tertiary underivative amines of the mass spectra peak for ion 126 for MDPV, PVP, naphyrone and pyrovalerone and ion 98 for MDPPP were the target ions selected for calculation parameters. These were selected, because the derivative ion amines provided ≤ 0.9 R^2 with more than 20% RSD for most examined samples.

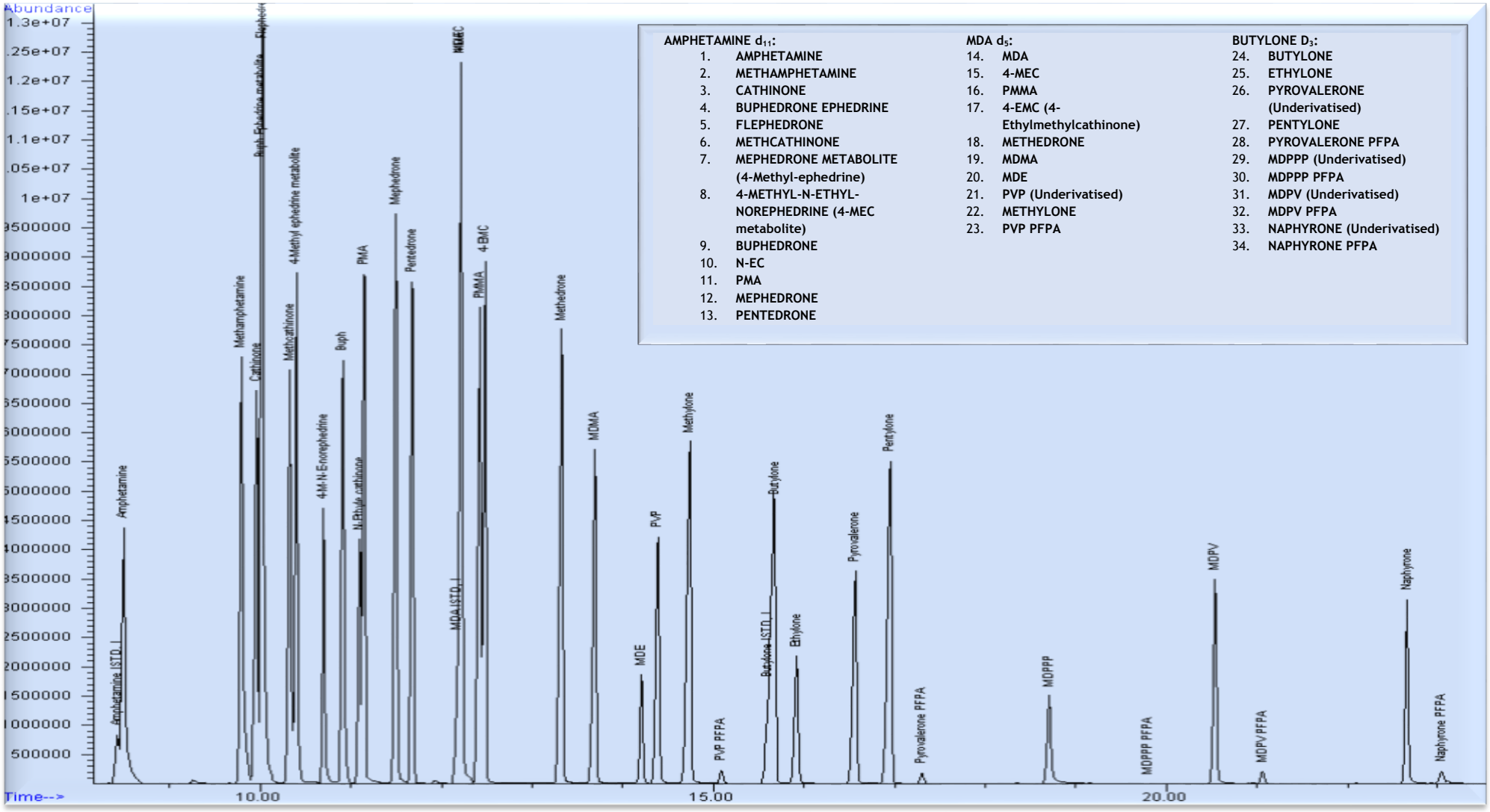


Figure 4-1: SIM chromatogram showing shape, separation and $_tR$ of ATS and SC peaks at a concentration of 2 µg mL^{-1} in DFU. Each substance from tertiary amines had two peaks, the earliest elution was underivatized and the additional peak was the tertiary amine derivative. The sequence number above was based on the first peak eluted until the last.

Table 4-1: SIM fragmentation patterns (m/z) and relative ion intensities (ratio %) with retention time ($_tR$). Quantification ions in bold. The remaining ions were used as qualifier ions with ratios (%).

Drug name	$_tR$	m/z	Ratio (%)	Drug name	$_tR$	m/z	Ratio (%)
AMPHETAMINE-d_{11}	8.422	**194**	100	PENTEDRONE	11.668	**232**	100
		128	72			190	59
		98	33			105	45
						337	3.90
AMPHETAMINE	8.486	**190**	100	MDA-d_5	12.191	**167**	100
		118	79			330	62
		91	36				
		65	9.2				
METHAMPHETAMINE	9.791	**204**	100	MDA	12.198	**135**	100
		160	31			162	47
		118	24			325	15
		91	14				
CATHINONE	9.937	**105**	100	4-MEC	12.220	**119**	100
		77	31			218	37
		51	6.9			190	30
		190	6.0			91	17
FLEPHEDRONE	10.024	204	100	PMMA	12.408	**121**	100
		123	59			204	159
		95	19			148	102
						160	42
BUPHEDRINE	10.030	**218**	100	4-EMC	12.472	**133**	100
		119	12			204	20
		308	2.6			160	10
METHCATHINONE	10.316	**105**	100	METHEDRONE	13.33	**135**	100
		204	102			77	8.0
		160	36			160	6.1
		77	30			204	9.1
4-METHYLEPHEDRINE	10.403	**204**	100	MDMA	13.679	**204**	100
		119	13			162	73
		160	18			135	43
		308	2.60			339	12
4-M-N-E-NOREPHEDRINE	10.706	**218**	100	MDEA	14.215	**218**	100
		119	20			353	8.5
		190	22			162	57
		322	2.50			135	25
BUPHEDRONE	10.905	**218**	100	PVP	14.384	**126**	100

		105	51			77	6.1
		77	21			105	3.2
		160	23				
N–EC	11.091	218	100	METHYLONE	14.716	**149**	100
		105	45			204	19
		190	40			160	11
		77	23			353	4.90
PMA	11.11	**121**	100	PVP PFPA	15.066	214	100
		148	42			70	36
		190	5			229	33
		311	7.4			214	18
MEPHEDRONE	11.482	**119**	100	MDPPP underivatised	18.703	**98**	100
		204	25			149	10
		91	20				
		160	14				
BUTYLONe-d_3	15.627	**221**	100	MDPPP PFPA	20.026	96	100
		370	20			245	36
		163	144			176	22
						216	43
BUTYLONE	15.667	**218**	100	MDPV underivatised	20.539	**126**	100
		149	480				
		160	35				
		367	20				
ETHYLONE	15.923	**190**	100	MDPV PFPA	21.052	124	100
		149	655			273	18
		218	198			149	41
		367	22			70	52
PYROVALERONE underivatised	16.564	**126**	100	NAPHYRONE underivatised	22.667	**126**	100
		91	4				
PENTYLONE	16.946	**149**	100	NAPHYRONE PFPA	23.063	124	100
		190	22			279	45
		232	19			70	56
		381	5				
PYROVALERONE PFPA	17.287	124	100				
		243	32				
		228	15				
		159	11				

4.3.2 Linearity

All selected analytes were plotted using the simple unweighted linear regression model least squares method, excluding PMA which was plotted using the quadratic method. This technique produced correlation coefficients R^2 > 0.990 for all analysed substances, with the exception of 4–methyl–n–ethyl–norephedrine, 4–MEC, MDPPP and naphyrone; see **Table 4-2** for the average results.

By using the accuracy approach to calculate each calibrator point, all substances fitted the constructed calibration curve for all seven concentration points (50 to 2000 ng mL^{-1}), excluding the following drugs:

- 4–methylephedrine, PMA, N–EC and PVP of six calibrator points from the concentrations of 100 to 2000 ng mL^{-1}.
- 4–MEC, ethylone, pyrovalerone, MDPPP, MDPV and naphyrone of five calibrator points from 250 to 2000 ng mL^{-1}.
- 4–Methyl–N–ethyl–norephedrine of four calibrator points from 500 to 2000 ng mL^{-1}.

4.3.3 Accuracy and precision

In **Table 4-3**, most examined substances were valid and fell within the SWGTOX ±≤20% criteria for bias and RSD.

All underivatised tertiary amines (MDPV, PVP, pyrovalerone, MDPPP and naphyrone) were above ±20% in accuracy and precision parameters at 250 and 750 ng mL^{-1}, and only valid at 1500 ng mL^{-1}. Derivatised tertiary amine ions were similarly assessed and were out of accepted range at all QCs.

The two metabolites of mephedrone and 4–MEC were inaccurate (bias ±≥20%) at a concentration of 250 ng mL^{-1}. 4–MEC and PMA were also slightly above 20% when intra–day precision was measured at 250 ng mL^{-1}. It can be concluded that 21 out of 29 compounds were valid at concentrations of 250, 750 and 1500 ng mL^{-1} and successfully passed the SWGTOX recommendation for accuracy and precision parameters. The average within-run precision results were 9.5, 9.2 and 5.9%, and

the average the between run results were 3.03, 3.64 and 2.66%. The average accuracy was 3.06, −0.01 and −0.19% for the low, medium and high QCs, respectively in each parameter. The method can be still beneficial for the detection of the eight unacceptable mixture substances for screening purposes, or even for quantification work at high concentration, such as 1500 ng mL^{-1}.

Table 4-2: Linearity study

Compound name	(R^2)	Compound name	(R^2)
AMPHETAMINE	0.999	PMA	0.993
METHAMPHETAMINE	0.998	MEPHEDRONE	0.996
CATHINONE	0.997	PENTEDRONE	0.998
FLEPHEDRONE	0.998	MDA	0.998
BUPHEDRINE	0.996	4−MEC	0.989 (Invalid)
METHCATHINONE	0.998	PMMA	0.997
4−METHYLEPHEDRINE	0.995	4−EMC	0.999
4−M−N−E−NOREPHEDRINE	0.988 (Invalid)	METHEDRONE	0.999
BUPHEDRONE	0.998	MDMA	0.999
N−EC	0.994	MDEA	0.996
PVP	0.997	PENTYLONE	0.999
METHYLONE	0.999	MDPPP	0.988 (Invalid)
BUTYLONE	0.999	MDPV	0.995
ETHYLONE	0.997	NAPHYRONE	0.988 (Invalid)
PYROVALERONE	0.996		

b

Table 4-3: Accuracy and precision results. The concentration unit of QCs in ng mL^{-1}

Drug name	QCs	Intra-day RSD	Inter-day RSD	Bias	Drug name	QCs	Intra-day RSD	Inter-day RSD	Bias
AMPHETAMINE	250	6.5%	1.93%	3.1%	PMMA	250	8.9%	3.02%	−1.6%
	750	5.1%	2.30%	1.7%		750	11%	1.39%	−3.4%
	1500	2.89%	1.26%	−0.6%		1500	3.15%	2.84%	1.4%
METHAMPHETAMINE	250	12%	4.09%	−6.1%	4-EMC	250	7.8%	3.64%	0.7%
	750	9.7%	4.71%	−2.7%		750	5.3%	2.38%	0.1%
	1500	5.8%	2.28%	−0.1%		1500	4.30%	1.58%	−2.6%
CATHINONE	250	10%	2.78%	4.5%	METHEDRONE	250	4.76%	1.26%	8.9%
	750	8.9%	3.00%	2.3%		750	4.61%	1.24%	1.0%
	1500	7.3%	4.21%	3.6%		1500	3.44%	0.71%	0.4%
FLEPHEDRONE	250	6.9%	2.68%	8.9%	MDMA	250	9.2%	2.58%	2.7%
	750	8.5%	2.03%	5.3%		750	9.4%	2.17%	−2.2%
	1500	1.85%	0.59%	−0.2%		1500	4.70%	1.15%	2.5%
BUPHEDRINE	250	12%	3.56%	4.7%	MDEA	250	19%	5.0%	−12%
	750	16%	7.1%	1.0%		750	15%	7.9%	−9.5%
	1500	6.1%	2.79%	0.8%		1500	16%	5.7%	1.6%
METHCATHINONE	250	9.4%	2.13%	10%	PVP (underivatised)	250	100% (Invalid)	59% (Invalid)	119% (Invalid)
	750	14%	7.5%	1.6%		750	81% (Invalid)	62% (Invalid)	35% (Invalid)
	1500	4.61%	2.95%	−0.9%		1500	2.00%	0.81%	2.9%
4-METHYLEPHEDRINE metabolite	250	33% (Invalid)	7.1%	23% (Invalid)	METHYLONE	250	6.3%	0.96%	6.5%
	750	13%	6.2%	−4.2%		750	5.4%	0.59%	0.5%
	1500	7.5%	2.11%	1.3%		1500	2.98%	0.46%	1.5%
4-MEC metabolite	250	46% (Invalid)	16%	42% (Invalid)	BUTYLONE	250	3.87%	1.15%	1.0%
	750	17%	7.0%	−7.5%		750	3.79%	0.86%	−0.8%
	1500	13%	4.90%	0.6%		1500	1.83%	0.62%	−1.3%

BUPHEDRONE	250	6.9%	2.70%	8.9%	**ETHYLONE**	250	15%	5.4%	−10%
	750	10%	6.0%	6.2%		750	13%	6.2%	−8.4%
	1500	4.29%	2.96%	−2.5%		1500	13%	3.56%	−0.2%
N-EC	250	17%	4.27%	6.5%	**PYROVALERONE (underivatised)**	250	85% (Invalid)	31% (Invalid)	143% (Invalid)
	750	14%	5.5%	1.2%		750	284% (Invalid)	267% (Invalid)	231% (Invalid)
	1500	11%	3.73%	−3.3%		1500	5.4%	2.78%	0.8%
PMA	250	23% (Invalid)	7.3%	−3.9%	**PENTYLONE**	250	4.95%	1.74%	2.6%
	750	13%	7.0%	−0.4%		750	4.37%	1.11%	1.3%
	1500	3.80%	1.64%	5.6%		1500	3.34%	2.91%	−5.2%
MEPHEDRONE	250	13%	5.9%	9.3%	**MDPPP (underivatised)**	250	116% (Invalid)	49% (Invalid)	1278% (Invalid)
	750	12%	4.25%	1.5%		750	352% (Invalid)	351% (Invalid)	1565% (Invalid)
	1500	9.1%	5.8%	1.4%		1500	8.5%	3.54%	1.8%
PENTEDRONE	250	12%	4.13%	9.5%	**MDPV (underivatised)**	250	108% (Invalid)	41% (Invalid)	383% (Invalid)
	750	11%	5.5%	2.6%		750	338% (Invalid)	319% (Invalid)	503% (Invalid)
	1500	7.7%	4.97%	−0.1%		1500	2.41%	0.75%	−0.3%
MDA	250	5.2%	1.62%	3.1%	**NAPHYRONE (underivatised)**	250	121% (Invalid)	48% (Invalid)	568% (Invalid)
	750	3.49%	0.98%	2.2%		750	341% (Invalid)	317% (Invalid)	713% (Invalid)
	1500	2.15%	1.85%	−0.4%					
4-MEC	250	21% (Invalid)	10%	5.9%					
	750	12%	5.5%	−3.4%		1500	4.70%	1.33%	−2.0%
	1500	10%	2.61%	−2.2%					

4.3.4 LOD and LLOQ

The S/N ratios of LOD and LLOQ fell between 0.5 and 10 ng mL^{-1}, and 5 and 50 ng mL^{-1}, respectively for all examined substances; see **Table 4-4**. The detection and quantification limit results show that the method had sufficient sensitivity for the detection and quantification of the analytes in human urine samples within all examined concentrations, excluding the compounds discussed in the linearity results.

Table 4-4: *S/N* ratio for LOD and LLOQ
*LLOQ (bias) were applied to 25 replicate calibrator points. All data values in ng mL^{-1}

Compound name	LOD	LLOQ	LLOQ (bias)*	Compound name	LOD	LLOQ	LLOQ (bias)
AMPHETAMINE	0.5	10	50	PMA	1	10	100
METHAMPHETAMINE	1	10	50	MEPHEDRONE	10	50	50
CATHINONE	5	25	50	PENTEDRONE	0.5	5	50
FLEPHEDRONE	0.5	10	50	MDA	5	25	50
BUPHEDRINE	5	50	100	4-MEC	5	50	250
METHCATHINONE	0.5	5	50	PMMA	0.5	10	50
4-METHYLEPHEDRINE	5	50	50	4-EMC	1	10	50
4-M-N-E-NOREPHEDRINE	5	50	500	METHEDRONE	1	10	50
BUPHEDRONE	1	10	50	MDMA	5	50	50
N-EC	1	25	100	MDEA	10	50	50
PVP	0.5	10	100	PENTYLONE	5	25	50
METHYLONE	5	50	50	MDPPP	10	50	250
BUTYLONE	1	10	50	MDPV	5	50	250
ETHYLONE	5	50	250	NAPHYRONE	5	50	250
PYROVALERONE	5	50	250				

4.3.5 Recovery (%)

The evaluation of recoveries was conducted at three QCs within the linear range (low-250, medium-750 and high-1500 ng mL^{-1}). All substances had high recovery

between 69 and 126%, excluding MDEA (54%). The results for recoveries with RSD are presented in **Table 4-5**.

Table 4-5: Recovery results (n= 4).

Drug name	Conc. (ng mL^{-1})	Mean of recovery% (RSD)	Drug name	Conc. (ng mL^{-1})	Mean of recovery% (RSD)
AMPHETAMINE	250	83 (10)	PMMA	250	89 (7.6)
	750	78 (2.24)		750	95 (4.96)
	1500	80 (1.97)		1500	89 (1.81)
METHAMPHETAMINE	250	90 (17)	4–EMC	250	81 (6.0)
	750	87 (3.67)		750	76 (4.46)
	1500	89 (4.68)		1500	84 (0.32)
CATHINONE	250	94 (8.8)	METHEDRONE	250	93 (8.2)
	750	113 (16)		750	89 (4.83)
	1500	107 (4.62)		1500	91 (2.21)
FLEPHEDRONE	250	94 (10)	MDMA	250	87 (11)
	750	113 (7.3)		750	94 (1.34)
	1500	98 (3.14)		1500	93 (3.37)
BUPHEDRINE	250	104 (14)	MDEA	250	54 (16)
	750	120 (15)		750	112 (6.8)
	1500	109 (2.64)		1500	98 (13)
METHCATHINONE	250	90 (12)	PVP (underivatised)	250	101 (5.2)
	750	108 (8.1)		750	93 (2.74)
	1500	94 (3.84)		1500	92 (5.3)
4–METHYLEPHEDRINE metabolite	250	121 (14)	METHYLONE	250	90 (11)
	750	114 (16)		750	90 (3.65)
	1500	111 (4.11)		1500	92 (7.3)
4–MEC metabolite	250	115 (15)	BUTYLONE	250	73 (8.5)
	750	120 (16)		750	71 (1.00)
	1500	126 (7.3)		1500	69 (1.63)
BUPHEDRONE	250	95 (12)	ETHYLONE	250	93 (8.8)
	750	119 (9.3)		750	120 (8.0)
	1500	96 (4.99)		1500	94 (16)
N–EC	250	87 (16)	PYROVALERONE (underivatised)	250	120 (6.9)
	750	103 (14)		750	123 (2.12)
	1500	99 (14)		1500	117 (3.19)
PMA	250	81 (17)	PENTYLONE	250	104 (7.8)
	750	118 (13)		750	101 (4.47)
	1500	120 (4.90)		1500	99 (1.34)
MEPHEDRONE	250	107 (7.1)	MDPPP (underivatised)	250	122 (4.01)
	750	111 (14)		750	120 (5.4)
	1500	111 (2.84)		1500	115 (7.4)
PENTEDRONE	250	114 (12)	MDPV (underivatised)	250	100 (7.5)
	750	103 (15)		750	130 (3.61)
	1500	111 (1.99)		1500	109 (10)
MDA	250	106 (8.1)	NAPHYRONE (underivatised)	250	108 (4.85)
	750	99 (2.10)		750	101 (5.0)
	1500	102 (2.59)		1500	110 (9.0)
4–MEC	250	72 (6.8)			
	750	118 (6.6)			
	1500	81 (12)			

4.3.6 Selectivity and interferences

No interferences were observed using all four groups of investigated analytes in SIM mode. No matrix interferences were observed using DFU; see **Figure 4-2**.

4.3.7 Carryover

No carryover was observed using the method with the urine blank sample.

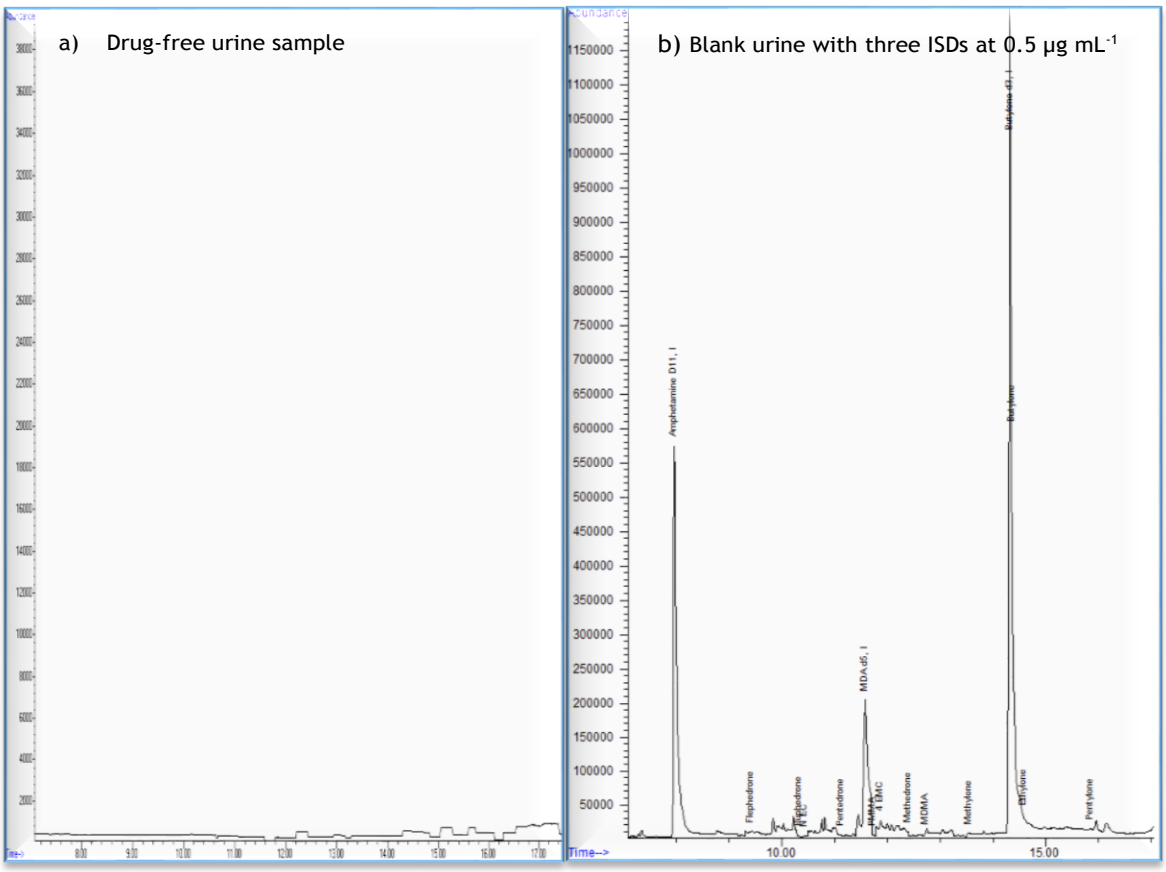


Figure 4-2: a) SIM chromatogram for DFU specimen. b) SIM chromatogram for DFU included ISD, amphetamine–d_{11}, MDA–d_5 and butylone–d_3.

4.4 Conclusion

The GC–MS method developed was valid for the simultaneous screening and quantification of 20 SC and ATS (amphetamine, methamphetamine, MDA, MDMA, MDEA, PMMA, cathinone, methcathinone, buphedrine, flephedrone, buphedrone, N–ethylcathinone, mephedrone, pentedrone, methedrone, methylone, butylone, ethylone, 4–EMC and pentylone) under SWGTOX guidelines in urine specimens. The nine drugs that were invalid were the five tertiarily amines (pyrovalerone, α–PVP, MDPPP, naphyrone and MDPV), two metabolites (4–MEC and mephedrone metabolites), PMA and 4–MEC. All examined substances were successfully extracted using SPE and reached recoveries of greater than or equate to 69%, except in the case of MDEA (recovery = 54%). No observation for endogenous or exogenous substances interfered with the target analytes. The sensitivity of the method was adequate for the detection of the mixtures concerned. The limits of quantitation were sufficient to quantify the adequate analytes specimens. Selectivity and carryover presented acceptable results. The repeatability and reproducibility of the method using accuracy and precision were satisfactory passed for the 21 inspected drugs.

Even though a large number of analytes were mixed in urine, the method achieved the acceptance criteria for the 20 drugs under the validation parameters of linearity, bias, RSD, recovery, carryover, selectivity, interferences, LOD and LLOQ. This technique provided a rigorous method for screening and confirmation purposes using GC–MS under a single procedure to examine urine samples for drug abuse testing of 20 mixture compounds in forensic toxicology laboratories. The technique that has the ability to quantify a large number of analytes in a single procedure is very important in order to meet deadlines and reduce consumable chemicals and materials.

5. Long-term stability of synthetic cathinones and amphetamine-type stimulants in urine using gas chromatography-mass spectrometry

5.1 Introduction

Forensic toxicology samples are exposed to different diverse conditions throughout transport, delivery, storage, handling and liquating between the urine collection and its analysis, or during the repetition of analysis. These conditions are different temperature, humidity, container type and light that may cause the concentration of the compounds not to reflect the real concentration. The drug stability impacts should be taken into consideration before the forensic toxicological samples analysis. The stability of ATS is extensively described in the literature, but the SC are not yet well understood. The determination of SC stability in biological matrices is needed as a result of expanding SC prevalence worldwide (refer to **sections 2.2.7** and **2.2.8**).

In chapter 4, methods were developed for the quantification of 29 substances in urine using SPE and GC–MS. The analytical methods were scientifically validated with SWGTOX (2013) for 20 ATS and SC compounds; the nine remaining drugs were also included in the stability study because the method was able to quantify the concentrations of 0.5 and 1 μg mL^{-1}. Therefore, the validated approach was used to determine the stability of SC and metabolites in human urine under three different temperatures: room temperature (RT), refrigerator (RF) and freezer (FZ) over 381 days.

To date, there are a limited number of papers that have investigated SC stability in urine. Guidelines (e.g. the Federal Workplace Drug Testing) necessitate that forensic toxicology laboratories keep all positive urine specimens for at least one year in freezing storage [463]. The long-term stability of SC in urine for up to six months have still not been reported. Additionally, SC have only been investigated for a small number of components. ATS drugs were studied in order to investigate if the SC breakdown products can interfere with the ATS by providing a false negative. The urine specimens were intended to be exposed to the ordinary light at RT. This was to match real conditions, as the samples may regularly sit on the

bench in the laboratory for several hours until examination. The samples may also sit during transportation or may be forgotten until a later time in the urine collection location or in laboratories. Drugs remain stable in the dark more so than in the light. Therefore, when a drug is stable in the light, it will also be stable in the dark. Several papers studied SC and ATS at RT in the dark. Hence, this is the first work that investigates the light condition of the selected drugs.

5.1.1 Synthetic cathinones stability

In Forensic Toxicology, the analysis of the sample in the screening method should not only inspect for target analytes but also metabolites. These products may appear because of the degradation that can happen in vivo or in vitro. The degradation products in vivo arise from enzymes, chemical processes or other physiological circumstances, such as oxidation, reduction, hydrolysis and de-esterification. The environmental conditions (in vitro) also affect the stability of substances, including pH, exposure to light, humidity, storage duration, the type of matrix, storage conditions, container type, preservatives and temperatures. After collection, samples must be stored in the laboratory based on policy. The policy typically states the conditions to prevent in vitro degradation of compounds, such as storage temperature, pH, type of tubes and preservatives. However, degradation may still occur under some cases and circumstances; for example, the specimens take several hours until the laboratories are received the samples for the analysis, and in that period of time parent or metabolite compounds may begin to break down.

Studies of NPS stability are limited or still under investigation [54, 213, 464]. Instabilities that might arise during transferring, storage, handling or analysis must be fully understood for the reliable interpretation of forensic toxicology investigations.

The breakdown of some NPS and SC in biological samples were briefly discussed by Soh and Elliott [54]. The tertiary amine of SC was more stable than the primary and secondary amine groups [459, 464]. Johnson et al. [465] studied the stability of four drugs in three matrices on days two, four, seven and 14 in whole blood,

plasma and urine for two synthetic cathinones (MDPV and mephedrone) and two piperazine-derived designer drugs (1-benzylpiperazine or BZN and 3-trifluoromethylphenyl or TFMPP). In the study, all drugs were stable in the freezer (FZ) at -20°C. Degradation of mephedrone at room temperature (RT) in the dark was initiated on day 2 in whole blood and on day 7 in urine and plasma. Mephedrone was stable in the refrigerator (RF) at 4°C in urine and plasma samples. Mephedrone in whole blood samples was unstable after seven days. MDPV was stable under all conditions and matrices over the period of the study. BZP was stable in all matrices and under all conditions examined, except the plasma sample at RT which lost 96% on day four and was undetected on day 14. TFMPP in plasma and whole blood degraded on day 4 and 7, respectively. TFMPP in urine samples was stable. See **Table 5-1**.

Table 5-1: Stability of selected cathinones

This table summarises Johnson et al. [465] paper on stability of Mephedrone, MDPV, BZP and TFMPP in urine, plasma and whole blood in human samples under three different conditions (-20, 4 and 22°C).

Name of Drug	On Day 2	On Day 4	On Day 7	On Day 14
Mephedrone	• Stable in urine and plasma samples under three different conditions • Unstable in whole blood at RT • Whole blood sample was stable at FZ and 4°C	• Stable in urine under three different conditions • Unstable in whole blood and plasma at RT • Stable in whole blood and plasma at RF and FZ	• Unstable at RT in three different matrices • Unstable in whole blood at RF • Stable in FZ in all matrices • Stable in urine and plasma at RF	• Unstable at RT in three different matrices • Stable at FZ in three different matrices • Stable in urine in RF • Unstable in whole blood and plasma at RF
MDPV	Stable	Stable	Stable	Stable
BZP	Stable	Stable except in plasma at RT	Stable except in plasma at RT	Stable except in plasma at RT
TFMPP	Stable	Stable except in plasma at RT	Stable except in plasma and whole blood at RT	Stable except plasma and whole blood at RT

Li et al. [466] studied the stability of eleven synthetic cathinones (mephedrone, buphedrone, flephedrone, 3-fluoromethcathinone (3-FMC), 3-

ethoxymethcathinone, methedrone, methylone, ethylone, butylone, MDPV and naphyrone) in equine plasma samples using LC tandem mass and liquid-liquid extraction over six months at -70°C, 30 days at -20°C, seven days at 4°C, 24 hours at 25°C and after three freeze/thaw cycles. The initial concentrations (day zero) were 0.5, 10 and 50 ng mL^{-1}. All drugs mentioned above were stable under all conditions, with the exception of flephedrone, 3-fluoromethcathinone and methedrone at RT after 24 hours.

Sorensen [213] examined the stability of many substances (cathinone, methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylone, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine) in whole blood and human liver in post-mortem samples for six days under RT and RF. This work was completed with and without preservative samples by using Venosafe tubes containing a fluoride-oxalate additive (pH 7.4) and a fluoride-citrate additive (pH 5.9). It was concluded that cathinone groups were unstable at RT after six days without preservatives, but stability improved after the acidification of the matrix. Ephedrines were stable under both conditions, because ephedrines have a hydroxyl group instead of a ketone group. However, pH played a significant role in minimising the degradation, especially when the samples were preserved under acidic conditions. The degradation was 30% on day six in cathinone, methcathinone, ethcathinone, mephedrone and flephedrone but when the whole blood samples were preserved with a fluoride-citrate buffer, the loss was reduced to 10%.

Concheiro et al. [129] studied the stability of 28 SC and metabolites at RT and 4°C for 72 hours in urine. The pH was 7.6 and liquid chromatography was coupled to high resolution mass spectrometry and solid phase cation exchange extraction (SOLA SCX). The study was completed using two concentrations (3 and 300 ng mL^{-1}). There was no preservative added to the urine. All compounds were stable after 3 freeze-thaw cycles after 72 hours at 4°C, except benzedrone and naphyrone that lost 33.3%. After 24 hours at RT, MDPPP, MDPBP, α-PVP, 4-MPBP and MDPV were stable, while the remaining 28 compounds had lost between 20% and 68%.

The stability, degradation products and pathways of mephedrone, flephedrone, 3-FMC , 2-FMC , methedrone, N-e thylcathinone (EtCAT) and N,N-dimethylcathinone (DMC) in alkaline solution were studied by Tsujikawa, Mikuma [464] using GC−MS in urine and blood samples with pH values of 4,7,10 and 12 at RT. These compounds were stable at pH 4 after 12 hours. The degradation increased as the pH was increased. The degradation was more than 80% at pH 12 for primary methcathinone compounds. Additionally, the pathways, degradation products and the effect of antioxidants were reported in this study. Antioxidants were more stable than non-antioxidant compounds [467].

A similar study was done by Togawa, Ohmori [468] at pH of 5, 7 and 9. Cathinone and methcathinone were stable at pH 5 and unstable at pH 7 and pH 9. The degradation of cathinone and methcathinone was pH dependent in urine as tested in 2001 by Paul and Cole [118]. They were the first to evaluate cathinone and methcathinone stability in urine over a three-month period. The compounds were stable at FZ temperatures, but stability only lasted 3 days at RF, and 79% was lost in 3 months for both samples. The stability of these substances at RT was not investigated. The first stability study of cathinone in plasma was done in 1989 by Morad [469]. The stability of cathinones in oral fluid samples were carried out in [470].

The stability of MDMA, 3-triflurомethylphenylpiperazine (3-TFMPP) and mephedrone were investigated at RT in post-mortem samples (tissue) after adding formaldehyde solution (5, 10 and 20% (v/v)) at three concentrations for pH values of 3.5,7 and 9.5, using HPLC with diode array detection. These samples were stored for 60 days for MDMA and 3-TFMPP and 28 days for mephedrone. Because of the formaldehyde, degradation products of N-methyl derivatives were detected, caused by the reaction of primary and secondary amine groups. These were unstable in formalin solutions and the degradation increased with increasing pH and formalin concentration. MDMA lost more in 5% formalin samples compared to 10 and 20% formalin samples, over a period of 60 days. 5% formalin samples of mephedrone and MDMA lost 82% and 25% respectively, over the period of this study. There was no degradation for 3-TFMPP after 24 hours, but 26% of the

concentration was observed on day 60. The degradation was 37% for mephedrone on day 28 and with pH 3.5 and 20% in formaldehyde [471].

The reasons for the effect of formaldehyde on the degradation product pathways at different formalin concentration of SC was briefly discussed in [472-474]. In brief, the Eschweiler–Clarke reaction occurs when secondary amine groups present in mephedrone, for example reacts in formaldehyde. The iminium hydrogenate will then form the methylated amine products, and the mephedrone continuously degrades, due to the carbonyl group stabilising the iminium ion.

Kerrigan and Glicksberg recently published three papers in 2017 evaluating the long-term stability of 22 cathinones over a period of six months using SPE and LC-Q/TOF-MS in blood and urine at pH 4 and 8. The evaluation was based on temperature (-20°C, 4°C, 20°C, and 32°C) and concentration (100 ng mL^{-1} and 1000 ng mL^{-1}). There was no significant difference observed when the concentration changed in all compounds examined. The variation in degradation for cathinones were highly dependent on target analyte, pH and temperature. The cathinones were stable in the FZ over the period of the study. Some cathinones were undetectable after 24 hours of storage. In short, under all conditions tested, unconfirmed cathinones, ring-substituted drugs, followed by methylenedioxyphenol-group were the most unstable, and the pyrrolidone-group cathinones were the most stable [475-477].

Al-Saffar et al. investigated buphedrone, mephedrone, methedrone, methylone, butylone, MDPV and naphyrone in urine [478]. The remaining drugs after a period of three months at FZ temperatures were 36.3% for buphedrone and between 62.1 and 106% for secondary amines with ring substituents and 78-96% for tertiary amines. The drugs remaining at RF temperatures were 0.9% for buphedrone, 2-15% for secondary amines with ring substituents and 85% and 30% for MDPV and naphyrone, respectively. All examined SC were undetected at RT, except MDPV which had 38% of the drug remaining.

Miller et al. investigated the stability of 10 SC (cathinone, methcathinone, naphyrone, 4-methylethcathinone, mephedrone, MDPV, PVP, buphedrone, methylone and *N*-ethylcathinone) over a period of a month in oral fluid under the

preservation using Quantisal™ and Oral-Eze™, as compared with unpreserved oral fluid samples that were stored under RT, RF (4°C) and FZ (−20°C), using ultra HPLC–MS-MS. All preserved and unpreserved samples that were kept at FZ temperatures were stable. At RT 71 to 100% of the samples were lost after one month, but at RF temperatures more than 88% was lost in unpreserved and Oral-Ez™ samples, whereas Quantisal™ oral fluid samples lost more than 34% [470].

5.1.2 Amphetamine-type stimulants stability

The long-term stability of amphetamine, methamphetamine, MDA, MDMA and other ephedrine derivatives were investigated in urine samples for two years by GC−MS. In this study, the work was carried out to evaluate sterilised and non-sterilised urine specimens under refrigerator (4°C) and frozen conditions (−20°C). No significant degradation of the substances was observed at any examined condition over the two years [479]. Amphetamine and methamphetamine were similarly stable under all examined conditions for a period of six [480] and 18 months at 4–8°C [481].

Peters et al. [482] measured the stability of amphetamine, methamphetamine, MDA, MDMA, MDEA, PMA, PMMA and other piperazine-derived designer substances in plasma. Stability was studied for three freeze/thaw cycles over three days, and no degradation was observed for any of the drugs. The samples were tested when left on the autosampler at RT for 32 hours, and no instability was observed.

Clauwaert et al. [25] reported a comprehensive stability study of MDA, MDMA and MDEA in urine, serum, water and whole blood at temperatures of −20, 4, and 20°C for more than 21 weeks. No degradation was reported for all examined analytes under the above conditions. Nevertheless, interferences from the degraded blood matrix prevented quantification of MDA and MDEA after five and 13 weeks, respectively under low concentrations at the conditions of 20 and 4°C.

Enantiomers of amphetamine, methamphetamine, MDA, MDMA and MDEA were stable in plasma over six months at frozen conditions and for the three freeze/thaw cycles [483, 484].

In the long-term storage and stability of amphetamine and methamphetamine in blood lost 38% after three years and 77% after one year, throughout the five-year study, respectively. However, RSD was greater than 30%, and this might be the reason for the loss, instead of degradation [485]. MDMA, MDA, MDEA and MBDB ((N-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine) in oral fluid within 10 weeks had lost 31% for MDA, 28% for MDMA, 38% for MDEA and 37% for MBDB [486].

5.1.3 Aim

To examine the long-term stability of the 7 ATS and 22 SC from chapter 4 including metabolites and a variety of structure compounds in human urine over 381 days when the specimens were stored at three temperatures in ordinary light: RT, RF (4°C) and dark: FZ (−20°C). The assessment of which included:

- To study the stability of RT, RF and FZ for all drugs examined in the period of 381 days at two concentrations 1 μg mL^{-1} and 0.5 μg mL^{-1}.
- To study the relative stabilities for each class.
- To investigate when SC will be totally undetectable.
- To confirm the ability of ATS to remain stable, even when SC are degraded in the urine sample.
- To assess autosampler stability over 72 hours.
- To estimate the half-lives of 14 drugs.
- To evaluate concentration and analyte dependence of selected SC.

5.2 Method and Materials

5.2.1 Chemicals and reagents

All 29 standards, three ISD (amphetamine-d11, MDA-d5 and butylone-d3), PFPA, materials and chemicals were purchased from the supplier mentioned in **sections 3.2.1.1** and **4.2.1.**

5.2.2 Sample preparation and storage

1.85 mL was taken from each drug's stock solution (100 µg mL^{-1}) and spiked into fresh DFU in order to prepare 185 mL and to achieve the final concentration of 1 µg mL^{-1}. 0.925 mL was transferred from the 100 µg mL^{-1} of each drug's stock solution and spiked to fresh DFU to obtain 185 mL for the final concentration 0.5 µg mL^{-1}. 185 µL was taken from the 100 µg mL^{-1} stock solution of each drug and spiked with fresh DFU to prepare 185 mL for the final concentration of 0.1 µg mL^{-1}. Each solution was inverted several times and shaken to make sure the spiked drug was homogeneously mixed with the drug-free urine.

Aliquots of 1 mL of urine were added to 180 individual 1.5 mL Eppendorf microcentrifuge tubes with safe-locks to obtain information on the stability of concertation at 1 µg mL^{-1}. A similar procedure was used to prepare the 0.5 and 0.1 µg mL^{-1} concentrations. A total of 540 Eppendorf tubes (1 mL of each) were prepared within three hours.

The samples were stored immediately after preparation at temperatures of RT, RF (4°C) and FZ (−20°C). The ambient (RT) and refrigerated samples were occasionally exposed to light. All spiked urine samples were exposed to light during routine laboratory work for sampling, preparation and analysis.

The experimental design is briefly described in **Figure 5-1**.

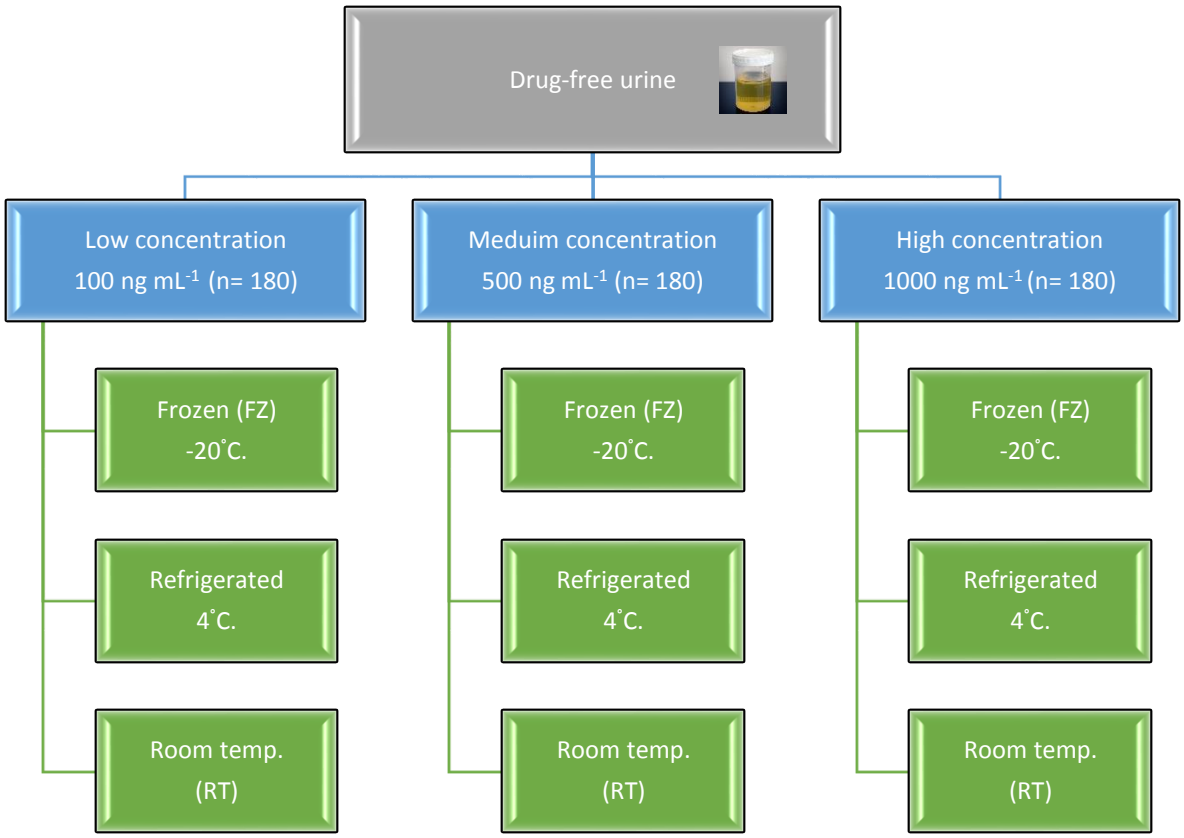


Figure 5-1: Experimental design indicating the conditions under which ATS and SC stability was investigated

5.2.3 Sample analysis

Specimens were analysed on days 0, 1, 2, 3, 7, 14, 21, 28, 42, 77, 108, 137, 172, 201, 319, 349 and 381. Testing occurred in three replicate samples in each concentration and condition with eight freshly prepared calibrator points and three QCs on each day of analysis. The calibration curves were plotted on each day with the calculation of accuracy and RSD for each concentration, QC and condition.

5.2.4 Calibrators, QCs and sample preparation procedure

Standards were freshly prepared on each day of the stability study. 100 µL was taken from each stock standard solution (100 µg mL^{-1}) to prepare a 5 mL volume

of the final concentration of 2 µg mL^{-1}. This mixture was diluted using DFU. The calibrator points were prepared using the method in chapter 4, **section 4.2.5.** QC3 and QC2 at 1 and 0.5 µg mL^{-1} were achieved by spiking 100 and 50 µL of working solution one (10 µg mL^{-1}) into 1 mL of DFU, respectively. 100 µL of working solution two (1 µg mL^{-1}) was added to 1 mL of DFU to prepare 0.1 µg mL^{-1}. All the mention were prepared daily.

5.2.5 Stability procedure

On each test day, 1 mL of calibrators at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 0.75, 1 and 2 µg mL^{-1}, QCs at 0.1, 0.5, and 1 µg mL^{-1}, RT samples at 0.1, 0.5, and 1 µg mL^{-1}, RF samples at 0.1, 0.5, and 1 µg mL^{-1}, FZ samples at 0.1, 0.5, and 1 µg mL^{-1} were added into 38 different culture tubes. The procedure from chapter 4 was then followed using SPE, PFPA and GC–MS (see **section 4.2.11** for the procedure). See **Figure 5-2** for the laboratory preparation work on each day of the stability study.

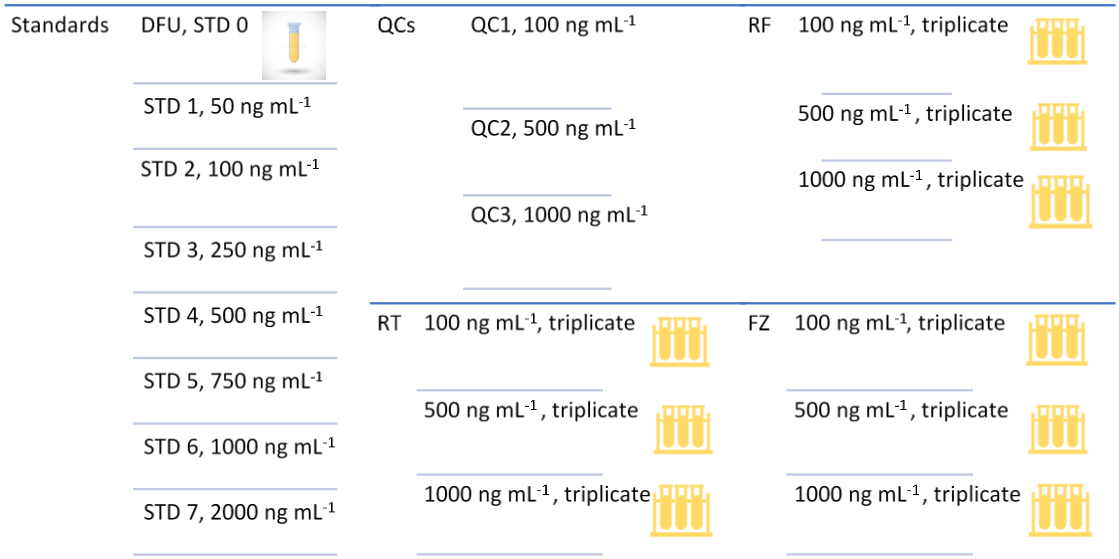


Figure 5-2: Laboratory preparation on the stability test days

5.2.6 Autosampler stability

Autosampler stability was assessed to determine if samples could be left in the autosampler for 24 hours without the target analytes concentration decreasing. It was also important to know if there were any other effects on the results if the samples stayed in the auto sampler for more than 24, 48 and 72 hours. The method lasts 25 min for one sample, and knowing how many samples can be run through the batch before the degradation initiates would be vital.

To examine the stability, the two QCs in urine were analysed at day zero for the evaluation of ATS and SC at concentrations of 500 and 1000 ng mL^{-1} in triplicate. The GC vials were at that time left on the autosampler and re-injected again after 24, 48 and 72 hours.

5.2.7 GC–MS conditions

See chapter 4, **section 4.2.15**.

5.3 Results and Discussion

Data stability was calculated using the standard design detailed by Hoffman et al. [20]. Accuracy parameter was selected to investigate stability. The studied compound was only considered unstable when its concentration was reduced by more than 20% of the initial concentration at day zero. The 20% bias was applied to avoid any confusion between the drug degradation and uncertainties. Single factor ANOVA was additionally used to determine the significant differences between tests (P = 0.05). The results in each concentration must be less than 20% of the RSD to be considered valid, otherwise it was reported as a Bad Response (B.R). The B.R can also mean that the relative ion ratios were less than 80% (Q. value) compared to the selected target ion.

In a laboratory, urine specimens must be immediately stored at FZ or RF after analysis is completed. However, urine specimens were allowed to be left at RT for 381 days for the purpose of assessing the stability. The stability study had several conditions and factors that may have affected the results, but all steps were taken to prevent this where possible.

All tertiary amine substances presented high RSD in most stability study days. Therefore, they were excluded from the analysis. The concentration of 100 ng mL^{-1} results were also excluded for the same reason.

5.3.1 Freezer stability study at −20°C

All tested drug groups were stable on all examination days, even after 381 days when the samples were stored at −20°C.

This is the first study of urine stability for cathinones over a long period of time under the stated conditions (see **Table 5-2**). The table shows that all drugs were stable on day 381, with similar stability findings on all other examined days.

Table 5-2: Freezer stability results on day 381 (n = 3)

Day number	Day 381					
Drug & Stability results	Mean	RSD	Bias	Mean	RSD	Bias
	(500 ng mL^{-1})			(1000 ng mL^{-1})		
AMPHETAMINE	421	4.08%	-16%	931	4.87%	-6.9%
METHAMPHETAMINE	488	1.27%	-2.34%	951	2.94%	-4.92%
CATHINONE	600	16%	20%	1182	8.8%	18%
FLEPHEDRONE	470	6.8%	-6.0%	1119	11%	12%
BUPHEDRINE	461	18%	-7.8%	962	7.0%	-3.79%
METHCATHINONE	458	6.2%	-8.5%	1094	12%	9.4%
4 METHYLEPHEDRINE met.	512	12%	2.34%	971	3.89%	-2.86%
4-MEC metabolite	484	14%	-3.27%	1056	4.49%	5.6%
BUPHEDRONE	431	6.8%	-14%	1054	8.4%	5.4%
N-EC	457	15%	-8.6%	990	7.3%	-1.00%
PMA	473	16%	-5.4%	912	3.72%	-8.8%
MEPHEDRONE	433	17%	-13%	984	6.4%	-1.56%
PENTEDRONE	404	12%	-19%	997	5.6%	-0.32%
MDA	504	16%	0.85%	885	3.29%	-11%
4-MEC	497	13%	-0.65%	950	9.1%	-5.0%
PMMA	536	17%	7.2%	966	3.60%	-3.45%
4-EMC	440	19%	-12%	1003	11%	0.30%
METHEDRONE	479	16%	-4.25%	923	5.2%	-7.7%
MDMA	467	18%	-6.6%	898	3.20%	-10%
MDEA	454	10%	-9.2%	878	5.1%	-12%
PVP	489	13%	-2.26%	911	4.11%	-8.9%
METHYLONE	479	17%	-4.28%	933	5.9%	-6.7%
BUTYLONE	503	17%	0.62%	965	4.66%	-3.52%
ETHYLONE	482	18%	-3.59%	904	5.1%	-9.6%
PYROVALERONE	518	16%	3.62%	911	4.30%	-8.9%
PENTYLONE	506	15%	1.17%	900	4.56%	-10%
MDPPP	512	11%	2.48%	1056	0.70%	5.6%
MDPV	526	16%	5.1%	939	4.48%	-6.1%
NAPHYRONE	498	19%	-0.39%	1048	7.8%	4.82%

5.3.2 Stability study at RT

ALL ATS drugs were stable in urine at RT from the initial day until day 349. On day 381, amphetamine and methamphetamine were stable, while the remaining ATS drug concentrations had decreased 24-44%. In general, most SC degraded after one day or two days, and the degradation was gradually increased to day 21 before being completely undetected on day 28 (see **Table 5-3** for the results).

More specifically, non-ring substitute cathinones required 24 hours (cathinone, methcathinone and N-EC), and 48 hours (buphedrone and pentedrone) to present a difference in the concentration (greater than 20%). All non-ring substitute drugs were undetected after 21 days, except for methcathinone, which only needed 14 days to entirely disappear.

Ring substituted compounds (flephedrone, mephedrone, 4-MEC, 4-EMC, methedrone) lost more than 19% concentration after 24 hours and were completely unobserved after 21 days, with the exception of methedrone which was undetected after 28 days.

Methylenedioxy-substituted substances were stable from day zero to day three for butylone and pentylone, but methylone and ethylone lost more than 38% and 40% after 24 and 48 hours, respectively at 1 μg mL^{-1}. These substances were undetected after 28 days.μ

Cathinone metabolites (buphedrone-ephedrine, 4-methylephedrine, 4-MEC metabolite) were all stable after 24 hours, except for 4-MEC metabolite which decreased by 39% after a day at 1 μg mL^{-1}. Concentrations fluctuated above the acceptable range of RSD and relative ion ratios from day 21 to 319. It was difficult to determine if this was from the breakdown of the products themselves or from the breakdown of other compound products. Pyrrolidine-type substances (tertiary amines, PVP, pyrovalerone, MDPPP, MDPV and naphyrone) achieved similarly poor responses in specimens with above 20% RSD from day 2 to 381. These drugs were underivatised and had one dominant mass spectra; the residual ions were less than 8%. Hence, the residual ions were unstable to be used as qualitative ions for

interpretation. Therefore, the stability results for this group were unknown, except for the first 24 hours when the drugs were stable.

5.3.3 Stability study at 4°C (refrigerator)

The ATS substances were stable in urine at RF over all examined days. Cathinones were only stable for 14 days excluding N-EC and the selected metabolites, which degraded after 48 hours. This group was primarily undetected after 77 days for N-EC and flephedrone, but diminished entirely after 349 days for all SC. See for **Table 5-4** the results.

Non-ring substitute cathinones were stable from day zero into day 14, except for N-EC which was stable in days zero and one, but then the degraded from day 21. By day 108, 137, 42, 172 and 77, cathinone, methcathinone, N-EC, buphedrone and pentedrone were undetectable, respectively. Ring substituted substances were stable until day 14, except flephedrone which only lasted three days. The breakdown products were observed and increased from day 21 to day 77 for mephedrone, and up to day 319 for 4-EMC and methedrone before totally disappearing. The RSD and the ion ratios of 4-MEC compound were more than 20% from day 21 to 381.

Methylenedioxy-substituted drugs were stable from day zero to day 14, but degraded from day 21 to 319, and were undetected from day 349 until the last day of the study.

SC metabolites were all stable for 24 hours before being degraded on day two, the uncertainties were above the acceptable recommendation. On most investigation days, tertiary amine substance results provided poor responses with similar errors.

The GC–MS was occasionally operated in full scan mode instead of SIM to look for breakdown products that could be identified. Unfortunately, the baseline of the GC–MS was high to catch peaks, and the ions were only determinable when the target quantitative and qualitative ions were selected and extracted.

A) Stability study at RT, 0.5 and 1 ug/mL

Group name		ATS							Non-ring substitute					Ring substituted					Methylenedioxy-substituted				Cathinone metabolites		
Days & Drugs	Condition	Amphetamine	Methamphetamine	PMA	MDA	PMMA	MDMA	MDE	Cathinone	Methcathinone	N-EC	Bupherone	Pentedrone	Flephedrone	Mephedrone	4-MEC	4-EMC	Methedrone	Methylone	Butylone	Ethylone	Pentylone	Buph-ephedrine	4-Methylephedrine	4-MEC metabolite
Day 0	R^2	1	1	0.999	0.999	0.997	0.999	0.999	0.999	1	0.998	0.999	0.999	1	0.999	0.994	0.999	0.999	0.999	1	0.998	0.999	0.999	0.998	0.998
	500 ng/mL	-4%	-4%	-3%	-7%	-2%	-4%	15%	13%	7%	16%	13%	13%	8%	14%	2%	-12%	-5%	-1%	-4%	5%	-4%	0%	10%	14%
	1000 ng/mL	0%	-4%	14%	-7%	2%	-1%	8%	4%	4%	16%	7%	13%	0%	6%	4%	-6%	-6%	-10%	-1%	6%	-1%	-8%	-5%	0%
	Stability	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST
Day 1	R^2	0.997	0.999	0.999	0.998	0.997	0.999	0.99	0.995	0.999	0.999	0.995	0.995	0.999	0.998	0.99	0.995	0.997	0.997	0.999	0.998	0.998	1	0.999	0.995
	500 ng/mL	-11%	-1%	-6%	-10%	-14%	-8%	0%	-20%	-22%	-10%	-7%	-12%	-17%	-12%	-4%	-21%	-19%	-16%	-8%	14%	-11%	-7%	-5%	-9%
	1000 ng/mL	-5%	-13%	15%	-11%	-18%	-8%	9%	-21%	-34%	-25%	-15%	-14%	-35%	-19%	-27%	-35%	-33%	-38%	-8%	-12%	-9%	-20%	-2%	-39%
	Stability	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST	ST	ST	UnST	ST	UnST	UnST	UnST	UnST	ST	ST	ST	ST	ST	UnST
Day 2	R^2	1	0.999	1	1	0.999	0.999	0.998	0.998	0.999	0.994	1	0.998	0.999	0.998	0.994	1	1	1	1	0.999	1	1	0.999	0.997
	500 ng/mL	-7%	-8%	-13%	-3%	-14%	-13%	-18%	-26%	-44%	-39%	-11%	-23%	-41%	-17%	-22%	-36%	-18%	-14%	-10%	-23%	-14%	-5%	-17%	-13%
	1000 ng/mL	-4%	-19%	-2%	-12%	-14%	-19%	B.R	-32%	-49%	-56%	-24%	-28%	-55%	-25%	-40%	-34%	-22%	-23%	-12%	-40%	-15%	-7%	-32%	-27%
	Stability	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	ST	UnST	ST	ST	UnST	UnST
Day 3	R^2	0.998	0.999	0.999	1	0.999	0.999	0.998	0.998	0.999	0.993	0.999	0.999	1	0.999	0.998	1	1	1	1	0.998	1	0.999	0.999	0.999
	500 ng/mL	1%	4%	-2%	-8%	-9%	-19%	B.R	-41%	-57%	-56%	-28%	-35%	-54%	-27%	-36%	-34%	-19%	-23%	-15%	-35%	-15%	-13%	-23%	-44%
	1000 ng/mL	2%	-18%	-7%	-10%	-17%	-16%	B.R	-53%	-69%	-71%	-42%	-50%	-71%	-43%	-42%	-43%	-24%	-30%	-18%	-43%	-19%	-15%	-26%	-49%
	Stability	ST	ST	ST	ST	ST	ST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	ST	UnST	ST	ST	UnST	UnST
Day 7	R^2	0.999	0.996	0.998	1	0.998	0.992	0.996	0.998	0.999	0.993	0.999	0.998	0.999	0.998	0.992	0.999	0.998	0.998	0.998	0.99	0.999	0.999	0.999	0.994
	500 ng/mL	-8%	-13%	-4%	-9%	B.R	B.R	B.R	-68%	-85%	-84%	-52%	-64%	-86%	-62%	-65%	-65%	-39%	-45%	-26%	-54%	-29%	-4%	-4%	-16%
	1000 ng/mL	0%	-9%	-10%	-13%	-15%	-11%	B.R	-81%	-95%	-92%	-69%	-83%	-96%	-76%	-57%	-78%	-46%	-55%	-33%	-53%	-41%	-15%	-36%	-38%
	Stability	ST	ST	ST	ST	ST	ST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	ST	UnST	UnST
Day 14	R^2	1	1	0.997	0.999	0.999	0.997	0.995	0.997	1	0.995	0.999	0.998	0.999	0.998	0.997	1	0.999	1	1	0.997	0.999	0.998	0.997	0.981
	500 ng/mL	-3%	-4%	4%	-10%	-9%	0%	0%	-90%	B.R	-44%	-76%	-87%	-95%	-83%	-63%	-86%	-59%	-68%	-39%	-58%	-48%	-20%	-41%	-40%
	1000 ng/mL	0%	-5%	-3%	-12%	2%	5%	15%	-95%	ND	-72%	-90%	-96%	ND	-93%	-72%	-95%	-74%	-82%	-51%	-68%	-63%	-18%	-30%	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	UnST	ND	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST
Day 21	R^2	0.999	0.999	0.999	0.999	0.997	0.999	0.993	1	0.998	0.998	0.999	0.999	0.999	0.998	0.997	0.997	0.999	0.999	1	0.994	0.999	0.998	0.998	0.998
	500 ng/mL	-4%	-8%	14%	-1%	B.R	13%	15%	ND	ND	ND	ND	ND	ND	ND	ND	ND	-74%	-79%	-50%	-67%	-58%	B.R	B.R	B.R
	1000 ng/mL	-1%	-12%	3%	-8%	5%	5%	8%	ND	ND	ND	ND	ND	ND	ND	ND	ND	-92%	-96%	-70%	-90%	-83%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	UnST	UnST	UnST	UnST	UnST	B.R	B.R	B.R
Day 28	R2	0.999	0.999	0.997	1	0.999	0.995	0.999	0.999	0.993	0.999	0.994	0.996	0.999	0.996	0.999	0.999	0.999	0.999	0.999	0.998	0.998	0.992	0.993	0.992
	500 ng/mL	-6%	-7%	10%	-9%	-17%	12%	6%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	13%	B.R	B.R
	1000 ng/mL	-3%	-7%	11%	-6%	14%	-5%	3%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ST	B.R	B.R
Day 42	R^2	0.998	0.997	0.994	0.995	0.998	0.998	0.993	0.994	0.997	0.967	0.995	0.998	0.998	0.998	0.995	0.997	0.998	0.999	0.998	0.991	0.999	0.998	0.993	0.996
	500 ng/mL	-14%	-9%	10%	-3%	-12%	-2%	2%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	-11%	-6%	1%	-17%	-7%	3%	9%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 77	R^2	1	0.999	0.998	1	1	0.999	0.998	0.998	0.997	0.998	0.997	0.996	0.998	0.996	0.999	0.999	0.999	1	1	1	0.999	1	0.997	0.999
	500 ng/mL	-8%	-12%	2%	-7%	-8%	9%	16%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	2%	-7%	5%	-8%	6%	7%	15%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 108	R^2	1	1	0.995	0.999	0.998	0.999	0.999	0.997	0.992	0.994	0.998	0.996	0.999	0.994	0.995	0.999	0.999	0.999	0.999	0.999	1	0.998	0.994	0.997
	500 ng/mL	-4%	16%	8%	-16%	-12%	-16%	-11%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	-3%	8%	10%	-15%	-7%	-9%	-8%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 137	R^2	0.999	0.999	0.999	0.998	0.999	0.999	0.99	0.997	0.994	0.997	0.997	0.992	0.997	0.995	1	0.999	0.995	0.993	0.999	0.991	0.998	0.995	0.998	0.997
	500 ng/mL	-15%	B.R	3%	-15%	-18%	-23%	-11%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	3%	-9%	-12%	-17%	9%	-2%	-4%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 172	R^2	1	0.999	0.983	0.992	0.999	0.997	0.979	0.999	1	0.995	1	0.992	0.992	0.999	0.983	0.962	0.997	0.996	0.995	0.964	0.992	0.994	0.999	0.98
	500 ng/mL	-10%	-1%	-15%	-3%	-12%	-7%	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	-10%	-13%	-13%	7%	-13%	-7%	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 201	R^2	1	0.997	0.992	0.998	0.993	1	0.915	0.989	0.995	0.991	0.998	0.99	1	0.981	0.992	0.996	1	1	1	0.987	0.999	0.997	0.995	0.993
	500 ng/mL	-7%	-5%	-17%	-13%	25%	-9%	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	1000 ng/mL	-6%	-11%	6%	-17%	12%	-11%	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R	B.R
Day 319	R^2	0.998	0.997	0.997	0.998	0.998	0.995	0.99	0.995	0.999	0.972	0.995	0.996	0.993	0.994	0.96	0.969	0.956	0.977	0.998	0.971	0.983	0.997	0.964	0.963
	500 ng/mL	-10%	B.R	-14%	-13%	B.R	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R
	1000 ng/mL	-4%	B.R	17%	-12%	-18%	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R
	Stability	ST	B.R	ST	ST	ST	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	B.R	B.R
Day 349	R^2	0.999	0.998	0.999	0.998	0.999	0.998	0.998	0.995	1	0.999	1	0.999	0.999	0.998	0.998	0.997	0.996	0.998	0.998	0.999	0.998	0.999	0.999	1
	500 ng/mL	-8%	-14%	-3%	B.R	2%	-1%	9%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1000 ng/mL	-15%	-16%	8%	7%	10%	8%	4%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Day 381	R^2	1	1	1	0.999	1	1	1	0.999	0.999	1	0.999	0.999	0.999	1	1	1	1	1	0.999	1	0.999	1	1	0.999
	500 ng/mL	-19%	10%	-32%	-26%	-24%	-32%	-44%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1000 ng/mL	-16%	0%	-26%	-28%	-18%	-30%	-30%	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Stability	ST	ST	UnST	UnST	UnST	UnST	UnST	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 5-3: Linearity and accuracy (bias) at RT for concentrations of 500 and 1000 ng mL^{-1} with stability condition for selected compounds. ST: the drug is stable, B.R: bad response, ND: the compound is undetected, UnST: the substance is unstable, but it can be detected. (n= 3)

A) Stability study at RF, 0.5 and 1 ug/mL

Group name		ATS							Non-ring substitute					Ring substituted						Methylenedioxy-substituted					Cathinone metabolites		
Days & Drugs	Condition	Amphetamine	Methamphetamine	PMA	MDA	PMMA	MDMA	MDE	Cathinone	Methcathinone	N-EC	Bupherone	Pentedrone	Flephedrone	Mephedrone	4-MEC	4-EMC	Methedrone	Methylone	Butylone	Ethylone	Pentylone	Buph-ephedrine	4-Methylephedrine	4-MEC metabolite
Day 0	500 ng/mL	-4%	-2%	-7%	-7%	3%	3%	-7%	12%	6%	0%	12%	14%	6%	15%	-7%	-12%	-1%	-2%	-5%	38%	-6%	1%	18%	17%
	1000 ng/mL	-1%	-8%	14%	-6%	1%	0%	7%	11%	7%	17%	11%	16%	4%	18%	-6%	-6%	-4%	-12%	0%	14%	-1%	-4%	0%	9%
	Stability	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST
Day 1	500 ng/mL	-6%	-7%	17%	-10%	-17%	-11%	-13%	-11%	-5%	-6%	-1%	-5%	-3%	-5%	-10%	-13%	-16%	-13%	-7%	5%	-8%	-11%	-15%	3%
	1000 ng/mL	-3%	-6%	1%	-11%	-15%	-3%	-3%	-11%	-6%	0%	-8%	-5%	-8%	-14%	-11%	-19%	-19%	-4%	-6%	-7%	-7%	-19%	-17%	5%
	Stability	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST
Day 2	500 ng/mL	-8%	-10%	-18%	-12%	B.R	-16%	B.R	-19%	-14%	-42%	-14%	-21%	-11%	-22%	-12%	-8%	-10%	-11%	-9%	B.R	-10%	-24%	-29%	-49%
	1000 ng/mL	-6%	-13%	-4%	-13%	-17%	-19%	B.R	-17%	-11%	-43%	-19%	-17%	-14%	-13%	-13%	10%	5%	5%	-6%	-19%	-7%	-20%	-23%	B.R
	Stability	ST	ST	ST	ST	ST	ST	B.R	ST	ST	UnST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST
Day 3	500 ng/mL	2%	-13%	-2%	-8%	B.R	-12%	B.R	-6%	-6%	-34%	-10%	-10%	3%	0%	-8%	-2%	-3%	-4%	-7%	B.R	0%	-16%	-28%	-60%
	1000 ng/mL	6%	-17%	-3%	-8%	B.R	-14%	B.R	-12%	-6%	-29%	-11%	-6%	-5%	1%	-8%	-3%	-4%	-5%	-4%	-18%	-4%	-24%	-30%	B.R
	Stability	ST	ST	ST	ST	B.R	ST	B.R	ST	ST	UnST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST
Day 7	500 ng/mL	-7%	-14%	-11%	-10%	B.R	-16%	B.R	-14%	-17%	-27%	-9%	-13%	-12%	-8%	-10%	-7%	-6%	-4%	-9%	-19%	-8%	-24%	-42%	-57%
	1000 ng/mL	1%	-18%	-11%	-10%	B.R	-7%	B.R	-15%	-20%	-72%	-11%	-15%	-21%	-14%	-10%	8%	9%	-1%	-5%	-14%	0%	-18%	-56%	B.R
	Stability	ST	ST	ST	ST	B.R	ST	B.R	ST	ST	UnST	ST	ST	UnST	ST	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST
Day 14	500 ng/mL	3%	0%	3%	-2%	-7%	-7%	-11%	-14%	-5%	-24%	-5%	-8%	-17%	-12%	-2%	-13%	-3%	-3%	-3%	-11%	-2%	B.R	B.R	B.R
	1000 ng/mL	5%	-4%	2%	-7%	1%	-1%	-2%	-17%	-14%	-24%	-9%	-11%	-26%	-12%	-7%	-12%	-3%	-4%	-2%	B.R	-4%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	ST	ST	UnST	ST	ST	UnST	ST	ST	ST	ST	ST	ST	ST	ST	B.R	B.R	B.R
Day 21	500 ng/mL	1%	B.R	7%	-5%	B.R	25%	B.R	-76%	-53%	-45%	-39%	-49%	-39%	-20%	B.R	-40%	-32%	-35%	-47%	-34%	-10%	B.R	B.R	B.R
	1000 ng/mL	3%	-18%	-3%	-2%	B.R	-9%	B.R	-70%	-36%	-83%	-44%	-53%	-65%	-59%	B.R	-51%	-34%	-55%	-24%	-81%	-24%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	B.R	ST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	B.R	B.R	B.R
Day 28	500 ng/mL	-4%	-7%	-18%	-4%	-7%	-10%	-12%	-41%	-20%	-53%	-22%	-36%	-64%	-39%	B.R	-40%	-19%	-20%	-7%	-12%	-17%	B.R	B.R	B.R
	1000 ng/mL	-1%	-8%	-13%	-7%	B.R	-11%	B.R	-51%	-17%	-61%	-28%	-40%	-70%	-42%	B.R	-43%	-21%	-26%	-7%	-21%	-16%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	B.R	UnST	UnST	UnST	ST	UnST	ST	B.R	B.R	B.R
Day 42	500 ng/mL	-12%	-8%	B.R	-17%	-11%	-19%	-13%	-49%	9%	-52%	-28%	-36%	-76%	-32%	B.R	-67%	-40%	-40%	-21%	-25%	-28%	8%	B.R	B.R
	1000 ng/mL	-9%	-15%	-14%	-15%	B.R	-15%	B.R	-61%	-27%	-75%	-54%	-66%	-88%	-59%	B.R	-76%	-54%	-58%	-30%	-53%	-38%	-33%	-17%	-24%
	Stability	ST	ST	ST	ST	ST	ST	ST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST
Day 77	500 ng/mL	-3%	2%	-13%	-4%	-6%	-11%	B.R	-78%	B.R	ND	-59%	-77%	ND	-75%	B.R	-76%	-46%	-51%	-18%	-52%	-32%	B.R	B.R	B.R
	1000 ng/mL	-1%	-5%	B.R	-8%	-5%	-10%	B.R	-85%	B.R	ND	-67%	-83%	ND	-81%	B.R	-81%	-49%	-58%	-20%	-55%	-36%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	B.R	UnST	B.R	ND	UnST	UnST	ND	UnST	B.R	UnST	UnST	UnST	UnST	UnST	UnST	B.R	B.R	B.R
Day 108	500 ng/mL	B.R	B.R	B.R	B.R	B.R	B.R	B.R	-88%	2%	ND	B.R	ND	ND	ND	B.R	B.R	B.R	B.R	B.R	B.R	B.R	B.R	B.R	B.R
	1000 ng/mL	-3%	-10%	B.R	B.R	B.R	B.R	B.R	-95%	-46%	ND	-91%	ND	ND	ND	B.R	-87%	-84%	-87%	-65%	-83%	-71%	-52%	-46%	-54%
	Stability	ST	ST	B.R	B.R	B.R	B.R	B.R	UnST	UnST	ND	UnST	ND	ND	ND	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST	UnST
Day 137	500 ng/mL	-8%	-1%	5%	-10%	B.R	10%	-2%	ND	-90%	ND	-62%	ND	ND	ND	B.R	-67%	-49%	-58%	-36%	-92%	-47%	-51%	B.R	-72%
	1000 ng/mL	-2%	-5%	-12%	-11%	11%	3%	B.R	ND	-94%	ND	-76%	ND	ND	ND	B.R	-84%	-65%	-73%	-40%	-78%	-55%	-43%	B.R	-51%
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	UnST	ND	UnST	ND	ND	ND	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	B.R	UnST
Day 172	500 ng/mL	-9%	-2%	B.R	11%	-10%	1%	B.R	ND	ND	ND	-88%	ND	ND	ND	B.R	ND	-66%	-73%	-36%	-83%	-49%	B.R	B.R	B.R
	1000 ng/mL	-4%	-7%	B.R	14%	0%	18%	5%	ND	ND	ND	-94%	ND	ND	ND	B.R	ND	-76%	-85%	-51%	-84%	-66%	B.R	B.R	B.R
	Stability	ST	ST	B.R	ST	ST	ST	ST	ND	ND	ND	UnST	ND	ND	ND	B.R	UnST	UnST	UnST	UnST	UnST	UnST	B.R	B.R	B.R
Day 201	500 ng/mL	0%	-7%	-18%	-3%	13%	-2%	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	-89%	-75%	-83%	-50%	-79%	-63%	B.R	B.R	B.R
	1000 ng/mL	1%	-14%	B.R	-19%	B.R	-5%	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	-95%	-87%	-91%	-61%	-79%	-72%	B.R	B.R	B.R
	Stability	ST	ST	ST	ST	ST	ST	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	UnST	UnST	UnST	UnST	UnST	UnST	B.R	B.R	B.R
Day 319	500 ng/mL	-1%	1%	B.R	-10%	B.R	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	-72%	-86%	-83%	-73%	B.R	-66%	-84%	B.R	B.R
	1000 ng/mL	4%	-6%	B.R	-4%	-17%	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	-86%	-91%	-91%	-75%	-85%	-90%	-91%	B.R	B.R
	Stability	ST	ST	B.R	ST	ST	B.R	B.R	ND	ND	ND	ND	ND	ND	ND	B.R	UnST	UnST	UnST	UnST	UnST	UnST	UnST	B.R	B.R
Day 349	500 ng/mL	-18%	-11%	-11%	-3%	-7%	-7%	-5%	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1000 ng/mL	-9%	-8%	9%	8%	9%	13%	13%	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND
Day 381	500 ng/mL	-13%	-2%	-12%	-3%	-9%	-9%	-10%	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1000 ng/mL	-6%	5%	-10%	-15%	-6%	-7%	-8%	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Stability	ST	ST	ST	ST	ST	ST	ST	ND	ND	ND	ND	ND	ND	ND	B.R	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 5-4: Accuracy (bias) at RF for concentrations of 500 and 1000 ng mL^{-1} with stability conditions for selected compounds.
ST: the drug is stable, B.R: Bad response, ND: the compound is undetected, UnST: the substance is unstable, but it can be detected. (n= 3)

5.3.4 Autosampler Stability

The results of autosampler stability for the 29 substances at 0.5 and 1 μg mL^{-1} on days zero through three in triplicate urine samples are shown in **Table 5-5.** All tested analytes were less than or equal the acceptable range of ±20% for the accuracy and RSD.

Therefore, the data proved that the ATS and SC with ISD are stable when left in the autosampler for at least three days after extraction. Additionally, the data showed that most SC lost more than 10% but less than 20% in concentration on day three, which indicates that these compounds could be become unstable after this day.

5.3.5 Concentration reliance

Concentration reliance was evaluated by associating the remaining percentage of the target ion at 500 ng mL^{-1} and 1000 ng mL^{-1}. One-way ANOVA was applied to compare the average concentration of each test day at each concentration and temperature. The statistical work was completed for each substance alone in each condition. No concentration reliance was observed in the stability study for any condition (P = 0.05) for all valid samples.

The ANOVA result was true for most values, but not all, because some data achieved bad response (B.R). Therefore, the concentration reliance was additionally assessed by trendline accuracy (%) results; these results show that most substances were following approximately the similar trendline for both concentrations on all stability test days with the similar degradation (see the results that were mentioned earlier on **Table 5-3** and **Table 5-4**).

Figure 5-3 and **Figure 5-4** illustrate that trendline changes are comparable for both concentrations. Overall, no significant difference was observed in the stability work between the two concentrations examined (500 ng mL^{-1} and 1000 ng mL^{-1}) using single factor ANOVA study.

Autosampler_day 0	Amphetamine	Methamphetamine	Cathinone	Flephedrone	Buph-ephedrine metabolite	Methcathinone	4-Methyl ephedrine metabolite	4-M-N-E-norephedrine	Buphedrone	N-Ethyle cathinone	PMA	Mephedrone	Pentedrone	MDA	4-MEC	PMMA	4-EMC	Methedrone	MDMA	MDE	Methylone	Butylone	Ethylone	Pentylone
R^2	1	1	0.999	0.999	1	0.999	1	0.999	0.999	1	1	1	0.999	0.999	1	1	1	1	1	1	1	0.999	1	0.999
500 ng/mL	438	481	652	506	468	479	471	495	454	476	425	444	431	446	422	439	444	429	408	415	426	453	421	420
500 ng/mL	404	493	490	444	374	426	482	412	398	380	436	352	350	469	534	548	353	441	427	440	439	452	442	533
500 ng/mL	422	490	657	460	541	468	582	544	443	516	558	502	432	598	534	621	522	567	566	507	571	604	583	565
The average	421	488	600	470	461	458	512	484	431	457	473	433	404	504	497	536	440	479	467	454	479	503	482	506
SD	17	6	95	32	84	28	61	66	29	70	74	76	47	82	65	92	84	76	86	47	80	88	88	76
RSD	4.1%	1.3%	15.8%	6.8%	18.2%	6.2%	11.9%	13.7%	6.8%	15.2%	15.7%	17.5%	11.6%	16.2%	13.0%	17.2%	19.2%	15.9%	18.4%	10.5%	16.7%	17.4%	18.3%	15.0%
Accuracy	-15.7%	-2.3%	19.9%	-6.0%	-7.8%	-8.5%	2.3%	-3.3%	-13.7%	-8.6%	-5.4%	-13.4%	-19.2%	0.8%	-0.6%	7.2%	-12.0%	-4.3%	-6.6%	-9.2%	-4.3%	0.6%	-3.6%	1.2%
1000 ng/mL	941	924	1209	1149	995	1121	979	1063	1058	966	910	987	985	872	939	957	1025	913	875	840	923	957	886	888
1000 ng/mL	882	948	1067	980	884	955	931	1006	963	933	880	920	948	865	870	936	885	882	889	867	885	924	869	866
1000 ng/mL	971	980	1270	1229	1007	1205	1005	1100	1141	1071	947	1046	1058	919	1042	1004	1099	976	930	927	993	1013	956	945
The average	931	951	1182	1119	962	1094	971	1056	1054	990	912	984	997	885	950	966	1003	923	898	878	933	965	904	900
SD	45	28	104	127	68	127	38	47	89	72	34	63	56	29	87	35	109	48	29	45	55	45	46	41
RSD	4.9%	2.9%	8.8%	11.4%	7.0%	11.6%	3.9%	4.5%	8.4%	7.3%	3.7%	6.4%	5.6%	3.3%	9.1%	3.6%	10.8%	5.2%	3.2%	5.1%	5.9%	4.7%	5.1%	4.6%
Accuracy	-6.9%	-4.9%	18.2%	11.9%	-3.8%	9.4%	-2.9%	5.6%	5.4%	-1.0%	-8.8%	-1.6%	-0.3%	-11.5%	-5.0%	-3.4%	0.3%	-7.7%	-10.2%	-12.2%	-6.7%	-3.5%	-9.6%	-10.0%
Autosampler_24h																								
500 ng/mL	441	487	544	505	480	485	507	561	473	474	422	426	464	449	430	463	467	432	424	426	432	476	435	434
500 ng/mL	407	523	563	440	386	422	509	462	405	373	433	335	362	458	440	465	348	441	434	450	442	474	434	442
500 ng/mL	426	502	622	458	546	466	614	590	448	501	556	492	465	586	549	575	516	566	579	623	579	640	589	576
The average	424	504	576	468	470	458	543	538	442	449	470	418	430	498	473	501	444	480	479	500	484	530	486	484
SD	17	18	41	34	80	32	62	67	35	67	74	79	59	76	66	64	86	75	87	107	82	96	89	80
RSD	4.0%	3.5%	7.0%	7.2%	17.1%	7.0%	11.3%	12.4%	7.8%	14.9%	15.8%	18.9%	13.7%	15.4%	13.9%	12.8%	19.5%	15.7%	18.2%	21.5%	17.0%	18.0%	18.3%	16.5%
Accuracy	-15.1%	0.8%	15.3%	-6.5%	-5.9%	-8.5%	8.6%	7.5%	-11.6%	-10.1%	-5.9%	-16.5%	-13.9%	-0.5%	-5.3%	0.2%	-11.2%	-4.1%	-4.2%	-0.1%	-3.1%	6.0%	-2.8%	-3.2%
1000 ng/mL	938	924	1186	1153	1004	1190	1000	1085	1080	1023	884	940	998	845	929	933	930	895	876	842	897	992	867	877
1000 ng/mL	889	974	1168	987	893	937	964	1041	1005	907	873	890	982	859	903	920	883	891	903	892	903	994	900	885
1000 ng/mL	966	983	1137	1243	1013	1308	1016	1115	1166	1100	941	1017	1075	912	1010	984	1001	968	940	942	986	1066	974	957
The average	931	960	1164	1128	970	1145	993	1080	1084	1010	900	949	1019	872	948	946	938	918	906	892	929	1017	914	906
SD	39	32	25	130	67	189	27	37	81	97	37	64	50	35	56	34	59	43	32	50	49	42	55	44
RSD	4.2%	3.3%	2.1%	11.5%	6.9%	16.5%	2.7%	3.4%	7.5%	9.6%	4.1%	6.8%	4.9%	4.0%	5.9%	3.6%	6.3%	4.7%	3.5%	5.6%	5.3%	4.1%	6.0%	4.9%
Accuracy	-6.9%	-4.0%	16.4%	12.8%	-3.0%	14.5%	-0.7%	8.0%	8.4%	1.0%	-10.0%	-5.1%	1.9%	-12.8%	-5.2%	-5.4%	-6.2%	-8.2%	-9.4%	-10.8%	-7.1%	1.7%	-8.6%	-9.4%
Autosampler_48h																								
500 ng/mL	439	487	636	512	479	499	499	554	464	454	414	419	450	442	400	444	430	421	416	425	426	493	416	422
500 ng/mL	405	521	577	442	382	435	500	453	396	372	424	330	380	454	444	451	438	431	431	446	438	487	443	432
500 ng/mL	434	516	551	468	553	457	607	578	446	506	554	481	460	591	574	570	516	564	586	534	581	667	498	574
The average	426	508	588	474	471	464	535	528	435	444	464	410	430	495	472	488	461	472	478	468	482	549	452	476
SD	18	18	43	35	86	32	62	66	36	68	79	76	44	83	91	71	47	80	94	58	86	102	42	85
RSD	4.2%	3.6%	7.4%	7.5%	18.2%	7.0%	11.6%	12.5%	8.2%	15.3%	16.9%	18.6%	10.2%	16.7%	19.2%	14.5%	10.3%	16.8%	19.6%	12.3%	17.8%	18.6%	9.3%	17.8%
Accuracy	-14.9%	1.6%	17.6%	-5.2%	-5.8%	-7.3%	7.1%	5.7%	-12.9%	-11.2%	-7.2%	-18.0%	-14.0%	-0.9%	-5.5%	-2.3%	-7.8%	-5.6%	-4.5%	-6.4%	-3.6%	9.8%	-9.5%	-4.8%
1000 ng/mL	960	985	1061	1265	1019	1231	990	1077	1154	1055	927	977	1102	917	942	957	980	948	942	947	968	1063	945	939
1000 ng/mL	885	947	1087	1008	898	973	945	1008	1000	894	859	849	954	874	851	912	864	873	900	907	888	998	885	872
1000 ng/mL	913	908	1092	1157	994	1102	948	1016	1044	917	854	889	1012	869	882	890	905	868	865	843	876	989	844	849
The average	919	947	1080	1143	970	1102	961	1034	1066	956	880	905	1022	886	892	920	916	896	902	899	911	1017	891	887
SD	38	39	17	129	64	129	25	38	80	87	41	66	75	26	46	34	59	45	39	52	50	40	51	47
RSD	4.2%	4.1%	1.6%	11.3%	6.6%	11.7%	2.7%	3.7%	7.5%	9.1%	4.6%	7.2%	7.3%	3.0%	5.2%	3.7%	6.4%	5.0%	4.3%	5.8%	5.5%	3.9%	5.7%	5.3%
Accuracy	-8.1%	-5.3%	8.0%	14.3%	-3.0%	10.2%	-3.9%	3.4%	6.6%	-4.4%	-12.0%	-9.5%	2.2%	-11.4%	-10.8%	-8.0%	-8.4%	-10.4%	-9.8%	-10.1%	-8.9%	1.7%	-10.9%	-11.3%
Autosampler_72h																								
500 ng/mL	414	474	601	455	525	424	554	518	422	470	518	442	442	565	499	468	487	532	484	525	546	555	472	542
500 ng/mL	473	455	591	412	355	392	461	408	359	412	397	397	343	420	397	420	415	403	412	430	410	463	408	407
500 ng/mL	409	456	562	480	447	453	452	507	421	382	383	379	428	412	379	415	403	387	395	405	395	465	385	392
The average	432	461	585	449	442	423	489	477	400	421	433	406	404	466	425	434	435	441	430	453	450	494	422	447
SD	36	11	20	34	85	30	57	61	36	44	74	32	54	86	65	29	46	79	47	63	83	52	45	83
RSD	8.3%	2.3%	3.5%	7.7%	19.3%	7.2%	11.6%	12.7%	8.9%	10.5%	17.2%	8.0%	13.2%	18.5%	15.2%	6.6%	10.5%	18.0%	11.0%	14.0%	18.4%	10.6%	10.7%	18.5%
Accuracy	-13.6%	-7.7%	17.0%	-10.2%	-11.6%	-15.4%	-2.2%	-4.5%	-19.9%	-15.7%	-13.4%	-18.8%	-19.2%	-6.9%	-15.0%	-13.1%	-13.0%	-11.9%	-14.0%	-9.3%	-9.9%	-1.2%	-15.7%	-10.6%
1000 ng/mL	919	929	1015	1207	972	1094	903	972	1066	907	883	899	1080	883	961	912	926	892	915	923	902	1041	902	877
1000 ng/mL	845	877	1048	975	852	864	862	906	919	774	822	784	941	857	852	857	817	817	894	884	819	969	831	812
1000 ng/mL	843	837	1093	1074	934	939	839	890	940	799	794	791	1012	796	860	825	818	785	819	795	786	918	778	770
The average	869	881	1052	1085	919	966	868	923	975	827	833	825	1011	845	891	864	853	831	876	867	836	976	837	820
SD	43	46	39	117	61	117	32	43	80	71	45	65	70	45	61	44	63	55	50	66	60	62	62	54
RSD	5.0%	5.2%	3.8%	10.7%	6.6%	12.2%	3.7%	4.7%	8.2%	8.6%	5.4%	7.8%	6.9%	5.3%	6.9%	5.1%	7.3%	6.6%	5.8%	7.6%	7.2%	6.3%	7.4%	6.6%
Accuracy	-13.1%	-11.9%	5.2%	8.5%	-8.1%	-3.4%	-13.2%	-7.7%	-2.5%	-17.3%	-16.7%	-17.5%	1.1%	-15.5%	-10.9%	-13.6%	-14.7%	-16.9%	-12.4%	-13.3%	-16.4%	-2.4%	-16.3%	-18.0%

Table 5-5: Autosampler stability study. Triplicate urine samples in each concentration of 0.5 and 1 µg mL^{-1} repeated after 24, 48 and 72 hours with calculation of R^2, mean (n= 3), SD, RSD and accuracy (bias).

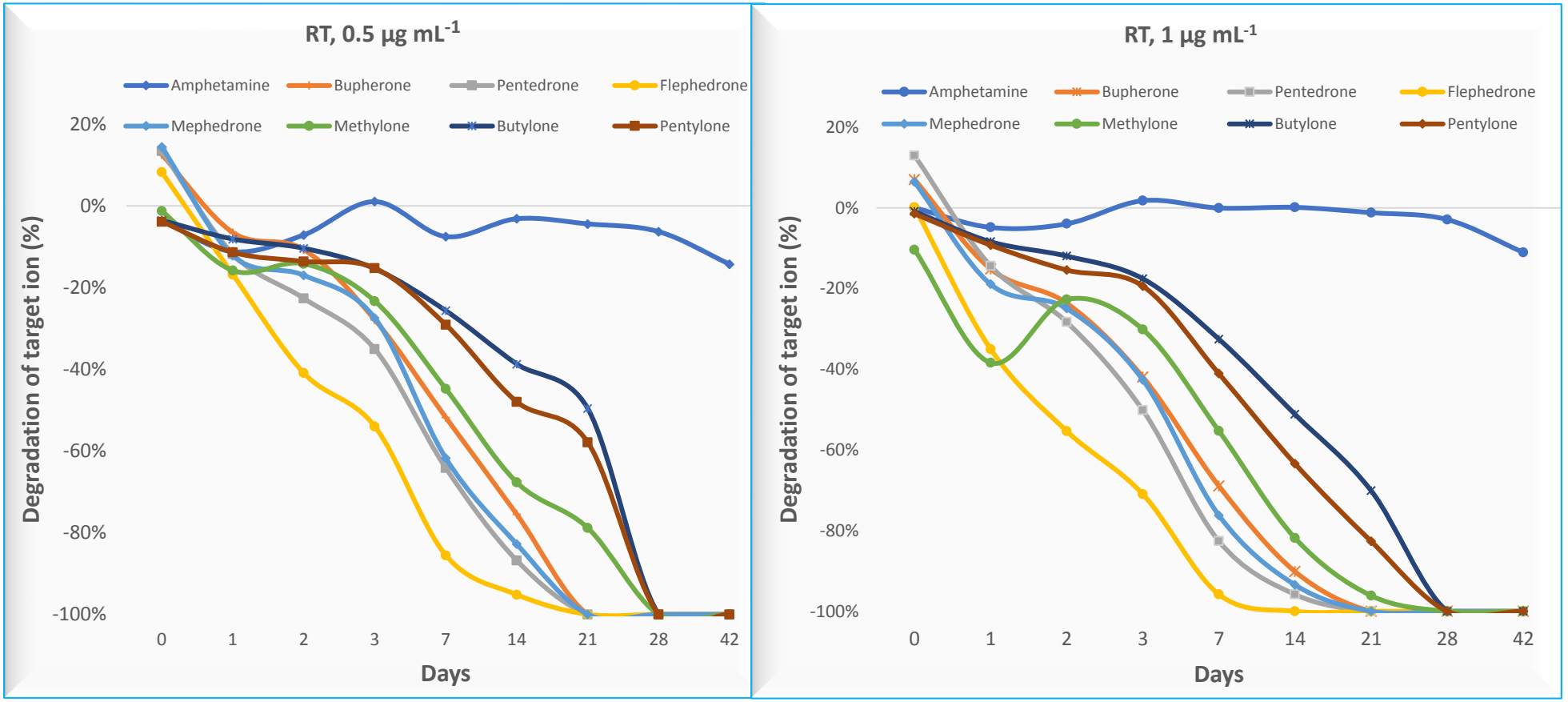


Figure 5-3: The degradation of selected ATS and SC at 500 and 1000 ng mL^{-1} at RT (n=3)

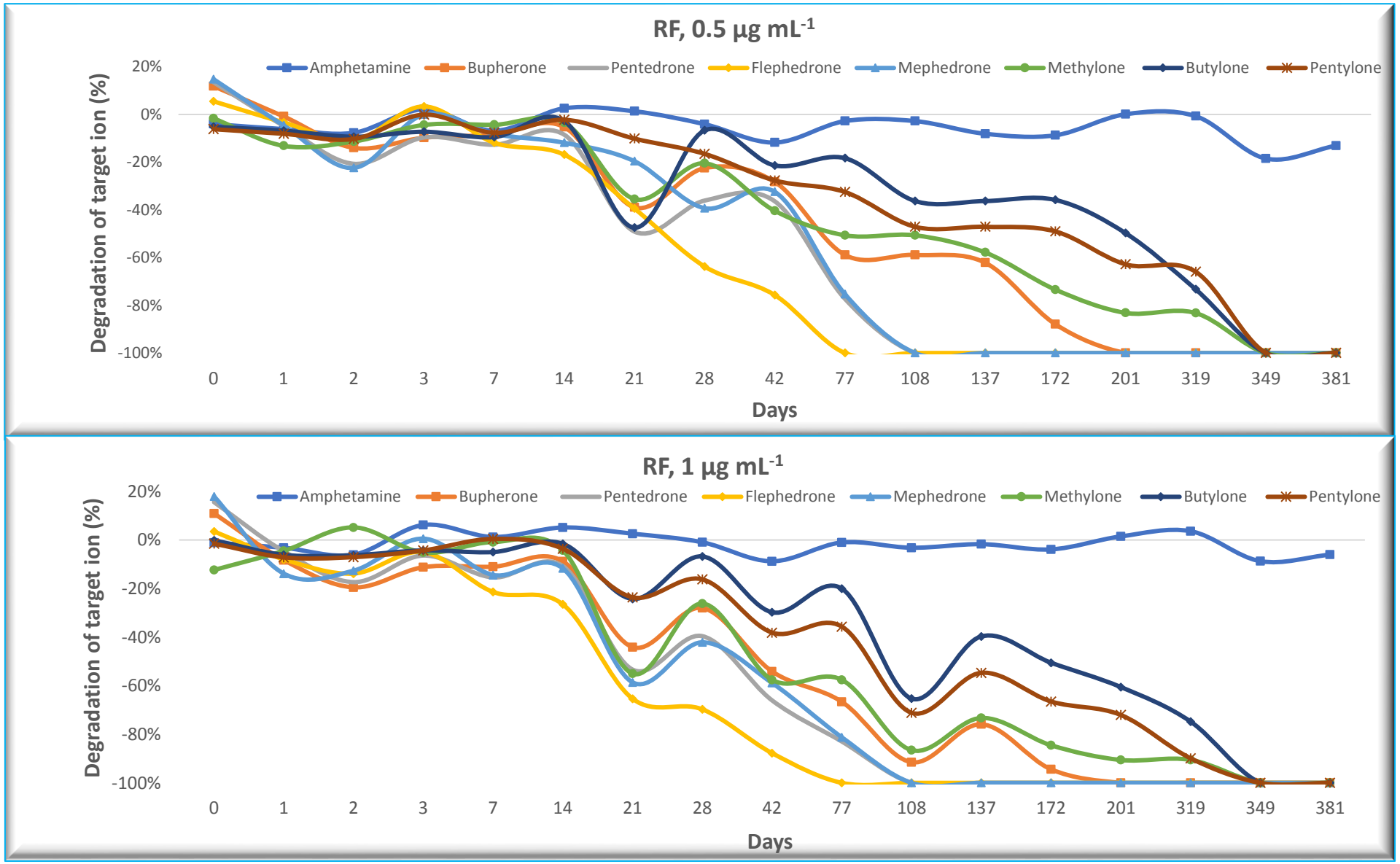


Figure 5-4: The degradation example for selected ATS and SC at 500 ng mL^{-1} and 1000 ng mL^{-1} at RF (n=3)

5.3.6 Half-life estimation

Half-lives of each unstable drug were estimated in each condition based on the concentration average of triplicate measurements at a specific time using the following equation:

Equation 5-1: Half-life equation

$$t_{1/2} = \frac{Ln2}{\gamma}$$

where Ln2 = 0.693, γ is the constant rate (γ = ln (the concentration of analyte after specific time) − ln (the initial concentration) ÷ (− the specific time)), $t_{1/2}$ is the half-life. For example, the initial concentration for the cathinone compound was 500 ng mL^{-1} (true value), the concentration of cathinone at RT after 14 days was 51 ng mL^{-1}, and the calculation can be applied as follows:

$$\gamma = In\,(51) - In\,(500)\ \div (-14) = 0.163$$

$$\text{So } t_{1/2} = \frac{0.693}{0.163} = 4.24\ days.$$

The results are shown in **Table 5-6**. The half-lives of the 14 cathinones demonstrate the significant differences between ambient temperature and refrigerator conditions, as well as the analyte and concentration-dependent variables. In this study the metabolites and tertiary amines groups were rejected, due to reasons stated in the previous sections.

The half-lives at RT for non-ring substitute, ring substituted and methylenedioxy-substituted cathinones in urine ranged from 1.78 to 6.9, 1.50 to 11 and 4.62 to 17 days respectively; and at RF 19 to 56, 16 to 39 (not including methedrone) and 94 to 169, respectively. The 4-MEC results were not known, due to high variation errors.

It should be noted that the constant rate had substantial estimation variation between the stability days, and little variation within stability days (RSD≤20%). In addition, the analyte dependence was determined by referencing the above

graphs and tables. As a result, it can be concluded that the uncertainties originated from several issues rather than instrument error. Consequently, the large variation errors in the constant rate were due to the effects of several factors such as light, pH, store condition and pre-sample preparation.

The samples were left covered at RT or RF but were not completely in the dark. The lights in the room were switched on and off on different occasions; this was similarly true for RF, as the refrigerator door was intermittently opened by other students. This procedure was designed to emulate real laboratory conditions.

Even though several studies proved that SC were significantly more stable in acidic urine [17, 21-23, 26, 30], this project was proposed to examine human urine samples at an average of healthy people pH (4.5-8).

The store condition also impacted such as humidity and temperature changes. The temperature in the room and refrigerator slightly fluctuated throughout the day. The interval time for sampling was slightly different for each stability day. Hence, these factors may contribute to increasing error variation in the constant rate.

5.3.7 Interferences and breakdown products

The products that resulted from SC degradation did not interfere the reliability of the quantification of the analytes, except for tertiary amines and metabolites. The breakdown products may be the reason for the increasing statistical variability of these compounds. The project was carried out using a mixture rather than each drug alone, and it was difficult to study the breakdown products in scan mode.

The scan mode was inadequate for interpreting the behaviour of the product, due to the complexity of the mixture and the background noise. No new endogenous interferences in blank urine were observed over the period of the study under the described conditions.

Table 5-6: Half-life estimation of 14 cathinones in urine in days (d) at RT and RF

Synthetic cathinones	RT		RF	
	500 ng mL^{-1}	1000 ng mL^{-1}	500 ng mL^{-1}	1000 ng mL^{-1}
CATHINONE	4.24 d	3.25 d	36 d	28 d
METHCATHINONE	2.56 d	1.78 d	19 d	21 d
N-EC	2.67 d	1.93	24 d	21 d
BUPHEDRONE	6.9 d	4.23 d	56 d	43 d
PENTEDRONE	4.78 d	3.03 d	36 d	30 d
FLEPHEDRONE	2.48 d	1.50 d	20 d	16 d
MEPHEDRONE	5.5 d	3.56 d	39 d	33 d
4-MEC	9.2 d	7.7 d	Not known	Not known
4-EMC	4.95 d	3.24 d	27 d	38 d
METHEDRONE	11 d	5.8 d	113 d	92 d
METHYLONE	9.1 d	4.62	119 d	94 d
BUTYLONE	23 d	12 d	169 d	161 d
ETHYLONE	13 d	6.4 d	89 d	116 d
PENTYLONE	17 d	7.1 d	129 d	109 d

5.4 Conclusion

Urine specimens must be protected from heat promptly after being collected and kept, where possible, under freezing temperatures. Otherwise, cathinones may gradually degrade until lost. ATS and SC in urine were assessed to measure decrease half-life, concentration, group and temperature stability. Long term stability lasted 381 days without degradation at −20°C was shown for ATS and SC. The ATS group was also stable at RF and RT throughout 349 days.

Most SC at RT had decreased concentrations of more than 20% after 24 or 48 hours, before completely disappearing in less than a month. Most SC at RF were unstable on day 21, after which the concentration of each drug was gradually decreased until undetected between the days 77 and 349. The last day (381) of the stability study was completed to confirm that all SC were undetected at RF.

The autosampler study has proven the stability of ATS and SC within 72 hours. The stability variations between and within SC groups were slightly different, particularly at RF. No significant difference in the results was noted for the concentrations of 500 ng mL^{-1} and 1000 ng mL^{-1}. No concentration-dependent variations were observed for all unstable SC drugs using one-way analysis of variance. Half-lives in 14 SC were estimated for each drug alone and within the groups. The analyte dependence was clearly observed for all examined cathinones using the half-life equation. The factors affecting the uncertainties were briefly discussed. In this project, human drug-free urine was used rather than commercial DFU, which had many advantages. Stability results for the metabolites and tertiary groups were rejected due to the reasons discussed above.

Urine positive samples are typically stored in the Freezer for at least one year. These tests might be repeated due to legal requirements, and this is the first work proving the stability of all examined substances in urine without the necessity for preservations. Additionally, this is the first work that demonstrates that ATS substances do not interfere with the breakdown products of SC under all studied conditions in urine specimens over a year.

6. Determination of amphetamine-type stimulants and synthetic cathinones in urine using solid phase microextraction in tips and gas chromatography-mass spectrometry

6.1 Introduction

SPME was developed in 1989 as an extraction method at the University of Waterloo (Ontario, Canada) with a number of advantages compared to traditional sampling techniques [487], including a reduced volume of sample, a shorter preparation time, a lower solvent volume required and an increased LOD [488]. When SPME was first discovered, coatings were mostly applied to natural and hydrophobic substances [289, 489]. Currently, innovative coatings have been developed to extract additional polar analytes with charged particles and molecules [490, 491]. The application of SPME is published on a consistent basis, which covers a wide range of coatings. The recent techniques involving sorbents and coatings are chemical grafting, sol-gel technology, electrospinning, liquid-phase deposition, hydrothermal methods, dipping and physical agglutinating and electrochemical methods [492].

The unique feature of SMPE is the ability of the system to yield similar quantities even when the sampling is repeated. This is because the reaction occurs based on equilibrium extraction with a small quantity of analytes (named negligible depletion) between the analyte and stationary phase. This is the ideal characteristic for identification of forensic toxicology drugs in matrices; by repeating every time without reducing the concentration of analytes [493-495].

At the present time, SPME has confirmed the validity of various bioanalytical and forensic toxicology examinations, involving in vivo and in vitro sampling for a number of diverse forensic matrix investigations [493, 496]. The comparison between the SPME in tips with other extraction methods is illustrated in **Table 6-1**.

Table 6-1: Comparison of SPME tips, SPME-TFME, SPE, SPME-traditional fibre and LLE

Extraction type & feature	SPME-tips [497]	SPME-TFME [497]	SPME-traditional fibre	LLE	SPE
Steps	Pre-treatment, two steps, extraction and desorption	Two steps, extraction and desorption	Pre-treatment, dilution for complex sample	multi-stage operations, time-consuming, labour-intensive	multi-stage operations, time-consuming, clotting, percolation
Volume	Small sample volume	Large sample volume to improve sensitivity	Large sample volume to improve sensitivity	Large sample volume to improve sensitivity	Large sample volume to improve sensitivity
Cost	Disposable, Lower cost per sample	Reusable, expensive	Reusable, expensive, fragile	Waste disposal of solvents	Disposable, waste disposal of solvents
Handling	Pipettor, robotic liquid handling system	Require dedicated commercial TFME robotic station	Manual and Automated	Manual	Manual and Automated
Theory	Non-exhaustive system	Non-exhaustive system	Non-exhaustive system	Exhaustive system	Exhaustive system
Sampling repetitions	The sampling can be repeated	The sampling can be repeated	Remeasure the same sample	No	No

Abbreviations: SPME-tips (solid-phase microextraction in tips), SPME-TFME (thin-film microextraction), SPE (solid-phase extraction), LLE (liquid-liquid extraction).

6.1.1 General principle of SPME

This work will focus on a liquid matrix and polymer coating fibres with a direct immersion mode, because only those were included in the thesis. An understanding of SPME theory provides direction for evolving and optimising this method [489, 498].

Extraction equilibrium happens between the sample and the fibre coating. The equilibrium reaction occurs during the adsorption and desorption of analyte by the fibre polymer coating with the consequent increase or decrease of the concentration depending on, for example the material used and the thickness of the fibre coating. The time required for the extraction is based on the distribution

coefficient. The higher the distribution coefficient ratio, the better extraction for the analytes with higher precision results. SPME extraction is considered complete when extraction equilibrium is reached between the analyte in the matrix and the fibre coating, which means that further extraction time will not increase the amount of analyte extracted.

The distribution equilibrium ratio is reached faster when the M.W and bp. of the analyte are high. Selectivity can be achieved by altering the type of polymer or the thickness of the coating to be compatible with the properties of the target analyte. Volatile substances match well with a thicker coating, but semi-volatile compounds have a preference for a thin coating polymer. The amount of analyte distributed between the coating fibre (for example PDMS) and the matrix (for example urine) during the extraction stage is dictated by **Equation 6-1**:

Equation 6-1: The amount of analyte distributed between PDMS and urine

$$K_{fs} = (C_{fibre} \div C_{sample})$$

where K_{fs} is the distribution constant between the fibre coating and the sample, and C is the equilibrium concentration of the analyte in both the fibre and the sample. It can be concluded from the equation that the distribution constant (K_{fs}) can be affected by temperature, pH, salts, ionic strength, coating type, solvents and agitation speed.

The final concentration of analyte increases after the extraction processes by increasing the volume of the sample and coating, see **Equation 6-2**:

Equation 6-2: The final amount (mass) of analyte adsorbed by coating (n)

$$n = (K_{fs}\, V_f\, V_s\, C_i) \div (K_{fs}\, V_f + V_s)$$

where V_f = volume of fibre coating, V_s = volume of sample and C_i = initial concentration.

The above equation indicates that a directly proportional association (linear relationship) between the amount of analyte extracted and the initial concentration of analyte after equilibrium is reached in the matrix.

When the sample volume is very large, thermodynamic theory can be used to estimate the extraction amount of analyte by using the following **Equation 6-3**:

Equation 6-3: Thermodynamic theory

$$n = K_{fs} V_e C_s$$

where V_e = volume of extraction and C_s = concentration of analyte. [489, 498, 499]

6.1.2 Literature review for ATS and SC applied to SPME fibre in tips

Numerous studies have been published addressing the practicality of SPME for the examination of amphetamine and related substances in a biological matrix in [500-509], and more recently in [510-515]. The compounds were specifically determined in urine coupled with SPME-GC–MS [506, 516-524]. The application of SPME for SC were reviewed in **section 2.5.4**. However, the literature shows that the new trends of SPME fibre tips are very limited. Google Scholar, PubMed and Science Direct were used to search the following terms: “solid phase microextraction” and “tip”, “tips”, “in tip”, “in tips”, “pipette tips”, “well”, “well plate”, “96 well plate” or “pipette in tips”. Twenty relevant articles were identified based on the above key words (see **Table 6-2**). Most of the coating fibres were manufactured in-house and were used in tip with a syringe. A comprehensive review for the multi–well–plate format of SPME tips was published elsewhere [497].

Table 6-2: General application of SPME in tips

Name of drugs	Matrix	Fibre coating type	Instrumentation name	Year	Ref.
Iminodiacetic acid	Natural water	Monolithic chelating	ICP-MS	2010	[525]
Pesticide residues	Cucumber	Polytetrafluoroethylene (PTFE)	GC–MS	2012	[526]
A peroxisome proliferator activated receptor modulator drug compound	Human plasma	PDMS-DVB	SPME automation (96-well) coupled with HPLC–MS-MS	2014	[527]
Ultra-trace perfluorinated compounds	Whole blood, water and milk	Wooden-tip	Ambient mass spectrometry	2014	[528]
Enkephalins	Human cerebrospinal fluid	Imprinted polymer	HPLC–ultraviolet (UV)	2015	[529]
Benzoylecgonine and cocaethylene, norfentanyl, and methadone and its metabolite EDDP (2-ethylidene-1, 5-dimethyl-3, 3-diphenylpyrrolidine)	Urine	SPME LC tips (C18)	LC-MS-MS (96-well array-automation)	2015	[530]
Copper	Serum	Carbon cloth	Micro sampling flame atomic absorption spectrometry	2015	[531]
MDPV, buphedrone, flephedrone, butylone, ethylone, mephedrone, methylone and methedrone	Serum and plasma	SPME LC tips (C18)	LC-MS (96-well array-automation)	2015	[532]
Cadmium	Water and WB	Modified magnetic nanoparticles of iron oxide Triton X114	Flame atomic absorption technique	2015	[533]
Metoprolol, propranolol, carbamazepine and diazepam	Dried blood	SPME LC tips (C18)	LC-MS-MS (96-well array-automation)	2016	[534]

Arsenic speciation	Water	Polymer, polystyrene polydimethyl siloxane	Electrothermal atomic absorption spectroscopy (ET-AAS)	2016	[535]
Mefenamic acid	Urine	A carbon nanotube–zinc sulfide	HPLC	2016	[536]
Dithizone–mercury	Water	Carbon xerogel	A Shimadzu UV–vis spectrophotometer	2016	[537]
Gallic acid	Orange juice samples	Molecularly imprinted silica monolithic	HPLC analysis	2017	[538]
Alkaloids flavonoids	Urine, feces and cell culture fluid samples	Sulfonated carbon nanotube-polymer	Scanning electron microscopy (SEM) images	2017	[539]
Silver-APDC (ammonium pyrollidine dithiocarbamate)	Fresh and waste water samples	Carbon cloth	Electrothermal atomic absorption spectroscopy (ET-AAS)	2017	[540]
Bisphenol A	Urine	Molecularly imprinted polymers	GC–MS	2017	[541]
Vanadium species	Water and food samples	Immobilized with tetraethylenepentamine	Atomic absorption spectrometer	2018	[542]
Inorganic antimony (Sb)	Environmental and food samples	Polystyrene oleic acid imidazole polymer	Atomic absorption spectrometer	2018	[543]
Antidepressants	Urine	Poly (ethylene dimethacrylate)	HP 1100 liquid chromatograph	2018	[544]

6.1.3 Aims

Because of an increased prevalence in the misuse of ATS and cathinones in specific geographical areas, more methods must be developed to provide quantification and green analytical chemistry, while adhering to the validation guidelines for obtaining a new forensic toxicology sample investigation technique. In addition, no previous work was found on SPME in tips to extract ATS and SC drugs.

Therefore, the aims of the study are as follows:

- To develop and validate a method for the simultaneous detection and quantification of amphetamine, methamphetamine, PMA, MDMA, mephedrone, buphedrone ephedrine metabolite, 4-methylephedrine (mephedrone metabolite) and pentylone in a human urine specimen, using SPME in tips followed by GC–MS analysis.

- To apply a clean, simple, fast, convenient, cheap, sensitive, selective sample preparation method with a microlitre scale while considering the need to reduce solvents, chemicals, reagents, waste, energy and environment impacts using SPME in tips and adhering to guidelines for method validation.

- To assess the new SPME pipette tips with fibres of C18, C18/SCX and PDMS/DVB.

- To compare this method with the SPE method discussed in chapter 4.

- To evaluate the method using five criteria (environment, safety, energy, health and waste) for the assessment of green analytical chemistry.

- To apply this method for quantification of real human urine samples collected from Saudi Arabia.

6.2 Materials and methods

6.2.1 Chemicals and reagents

All nine reference standard substances at a concentration of 1 mg mL^{-1}, three ISD and PFPA were purchased from the supplier mentioned in **section 3.2.1.1**.

The substances were four ATS of amphetamine, methamphetamine, PMA, MDMA, five SC of cathinone, mephedrone, buphedrine, 4-methylephedrine, pentylone, three ISD of amphetamine-d_{11} (1 mg mL^{-1}), cathinone-d_5 (0.1 mg mL^{-1}), pentylone-d_3 (0.1 mg mL^{-1}).

Pipette tips of PDMS-DVB (IonSense® PDMS/DVB SPME-in Tips) and C18 (IonSense® C18 SPME-in Tips) coating fibres, vial kits in the size of 0.3 mL (certified vial kit, low adsorption (LA) QsertVial™ volume 0.3 mL, QsertVial, clear glass vial, natural PTFE/silicone septa (with slit), thread 9 mm), 1.2 mL (certified vial kit, low adsorption (LA) MRQ30 CD™ vial volume 1.2 mL, MRQ30 Vial, clear glass vial, natural PTFE/silicone septa (with slit), thread 9 mm, pkg of 100), 0.7 mL (crimp top microvial, requiring a 8 mm seal volume 0.7 mL, amber glass vial, O.D. × H 7 mm × 40 mm , flat bottom, pkg of 100) and formic acid (FA) were obtained from Sigma-Aldrich, Gillingham, UK. The new pipette SPME tips were coated with fibres of mixed mode (C18-SCX) and were provided by Sigma Aldrich, due to unavailability on the market.

Microcentrifuge tubes, Eppendorf® (1.5 mL), acetonitrile, acetone, 2-propanol, sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from VWR International Ltd (Lutterworth, UK). Deionised water was generated from an Ultrapure water purification system (Merck Direct QR 3UV water deionizer). Other materials and grade chemicals were obtained from the supplier mentioned in **section 3.2.1.1**.

6.2.2 Drug-free urine (DFU)

Urine samples were obtained from healthy volunteers under ethical approval statement. All specimens were tested using scan and SIM mode to confirm

the absence of drugs in the urine using the procedure demonstrated in chapter 4. All DFU were stored at 4°C in the RF.

6.2.3 Ethics statement

All procedures, written informed consent from all subjects and applications within this study were completed based on the guidelines obtained from the College of MVLS Ethics Committee for Non-Clinical Research Involving Human Subjects at the University of Glasgow. The protocol was reviewed and approved by the MVLS College Ethics Committee, University of Glasgow (200160055) (See Appendix 2). The urine samples that were collected in Saudi Arabia (SA) were also ethically approved by the Research Committee at Security Forces Hospital (SFH), Riyadh, SA (16-190-24) (See Appendix 3). Both University of Glasgow and SFH committees reviewed the research proposal and agreed that there was no objection on ethical grounds.

Drug-Free Urine (DFU) samples were collected under an ethical approval procedure. The procedure was reviewed and approved by the MVLS College Ethics Committee, University of Glasgow (200160020).

6.2.4 Preparation of drug standards

Standards at a concentration of 1 mg mL^{-1} for all eight drugs (amphetamine, methamphetamine, PMA, MDMA, mephedrone, buphedrine, 4-methylephedrine and pentylone) were diluted in MeOH to prepare a stock solution of 100 µg mL^{-1} (See **section 4.2.3** for the procedure used for preparation).

6.2.5 Preparation of ISD

ISD of amphetamine-d_{11} at 1 mg mL^{-1} were diluted to 100 µg mL^{-1}. The amphetamine-d_{11} of 100 µg mL^{-1}, cathinone-d_5 (100 µg mL^{-1}) and pentylone-d_3 (100 µg mL^{-1}) were diluted to 10 µg mL^{-1} as a mixture. See **section 4.2.3 and 4.2.4** for the full procedure used for the preparation.

6.2.6 Preparation of working solutions

The mixture working solutions of eight drugs were diluted using DFU to reach 2 µg mL^{-1} for the validation work; and MeOH to reach 10 µg mL^{-1} for the development work. The stock standards mentioned above were similarly prepared following the procedure stated in **section 3.2.1.12** and **4.2.5.**

6.2.7 Laboratory preparation for the method development work

Pipette tips of SPME were evaluted for three types of fibres (PDMS, C18 and mixed mode) in DFU using the following parameters: size of vials, sample volume, pH of buffer, addition of salts, addition of derivatisation reagent, ionic strength, solvent type and volume, extraction time, agitation speed in the period of extraction stage, desorption time, agitation speed in the period of desorption stage and finally increasing the temperature before the extraction stage. Each parameter was assessed alone as a single factor while the other factors were kept constant.

The parameters above were optimised and absolute recoveries were calculated [545] . During the optimisation of analytes 1 mL of urine was used 1 µg mL^{-1}. 50 µL of amphetamine-d_{11} at a concentration of 10 µg mL^{-1} was added to each evaluated sample prior the evaporation stage.

Duplicate tubes of unextracted MeOH contain a mixture of the eight substances (final concentration= 1 µg mL^{-1}) with amphetamine-d_{11} (final concentration= 0.5 µg mL^{-1}). They were prepared each day and analysed together with the samples to increase the accuracy of the results during the development work.

The calculation recovery method was mentioned in chapter 3, **section 3.2.2.6**. The evaluation rule was very simple: the uppermost percentage recovery achieved was considered the best results in each evaluated parameter. The parameters were developed until the highest recovery and sensitivity possible for the instrument was achieved.

The method development processing stages for optimisation of each parameter followed the procedures used in most articles published for general SPME method development. The design protocol that was discussed in the hand book sample preparation, hand book of solid phase microextraction and the book of solid phase microextraction method development was followed more specifically [202, 499, 546].

6.2.7.1 pH

The investigation began by evaluating three fibres using urine samples adjusted to a pH of three, five, seven, nine and 11. A triplicate pH buffered of 1 mL urine samples with 100 µL of drug mixtures (10 µg mL^{-1}) was tested. The total number of samples was 47 (3 (repetition) × 3 (fibres) × 5 (pH)) + 2 unextracted tubes). The preparation method of each selected pH value was calibrated as shown in **Table 6-3**.

The introductory procedure is described as follows. The three types of fibres were conditioned for 20 minutes in MeOH and distilled water (50:50). 900 µL of adjusted pH urine and 100 µL of drug mixtures in MeOH (10 µg mL^{-1}) were added to 1.2 vials. The fibres were then inserted for extraction with an agitation speed of 1000 rpm for 30 minutes. After, the fibres were inserted in the vials of 0.3 mL which had 100 µL of 0.5 mL of (NH_4OH (28% solution, v/v) + 99.5 mL of MeOH) + 50 µL of ISD amphetamine-d_{11} for the desorption step with an agitation speed of 1000 rpm for 30 minutes. After this, 10 µL of acidified MeOH was added (1 % HCL + MeOH). The samples were gently evaporated using a hot block with nitrogen gas at 33°C. 50 µL of PFPA:EtOAc (2:1) was added, the tubes were capped and mixed for 3-5 seconds and then left for 20 minutes at 60°C for completion of the reaction. The specimens were evaporated again at RT and reconstituted by adding 50 µL of EtOAc. Finally, the contents were injected into GC–MS for analysis. It should be noted that the optimum results from each step were used in the next step, and so forth.

Table 6-3: An example preparation method for adjusting the selected pH values in urine samples using pH electrodes.

Note: phosphate buffer solution was also prepared and investigated based on the desired pH.

pH	The preparation method
3	Formic acid was added to 25 mL DFU until the desired pH was reached.
5	Formic acid + 0.1 M HCL were added to 25 mL DFU until the desired pH was reached.
7	The original urine was below 7, so 25% (w/v) NaOH was added to 25 mL DFU until the desired pH was reached.
9	25% (w/v) NaOH was added to 25 mL DFU until the desired pH was reached.
11	25% (w/v) NaOH was added to 25 mL DFU until the desired pH was reached.

6.2.7.2 Ionic strength and salts additive

The introductory procedure above was applied to evaluate the ionic strength and salts additive. Examples of laboratory work days were:

A 1 mL duplicate of the drug urine samples mixture was tested including 100 µL of 5%, 10% and 25% (w/v) NaOH and KOH in each vial (total of samples = 36 samples + 2 unextracted); ((3 fibres) × (2 duplicate) × (3 NaOH)) + ((3 fibres) × (2 duplicate) × (3 KOH)).

Duplicate vials of 0.1, 0.25, 0.5, 0.75 and 1 g of NaCl were investigated separately (total of samples = 30 samples + 2 unextracted); ((3 fibres) × (2 duplicate) × (5 NaCl)).

In another separate study, 100 µL of 5%, 10% and 25% w/v NaOH was added together with 0.1, 0.25, 0.5, 0.75 and 1 g of NaCl ((3 fibres) × (2 duplicate) × (3 NaOH) × (5 NaCl) = 90 samples + 2 unextracted). This procedure was repeated again on the following two days for clarification of the results.

6.2.7.3 Temperature

Three fibres in tips were examined in triplicate using the temperatures of 60°C and room temperature before the extraction step.

6.2.7.4 Vial types

1.2 mL kits (**Figure 6-1**) and 1.5 mL Eppendorf vials were evaluated using duplicate specimens for each fibre. The linearity study was used for the assessment of the two vials at 50, 100, 250, 500, 750, 1000, 2000 ng mL^{-1} (duplicate specimens, total samples = 84 (2 (duplicate) × 7 (points) × 3 (fibres) × 2 (vials)).

Figure 6-1: 1.2 mL vial kit used during the development stage.

6.2.7.5 Derivatisation reagent

The PFPA derivatisation reagent was used for the assessment. Duplicate specimens containing the PFPA derivative were added prior, throughout and after the extraction stage. The PFPA was also added after the evaporation step.

During pre–extraction derivatisation, the 50 µL of PFPA was added to the sample containing the mixture of eight drugs and left for 15 minutes to

react. The extraction and desorption steps then took place after the compounds were derivatised.

Throughout extraction and derivatisation, which is also named simultaneous extraction and derivatisation, 50 µL of PFPA was added to the three types of fibre coatings which were then exposed to the mixture in the urine samples to permit derivatisation and extraction processes to continuously occur in the fibre coatings.

The 50 µL of PFPA was also added to the vial in desorption step (after the extraction stage).

6.2.7.6 Sample volume

Volumes of 1000, 500 and 100 µL urine samples at 1 µg mL^{-1} were measured with three replicated vials at each volume. 100, 50 and 10 µL of (10 µg mL^{-1}) the drug mixtures were added to 1000, 500 and 100 µL urine samples, respectively. The introductory procedure was applied using the optimum results obtained from the above sections.

6.2.7.7 Type of solvents in desorption stage

The evaluation of solvent types for the assessment of the desorption phase was applied to NH_4OH:MeOH (0.5:99.5, (v/v)), MeOH, EtOAc, IPA, NH_4OH:MeOH (2:98 (v/v)), DCM:IPA: NH_4OH (78:20:2, (v/v/v)), acetonitrile, and acetone: water (20:80 (v/v)). The urine samples were triplicated on two different test days (samples per day = 3 (replicate) × 3 (fibres) × 8 (solvents) = 72 + 2 (unextracted)).

6.2.7.8 Extraction time and agitation speed

On the first test day, triplicate spiked DFU specimens were tested for the assessment of agitation speed using durations of 15, 30, 60 and 120 minutes at a speed of 1000 rpm (all fibres were involved = 36 + 2 unextracted samples). On the second test day, durations of 5, 15, 30 and 60 minutes

were used with an agitation speed of 1500 rpm (12 (PDMS) + 8 (C18 and mixed mode) fibres + 2 unextracted samples). On the third test day, times of 30 and 60, 90, 120 and 180 minutes with an agitation speed of 2000 rpm were used (15 (PDMS) + 10 (C18 and mixed mode) fibres + 2 unextracted samples). The PDMS/DVB fibre tips only were triplicated on the second and third test days. The optimum procedure was used during and between the processing work for each fibre.

6.2.7.9 Desorption time and agitation speed

Triplicate urine specimens for PDMS/DVB and duplicate urine samples for mixed mode and C18 fibres were applied and repeated on three test days. On the first day, the shaker for agitation was set to 1000 rpm for 15, 30, 45, 60 and 90 minutes (total of samples = 37 (3 (triplicate) × 1 (rpm) × 5 (times) + (10 for mixed mode) + (10 for C18) + 2 unextracted).

On the second day, the samples were assessed at a speed of 1500 rpm and times of 20, 30, 40 and 50 minutes (total of samples = 30 (3 (triplicate) × 1 (rpm) × 4 (times) + (8 for mixed mode) + (8 for C18) + 2 unextracted). On the third day, a speed of 2000 rpm and times of 1, 5, 10 and 20 minutes were used (total of samples = 30 (3 (triplicate) × 1 (rpm) × 4 (times) + (8 for mixed mode) + (8 for C18) + 2 unextracted).

6.2.8 Lab preparation for the method validation

The new method was fully validated using the detection and quantification of the eight target analytes in urine. The validation guideline was Scientific Working Group for Forensic Toxicology (SWGTOX) [3]. The validation parameters were linearity, LOD, LLOQ, interferences, selectivity, carryover, accuracy and precision.

The optimised procedure was used as the sample preparation method for the determination of the following method validation parameters.

6.2.8.1 Linearity method

A mixture of all drugs tested was diluted using DFU to obtain 2 µg mL^{-1} (this was used as a highest point concentration for calibration assessment). The concentration points of 50, 100, 250, 500, 750 and 1000 ng mL^{-1} were freshly prepared using a technique similar to that in **section 4.2.5**. Calibration curves for each substance in the mixtures were plotted using a best fit straight-line method to calculate the linear regression (R^2) of the target ion substances with respect to the target ion ISD compounds using the procedure previously mentioned in chapter 3 with **Equation 3–2**. Linearity was measured by repeating eight samples four times each day to obtain eight concentration points including the DFU in each calibration curve. This procedure was repeated on five consecutive days to plot 20 calibration curves in total for each substance. The accuracy (bias) and RSD were calculated and should be between ± 20% error for each concentration point.

6.2.8.2 Accuracy and precision methods

Three QCs at concentrations of 250, 850 and 1500 ng mL^{-1} were prepared using a protocol similar to that detailed in **section 4.2.7**. The QCs were tested by replicating the samples four times every day on five consecutive days. These samples were analysed on the same days as linearity study mentioned above. Bias (%) was calculated using the grand average calculated concentration of **Equation 3–5.** Within-run and between-run precision were calculated using **Equation 4–2** and **Equation 4–3**. After

calculation, each analyte must be within ±20% (bias) to the true concentration value analysed in order to consider the method valid for the bias study. Each analyte must be less than or equal 20% error for the RSD to consider the method valid for the precision study.

6.2.8.3 LOD and LLOQ methods

The preparation was completed following the procedure configured in **section 4.2.9**. The outcome of LOD and LOQ were known using a signal-to-noise *(S/N)* ratio obtained from the instrument software explained in **sections 3.2.2.4** and **4.2.9**. The drug mixtures in the DFU specimens were repeated ten times in two days including five in each day at concentrations of 200, 100, 50, 25, 10, 5 and 1 ng mL^{-1}. The LLOQ was evaluated using the linearity points by calculating the average concentration of each point with ≤20% and ±≤20% error of RSD and accuracy, respectively.

6.2.8.4 Interferences and selectivity methods

The method of interference studies was given in **section 4.2.14**, where it was assessed by using the instrument chromatogram to observe if there were peaks that interfere with the target analyte peaks. This was achieved using ten different DFU samples without the addition of an ISD via the SIM mode.

The selectivity study was completed by injecting DFU samples that contained twenty-one similar chemical structure drugs to those intended to be checked in order to complete the validation work. Those drugs are cathinone, 4-methyl-N-ethyl-norephedrine, N-ethylecathinone, methcathinone, PMA, MDEA, methedrone, methylone, pentedrone, flephedrone, MDPPP, butylone, pyrovalerone, MDPV, ethylone, MDA, bupherone, PMMA, 4-EMC , α-PVP and naphyrone at 2 μg mL^{-1}.

6.2.8.5 Carryover

Carryover was investigated by examining DFU specimen results after the sample was injected after the target analytes had a concentration of 2 μg

mL^{-1}. This procedure was used as a routine in each day of the validation work.

6.2.9 Application of the method

After the method was validated, it was applied to three positive real urine specimens that were collected from SA. These samples were confirmed as positive using this method and the SPE method stated in chapter 4. This application was completed to verify the robustness of the method for real-life urine samples.

Three positive samples were extracted and analysed using the optimised methods of PDMS/DVB SPME fibre in tips and GC–MS as stated previously in this chapter. The samples were repeated three times for each specimen to compare SPE and SPME. Two procedures were applied as follows.

Triplicate of the three urine samples were extracted and analysed by SPE and SPME coupled with GC–MS with the calibrator points and QCs using the procedures stated in chapter 4 and 6, respectively. After the completion of the analysis, the concentration result for each sample was reported for comparison purposes only.

6.2.10 Green analytical chemistry method

In general, there are five criteria that should be assessed for the evaluation of GAC: environment, safety, energy, health and waste [547-549]. Based on the above, SPME in tips, SPE and LLE procedures were compared and discussed in chapters 6, 5 and 4, respectively. The GAC criteria were evaluated for the analysis of ATS and SC during sample preparation stages only. The explanation of the method used here for the assessment of GAC was recently published in 2018 in a paper by Płotka-Wasylka [188].

6.2.11 Fragmentation patterns and retention time ($_tR$)

The interpretation of the results was by the retention time ($_tR$) of each target analyte compound with at least three fragmentation ions including

their relative ion intensities ratio (%). $_tR$ should not fluctuate more than ±1%. The relative abundance ions ratio should not be greater than ±10% for ions with the relative intensities > 50%.

6.2.12 GC−MS method

This method was completed using the procedure described in **section 3.2.2.12** with the exception of oven temperature programme; 70°C to 200°C at a rate of 11°C/minute (hold for 4 minutes), and 200°C to 280°C at a rate of 10°C/minutes (hold for 1 minute).

6.3 Results and discussion

6.3.1 Unextracted responses of the mixture of eight drugs in GC−MS

The methods demonstrated in chapter 4 were applied to the eight target analyte drugs using PFPA and unextracted tubes. The $_tR$ of the eight drugs with ions ratio were identified. After the method provided good responses and peaks with adequate separation, development work for the evaluation of the SMPE tips was initiated. See **Figure 6-2**.

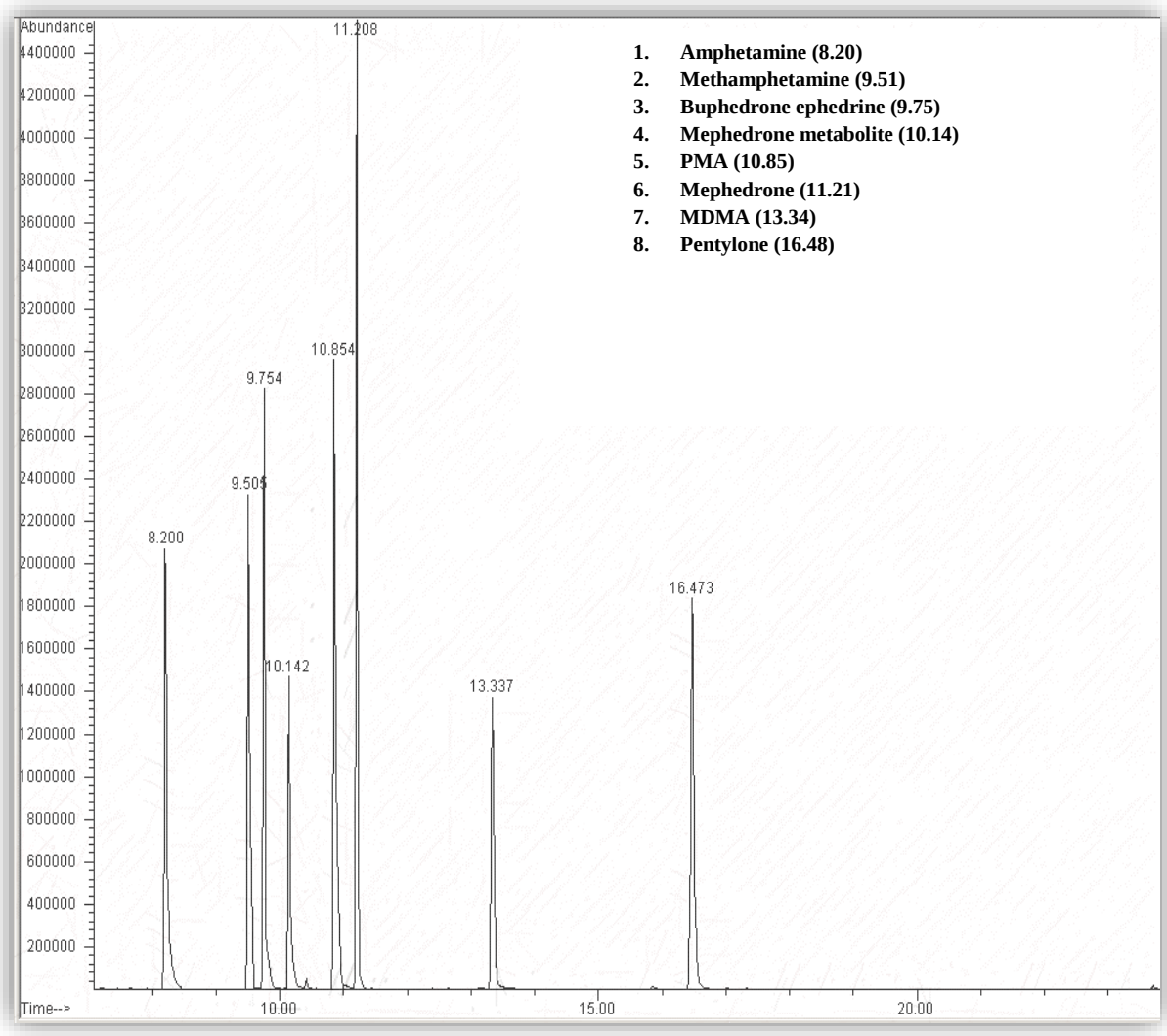


Figure 6-2: Unextracted separation chromatogram peaks for eight SC and ATS substances at 2 µg mL^{-1}

6.3.2 Method development

The SPME procedure is uncomplicated and comprises the performance of two stages: the absorption stage (partitioning of target analytes between the urine sample and the polymeric fibre coating in tips) and the desorption stage (desorption of the extracted compounds using back extraction solvents). Several parameters characteristically must be optimised with the purpose of reaching high sensitively, selectivity, recovery, repeatability and reproducibility.

The extraction of analytes can be increased by optimising and altering the method development parameters within the sample conditions [359]. Method development strategy was followed as detailed in **Figure 6-3**. The plan was designing such that the procedure would be modified to the optimum results in each step. This plan was used to save time, material, money, as well as accomplishing the above goals.

The retention time ($_tR$) with ion ratio (%) for each drug was determined and reported (see **Table 6-4**). The results presented successful separations and detections for PMDS/DVB−SPME fibre in tips.

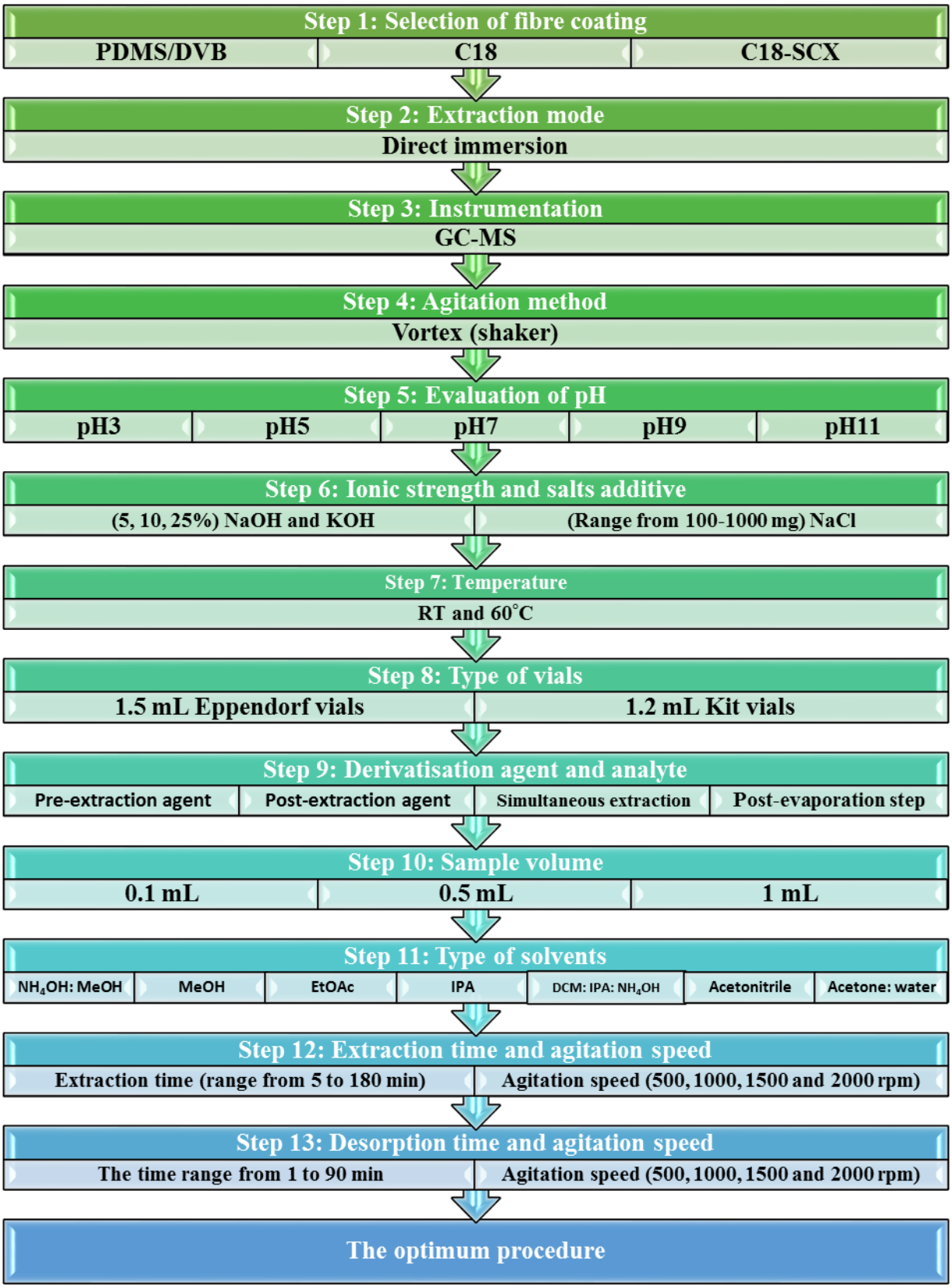


Figure 6-3: Optimisation processing stages in this thesis for the SPME method development work

Table 6-4: Retention time ($_tR$) and ion fragmentation (m/z) with ratio (%), the target ions in bold, the continuing ions were used for confirmation with their ratio (%).

Drug name	$_tR$	m/z	Ratio (%)	Drug name	$_tR$	m/z	Ratio (%)
Amphetamine-d_{11}	8.422	**194**	100	4-Methylephedrine (mephedrone metabolite)	10.158	**204**	100
		128	72			119	13
		98	33			160	20
						308	3
Amphetamine	8.486	**190**	100	PMA	10.854	**121**	100
		118	79			148	42
		91	36			190	5
		65	9			311	7
Methamphetamine	9.505	**204**	100	Mephedrone	11.215	**119**	100
		160	31			204	25
		118	24			91	20
		91	14			160	14
Pentylone-d_3	16.339	**193**	100	MDMA	13.315	204	100
		235	86			162	73
		149	380			**135**	43
						339	12
Buphedrine (buphedrone metabolite)	9.770	**218**	100	Pentylone	16.442	149	100
		119	12			**190**	22
		308	3			232	19
		160	18			381	5

6.3.2.1 Step 1: Selection of the fibre coating

During SPME method development, the appropriate selection of coated materials is crucial. SPME coated fibres have been reviewed elsewhere [492, 550, 551].

The selection of the fibre coating should be as the first step in SPME method development [289, 552]. Each fibre coating type with unique thickness, length, polarity and volatility of the fibre delivers different reactions and mechanisms in each analyte and in each matrix. The extraction efficiency is reliant on the distribution coefficient between type of sample, analyte and the fibre coating [289]. The distribution coefficient of the target analyte penetrates (absorption) the volume of polymer coating within a specified time. Enhancing the penetration of the entire analyte into the fibre depends on the compatibility of the polarity of the coating fibre and analyte, called "like dissolves like" (see **Figure 6-4** for general selection guide associated to ATS and SC) [546, 553-555].

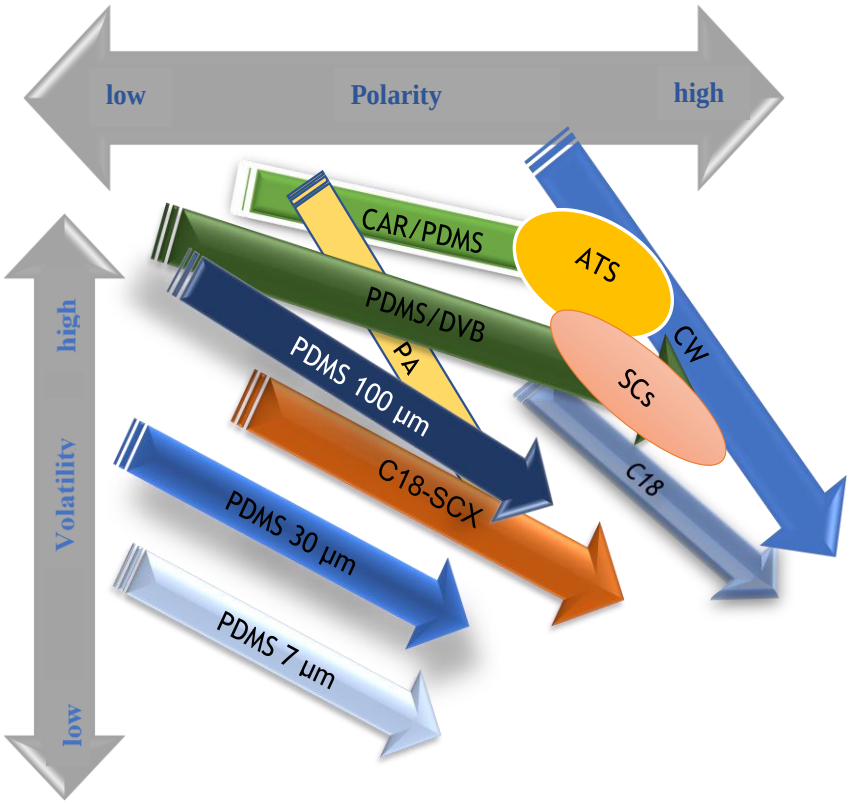


Figure 6-4: Estimation guide for the selection of fibre coating [546]

Since ATS and SC are polar and semi-volatile compounds, affinities are high in the polar and semi-volatile fibre coating, such as PDMS-DVB (bipolar), PA, C18 and C18-SCX [553-555]. In general, nonpolar substances have high affinity to adsorption on nonpolar coated fibres; the thicker the fibre coating, the higher the capacity to extract the volume of organic compounds. Conversely, a lower film thickness allows the compound to be effortlessly desorbed in the bound phase, specifically at higher temperatures with higher boiling points. This is because the diffusion of the compounds over a smaller thickness coating occurs much easier than the diffusion in a thicker coating [518]. However, the non-polar PDMS provides excellent extraction for amphetamines. This is because of the effects of other factors such as the thickness of the coating and the molecular mass of the analyte [500-502, 518, 546]. Generally, a thicker coating fibre needs a longer extraction time, but the recoveries are higher [358]. Additional discussion on the selection of coating fibres in SPME was reviewed by Graham et al. [556].

From the above discussion, it is evident that selecting the right fibre without investigating the affinity and behavior of the coating fibre with the target analytes is complex, particularly in the case of mixtures or new materials. The new fibre tips that were evaluated were slightly different in physiochemical properties, such as the size and length of the coating fibres. Therefore, it is very important to assess the sensitivity of these fibres. Unfortunately, the fibre tips on the market are accessible from a single source (Supelco) with availability of only two coated fibres PDMS/DVB and C18. The coated fibres of C18-SCX were kindly donated by Sigma Aldrich, because the product is still under investigation (not available on the market).

PDMS−DVB was designed for the extraction neutral fractions of the analytes with medium to high polarity and semi-volatile to volatile compounds, such as amphetamines [552, 557]. Several articles carefully chose PDMS/DVB as the fibre for the extraction of amphetamines [522, 555, 558] and cathinones [413]. For example, PDMS/DVB, PDMS, CAR/PDMS and CW/TPR were tested for amphetamine and methamphetamine in serum using LC MS, and the conclusion was that the PDMS/DVB was the favoured extraction fibre in terms of extraction efficiency, response and sensitivity [559]. Therefore, as expected, the results show that the

PDMS/DVB provides high sensitivity with great responses in GC–MS compared to the other tested fibre coating tips (see **Figure 6-5** for drug separation peaks in chromatograph).

C18 provided great separation characteristics and it is extensively used in chromatography or SPE as a stationary phase for medium and non-polar substances [560]. However, it had very poor responses in this study under all examined drugs and under all developing processing stages.

The mixed mode SPME–C18/SCX, which is a mixture of ion strong cationic exchange and hydrophobic coating phases, was introduced for the improvement of the extraction efficiency [561, 562]. It was used for the extraction of normal and charged fractions of the target analytes. It has high affinity for the extraction of ATS because the natural and ionic fractions of ATS can be absorbed on the fibre coating. Good responses were not found within this study. Additionally, accuracy and precision, recoveries or even good selectivity for all drugs tested under all the developing processing stages using C18/SCX were not adequate [511, 515, 561].

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

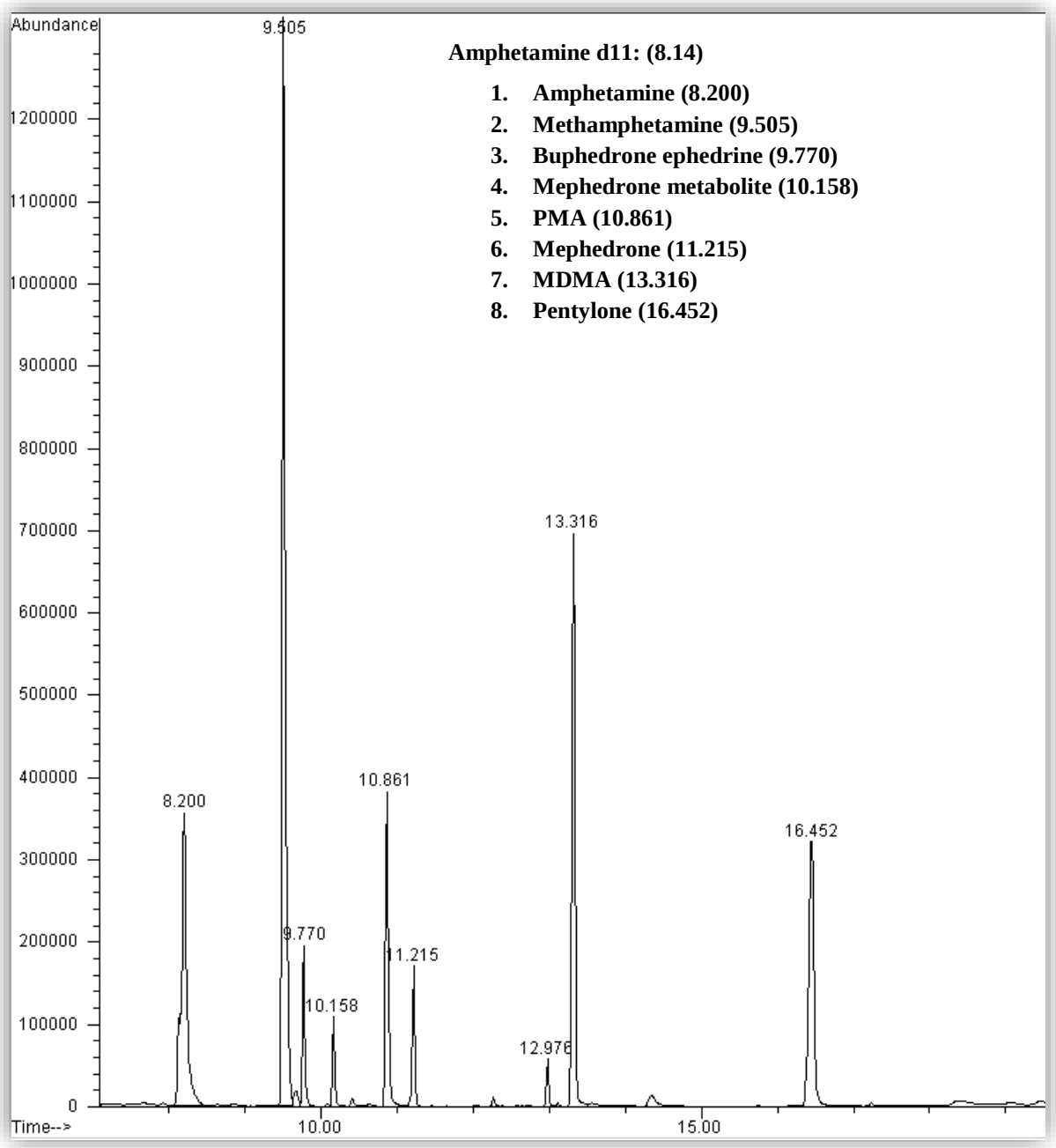


Figure 6-5: A) SIM chromatogram for eight stimulant drugs: SPME-PDMS/DVB fibre tips and PFPA derivative; this is after the optimum conditions were applied at a concentration of 1 µg mL^{-1} in a urine sample.

6.3.2.2 Step 2: Extraction mode

There are two commonly used SPME extraction methods: direct immersion and headspace. Even though the headspace mode is more suitable for complex, high to medium volatility and low to medium polarity samples and analytes, it cannot be utilised for SPME in tips because the headspace mode requires high temperature to enable evaporation. This step cannot be achieved for the fibre in tips because high temperatures cannot be withstood. Therefore, the direct immersion only was used for the following method development steps.

6.3.2.3 Step 3: Instrumentation

GC–MS is the most common technique used extensively for a wide range of mixture drugs of interest; for example, the quantitative analysis of ATS and SC in forensic toxicology laboratories.

6.3.2.4 Step 4: Agitation methods

The agitation mechanism is a very important parameter for the development of SPME. The effectiveness of the agitation method determines equilibration time of the aqueous specimen. This is significant in order to yield efficient extraction based on kinetic theory [489]. Suitable agitation techniques accelerate the transfer of mass analytes from the sample to the fibre coating providing shorter equilibrium reaction time and greater extraction of the analytes [359, 563].

For a liquid matrix such as urine, the most widespread agitation methods used within the literature for SPME were a shaking (vortex/ moving vial), magnetic stirring (a stirring bar in the vial), sonicating and fibre moving. All methods required temperature to reach equilibrium, with the exception of the shaker [546].

The most effective technique is direct sonication, which provides extraction times as short as 30 seconds [361]. However, it heats the sample, which in some cases destroys the fibre, and may also destroy the analytes [564]. Triplicate urine

samples were examined at RT and 60°C for 10 minutes using the direct sonication method throughout the extraction stage. The results were poor for all fibres in all investigated samples. Stirring bar and fibre movement techniques also require sufficient space in the vail and high temperatures, thus were not evaluated within this development study.

In-tip SPME (automation) fixed with a Tomtec Quadra 96 workstation significantly decreased agitation by simply operating aspiration and dispense functions through several cycles in the system [565].

As a result, the single remaining method that can be safely and effectively performed is a shaker vortex (IKA VIBRAX VXR shaker). Therefore, this technique was applied to the following steps.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

6.3.2.5 Step 5: pH evaluation

The pH of the sample is vital. For slightly basic compounds, such as amines, the undissociated form must be maintained [566]. It is also very important that the analytes are completely transformed into natural form, because SPME can extract only natural compounds [363]. Therefore, the requirement of pH values to be adjusted is key to increasing sensitivity. Low pH values improve recoveries for acidic species, while high pH values improve the extraction efficiency of basic compounds. Basic molecules are protonated at low pH values, and acidic molecules are ionised at high pH values. The optimum pH for extraction is within the stability range of the fibre coatings and the target analytes [546, 561].

The sample pH is theoretically described by the extraction equilibrium and acid–base equilibrium [567]. For ATS and SC (amphetamines pKa (8.8-10.4) [568], cathinones pka (7.4 and 9.5) [569]), the value of pH > 10 is essential, because of the high value of ATS and SC acid dissociation constants. Basic pH values enhance the recovery of basic analytes [570]. Overall, it is

expected that to deliver an efficient extraction, it is essential to make the contents 2 or 3 units above or below the pKa of the analyte of interest [571, 572] .

The three repeated specimens in each fibre coating in tip were adjusted to pH values of 3, 5, 7, 9 and 11. Poor responses and detections were observed for C18 and C18/SCX modes for all pH values, whereas PDMS/DVB was the only fibre that provided great responses and detections with good recovery for a pH value of 11 (see **Figure 6-6**). It can be noted that the PDMS/DVB pH results here is compatible with the evidence of the discussion above.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

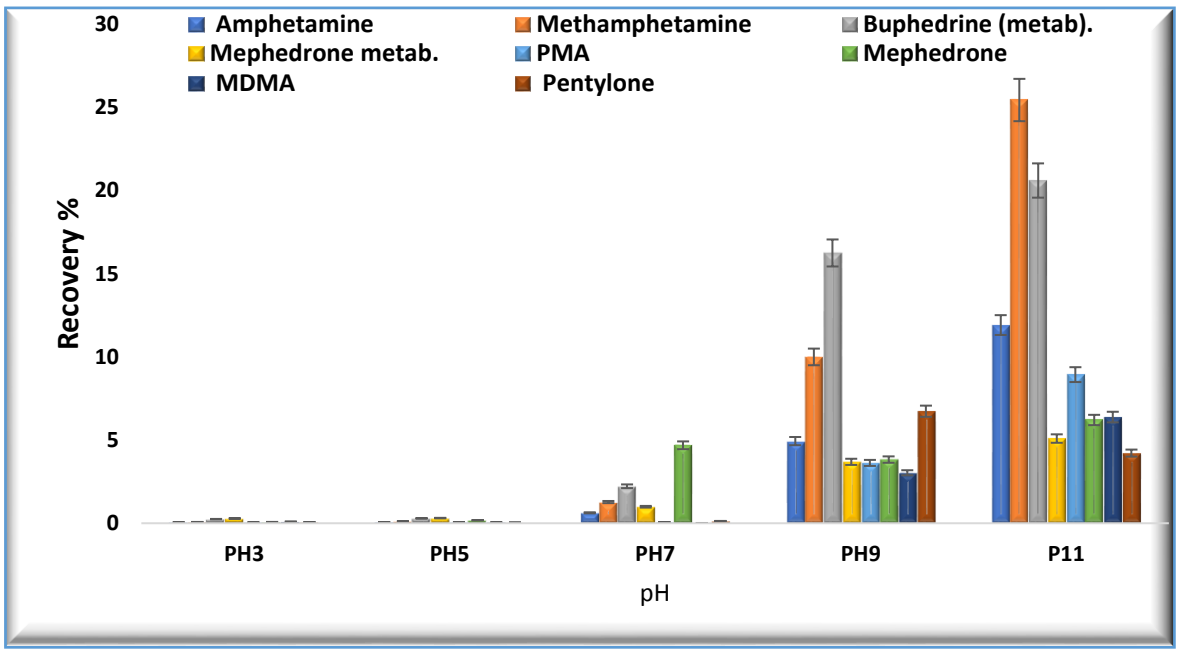


Figure 6-6: The pH buffering results of an averaged triplicate in PDMS/DVB-SPME tips in urine at 1 µg mL^{-1}

6.3.2.6 Step 6: Ionic strength and salts additive

The addition of inorganic salts increases the ionic strength of the matrix. Organic substances then become less soluble and the partition coefficients are achieved [564]. The distribution constant increases when salts are added. For example, with the addition of NaOH or NaCl, the aqueous

solubility of most compounds decreases and causes the target analytes to be distributed faster from the matrix to the fibre coating, as a result the extraction efficiency is improved [289]. The effect of the additive salts varies according to the nature of the targeted analytes and the matrices. The addition of salts can boost or diminish the amount of analytes extracted, depending on concentrations, therefore, it is important that the parameter be experimentally investigated [218, 546].

The results showed that the two coating fibres (C18 and C18/SCX) provided poor responses and detections in the experiment and similar results were achieved in the following sections.

The recovery of PDMS/DVB fibre coating improved when 10% NaOH and 0.5 NaCl were mixed with the contents of the samples. (see **Figure 6-7**, **Figure 6-8** and **Figure 6-9** for the example recovery results). Accordingly, the 10% NaOH and 0.5 NaCl were selected as the optimum conditions for the evaluation of the ionic strength and salts additive.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

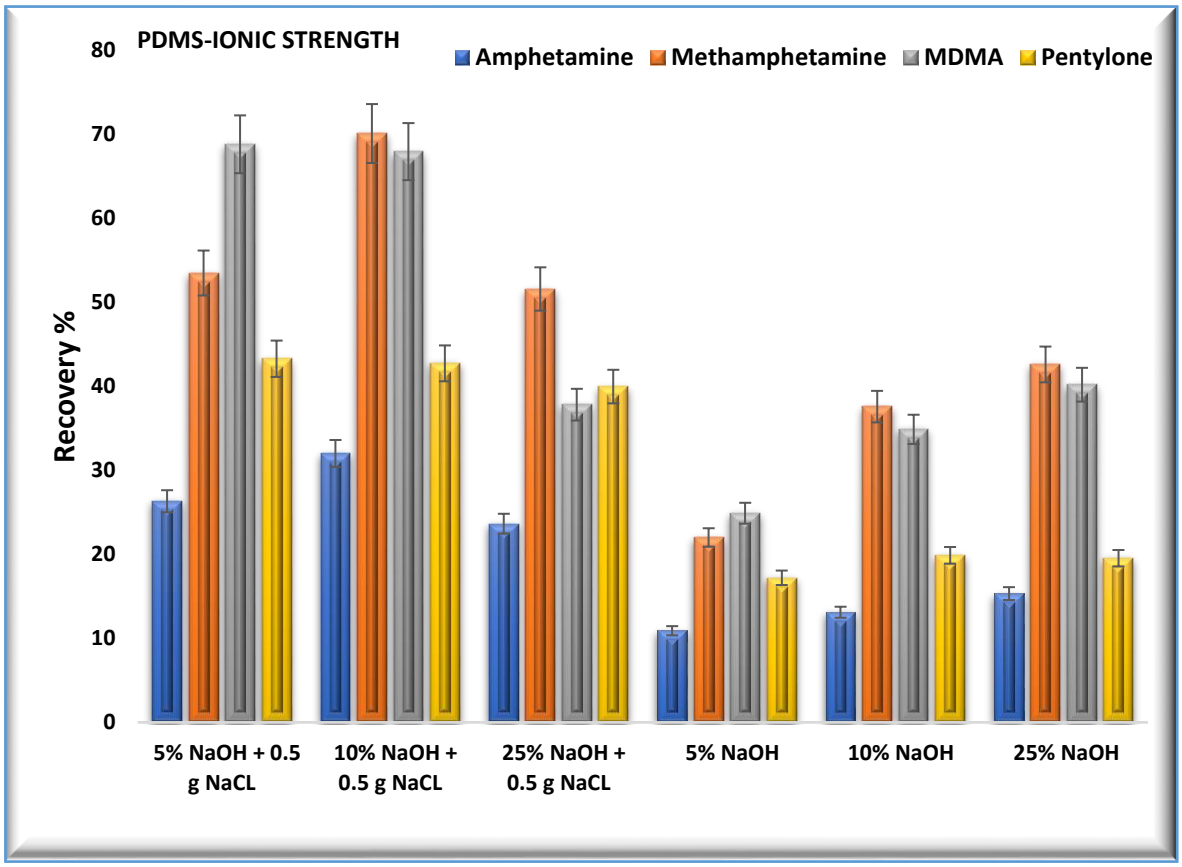


Figure 6-7: Example average recovery (%) (n=3) results for evaluation of the effects of the ionic strength with addition of salts applied to PDMS/DVB-SPME tips in selected drugs in urine at 1 µg mL^{-1}

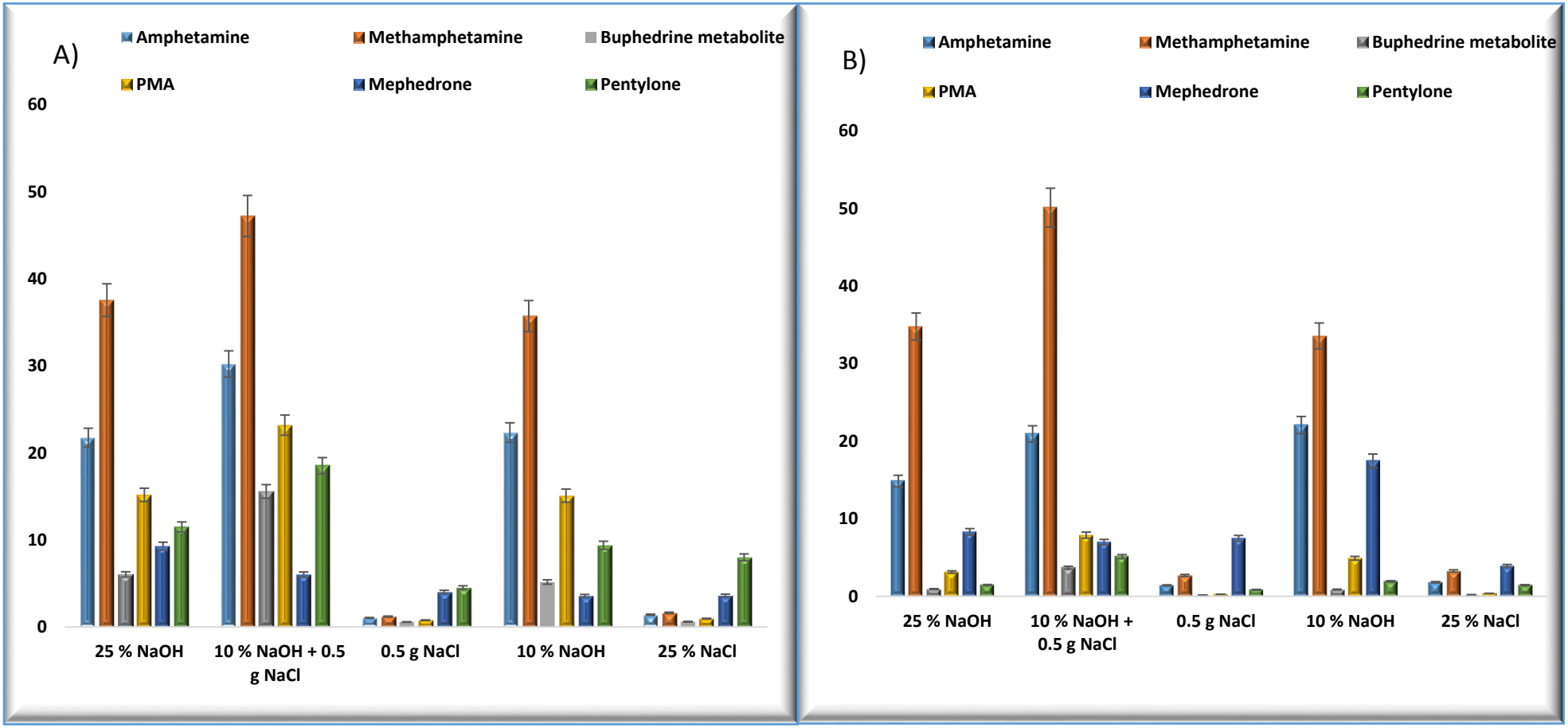


Figure 6-8: Comparison of average recovery (%) results (n=3) for assessment of the effect of ionic strength and additive of salts on PDMS/DVB-SPME tips on two different days (A and B)

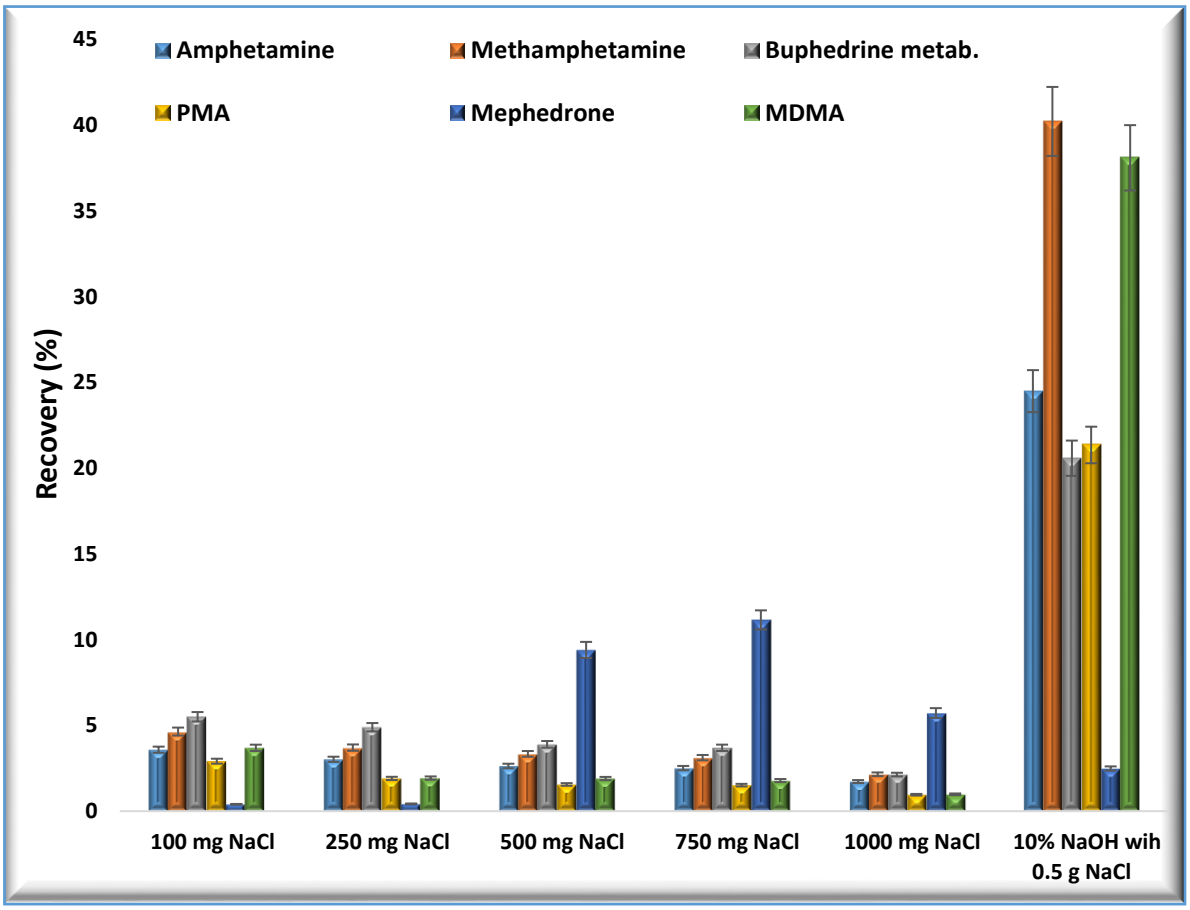


Figure 6-9: Percentage of average recovery example results (n=3) when only additive salts were applied to selected compounds on PDMS/DVB-SPME tips in urine at 1 µg mL^{-1}

6.3.2.7 Step 7: Temperature

The assessment of temperature during the extraction stage was an essential parameter to obtain high recovery, specifically in the headspace mode. Increasing the temperature in the tested vial increases the following: analyte diffusion coefficient, extraction rate, mass analyte to be transferred into the coating and shorter equilibrium time. The higher the temperature is, the lower the distribution constant in the sample matrix fibre coating, which leads to reduced sensitivity and recovery at equilibrium [546]. In general, higher temperatures should be applied to high molecular weight and less volatile substances [573].

The effect of temperature on methamphetamine compounds and recovery for SPME was evaluated for four temperatures (22, 40, 60 and 73°C). It was concluded that the highest recovery was achieved when the temperature was lowest, yet the extraction time was the longest [552]. In addition, it should be noted that

extremely high temperature damages the fibre coating and consequently eliminates the capability to absorb the analytes, causing the target analytes to be degraded [574].

Even though Supelco was introduced, the fibre in tips was not recommended for increased temperature conditions, because it was developed for ambient temperature. Three fibres in tips on triplicate samples were evaluated in each fibre using pre-equilibrium temperatures of 60°C and RT. Poor detections and recoveries were achieved in most tested drugs for the samples that were set at 60°C, while the RT samples provided better detections and recoveries.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

6.3.2.8 Step 8: Type of vials

The 1.2 mL vial kit that was purchased from Supelco was expensive, while the 1.5 mL microcentrifuge Eppendorf vial (conical bottom) was very inexpensive. The silicone septa (with slit), which was created specifically for SPME fibre in tips, cannot fulfil with the requirements for high agitation speeds in a shaker, as the tips had fallen when the shaker was set to 2000 rpm or more. This situation rarely occurred with Eppendorf vials.

These vials were investigated using linearity and recovery parameters. The results are illustrated in **Figure 6-10** showing that the linearity study was slightly better using Eppendorf vials, whereas the opposite was true for the recovery results. Since the Eppendorf has more advantages than kit vial and since they both provided similar results, the Eppendorf was selected for future work.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

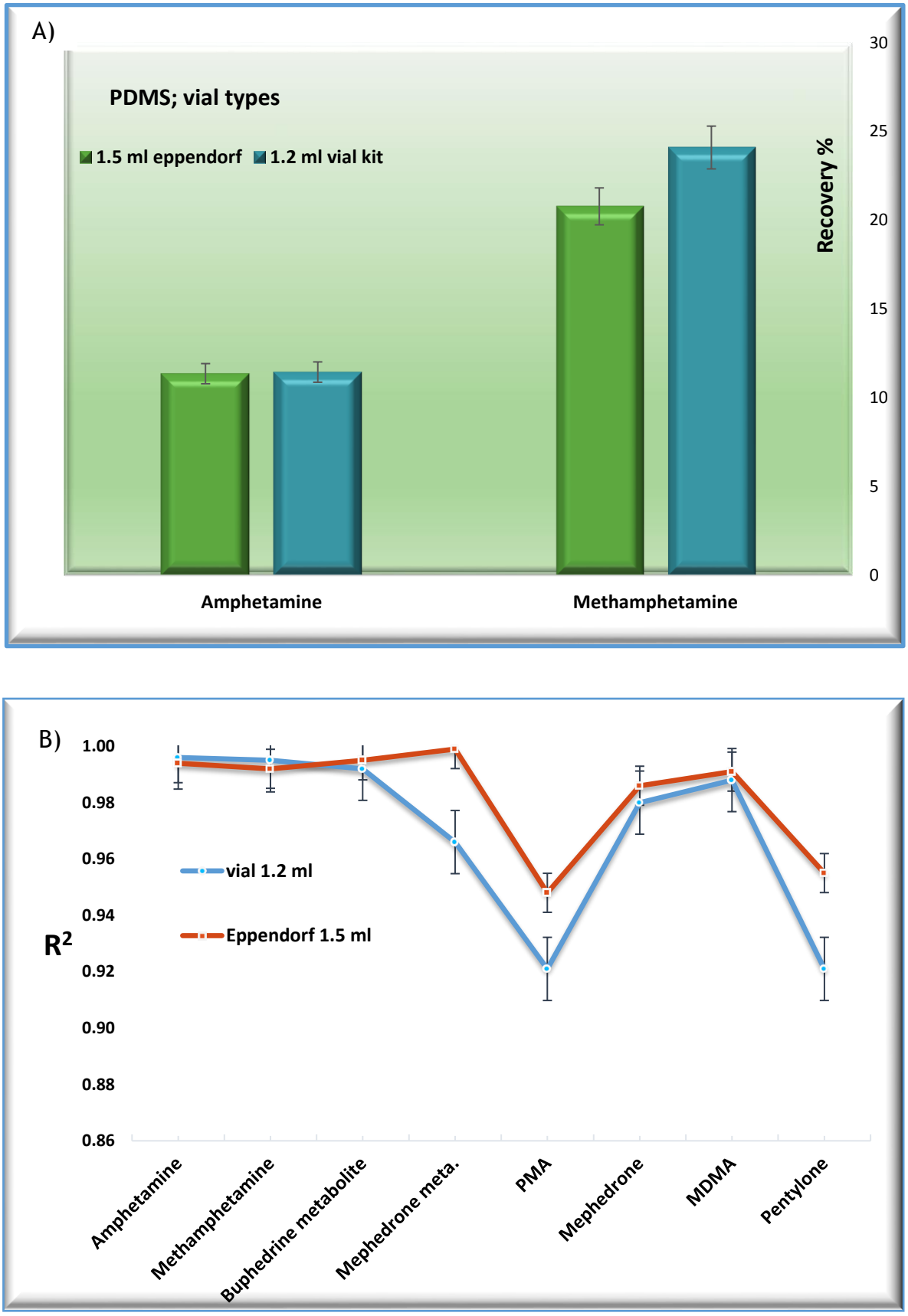


Figure 6-10: A) the comparison of the average recovery (%) (n=2) results for amphetamine and methamphetamine, B) the average linearity (R^2) (n=2) using PDMS/DVB-SPME tips

6.3.2.9 Step 9: Derivatisation reagent

The derivatisation agents were discussed in **section 3.1.2**. The reagents were normally used for the development assessment of SPME when added before, within or after the extraction stage. For example, when exposed to the fibre coating during the extraction step or prior, the extraction efficiency and the distribution constant (partition ratio) for the less volatile compounds will increase. This approach is commonly applied to the headspace mode to increase the volatility [556]. However, the four strategies were assessed, mentioned in **section 6.2.7.5**.

The results presented poor responses and detection for all drugs examined with the exception of adding PFPA after the evaporation step (see **Figure 6-11**).

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

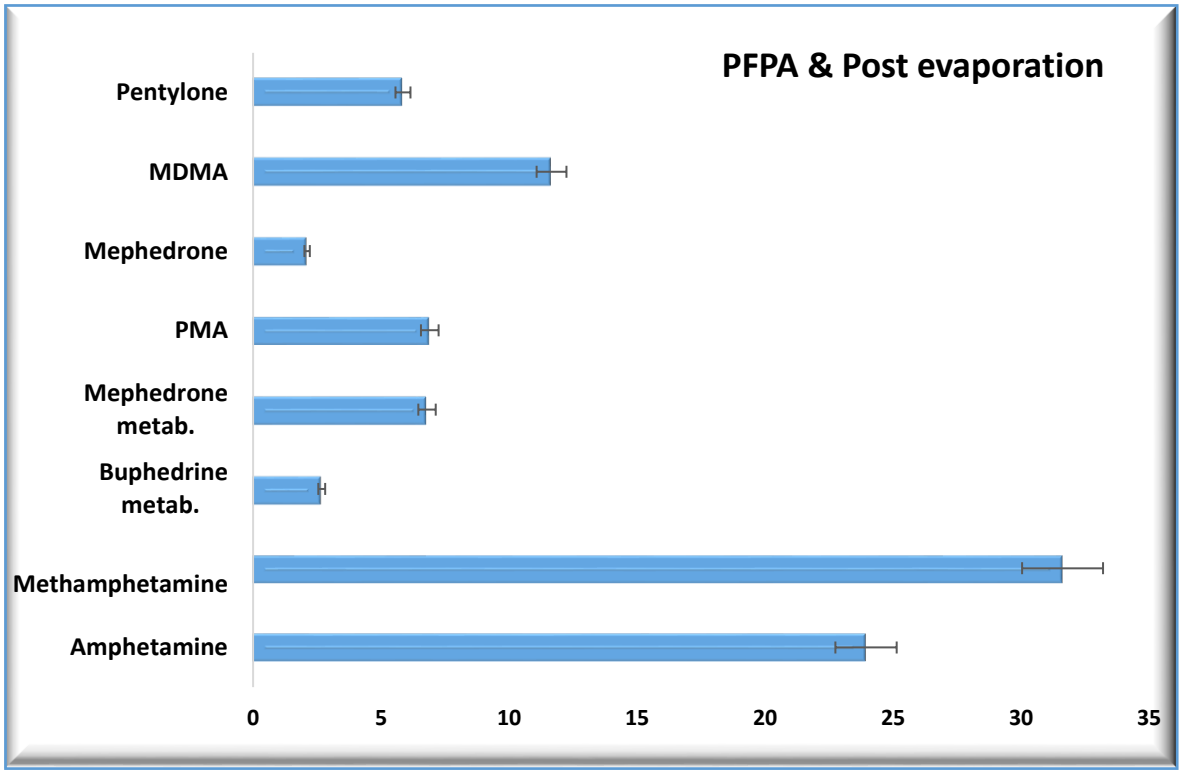


Figure 6-11: The average recovery results (%) for selected drugs when PFPA was added after the evaporation step in urine PDMS/DVB-SPME tips (n=2)

6.3.2.10 Step 10: Sample volume

From **Equation 6-2**, the amount of target analyte adsorbed increases when the sample volume size increases. Increasing the distribution constant (K_{fs}) can be accomplished by increasing the volume of the sample, which increases the amount the extracted analytes [202, 546]. However, the current scope limits the volume of the sample to maximum of 1 mL. This is to match the size of the sample vial and the vortex shaker, because the tips of SPME were introduced to fit with specific sizes of vials. In addition, reducing the sample volume is always preferable for the analysis of forensic investigation matrices due to availability constraints, minimising waste and eliminating contamination.

As expected, the recovery results (%) shows that a volume of 1 mL provided high extraction efficiency compared with lower urine sample volumes. See **Figure 6-12** for the results.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

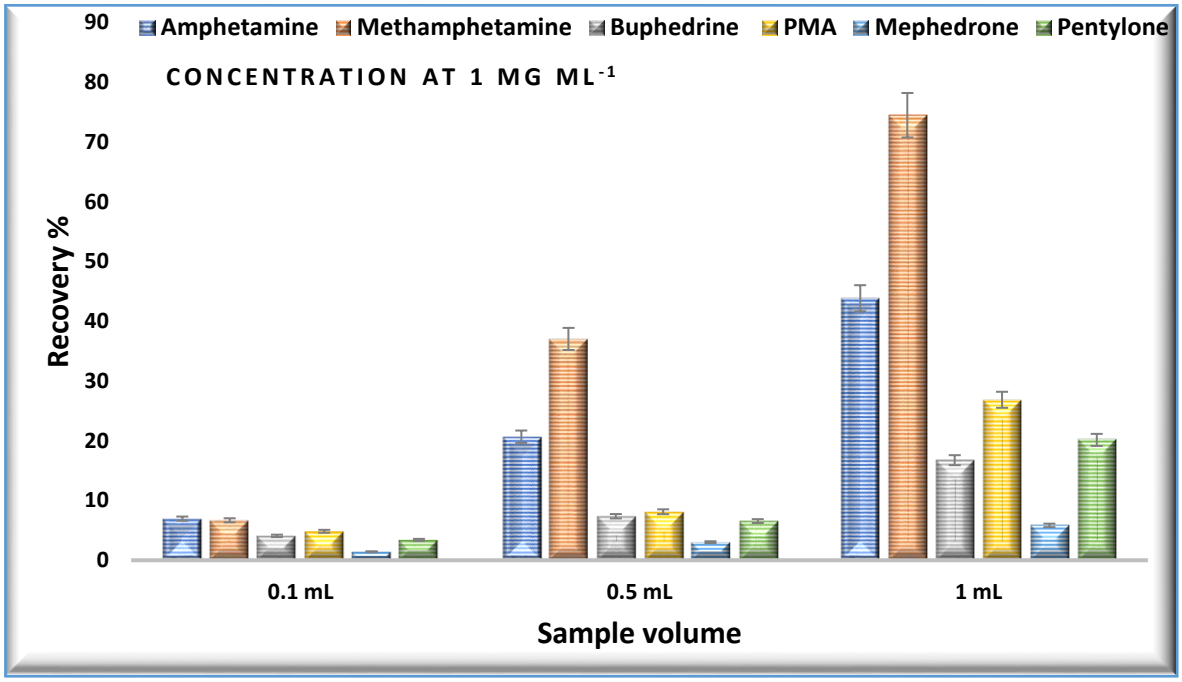


Figure 6-12: The average recovery results (%) of sample volumes 0.1, 0.5 and 1 mL on PDMS/DVB–SPME in tips for selected drugs using triplicate urine specimens (n=3)

6.3.2.11 Step 11: Type of solvents

Desorption is a very important stage, where the target analytes are desorbed from the coating fibres into the separation and detection system for the analysis. The desorption of analytes can occur directly during the static mobile phase or dynamically with another suitable solvent in the HPLC or LC interface using the SPME desorption chamber [202, 499, 546]. In tube SPME, the desorption is performed directly within the mobile phase in the extraction capillary over a specified time. In GC, the desorption occurs thermally in the injector port [388]. In tips SPME, the desorption stage occurs using either the direct desorption technique or a desorption solvent followed by evaporation, derivatisation and reconstitution [202]. Clearly, the desorption solvent followed by evaporation, derivatisation and reconstitution is one approach that can be utilised in GC, because the direct desorption technique is used in LC, HPLC or GC for automation. Therefore, the reversed-phase solvent is the only approach that can be used in this manual process in tips SPME coupled with GC–MS.

In this work, the amount of solvent was not evaluated, since it is recommended that the solvent should be kept at a minimum. This is to reduce its effects during the desorption step, because it can decrease the sensitivity and increase the chance that the analytes are desorbed into the wall of the vial [202]. For these reasons, a 0.3 mL certified glass vial kit, with low absorption and a septum cap with slit were operated during the desorption stage to reduce the possibility of the analytes desorbed into the wall of the vial or into the septum.

The 60 µL of solvent was sufficient to fully cover the fibre coating in tip; therefore, this volume was applied throughout the project.

The example results of tested drugs and solvents are shown in **Figure 6-13** and in **Table 6-5** for day one, and **Figure 6-14** with **Table 6-6** for day two. The recovery results of mephedrone were above 20% RSD on both days, the buphedrine (buphedrone metabolites) was above 20% on day two, which is unacceptable, and the remaining recovery drug results were below 20%.

According to single factor ANOVA illustrated in **Table 6-7**, there were no significant differences between the mean recoveries in each drug and solvent tested with the exception of buphedrine, which means that there was no difference in the recovery results when different solvents were used. Consequently, MeOH was selected as the optimum solvent to be used for the experiments and investigations. Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

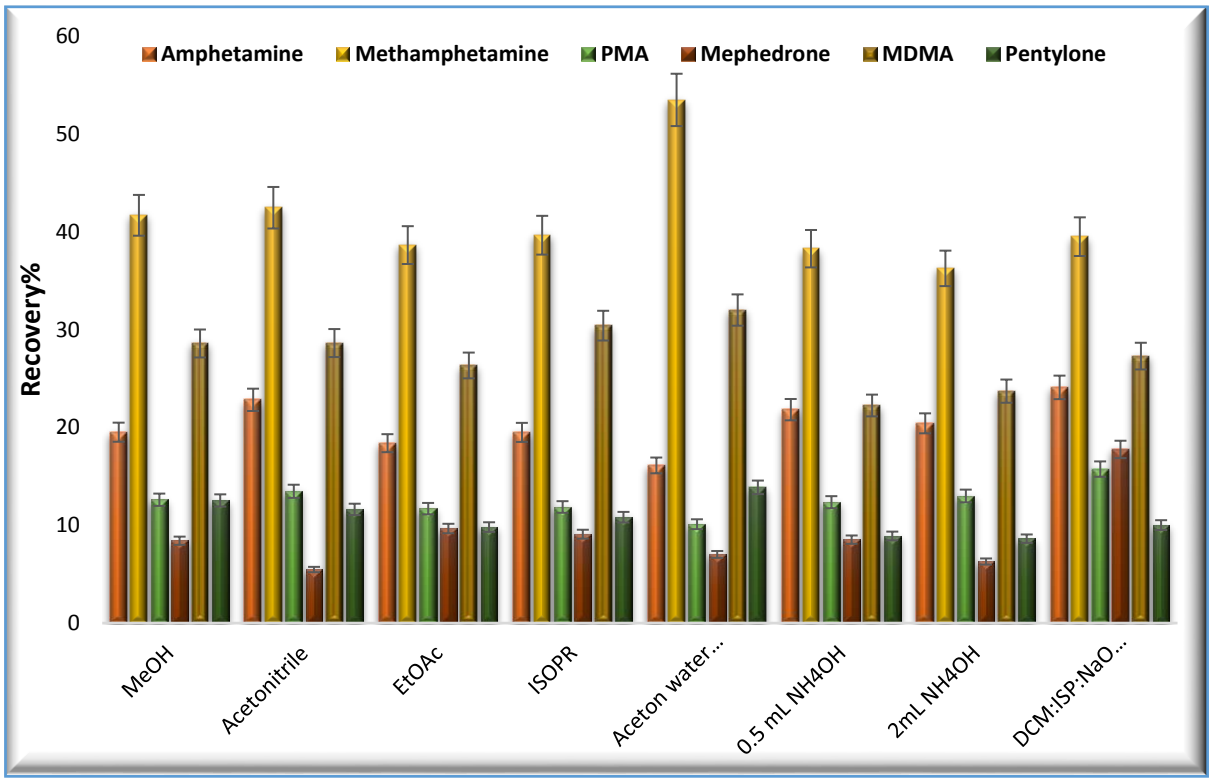


Figure 6-13: The average recovery results (%) of triplicate urine PDMS/DVB-SPME tips for the evaluation of solvent type on day one

Table 6-5: This table demonstrates the mean of all recoveries under all solvents in each selected drug with SD, RSD and confidence limit 95%.
The values here were calculated from the above figure.

Drug Name	Amphetamine	Methamphetamine	PMA	Mephedrone	MDMA	Pentylone
Mean of recoveries % with Confidence limit (95%)	20 ± 2.13	41 ± 4.44	13 ± 1.36	9.0 ± 3.18	27 ± 2.73	11 ± 1.52
SD	2.55	5.3	1.62	3.80	3.26	1.81
RSD	13%	13%	13%	**42%**	12%	17%

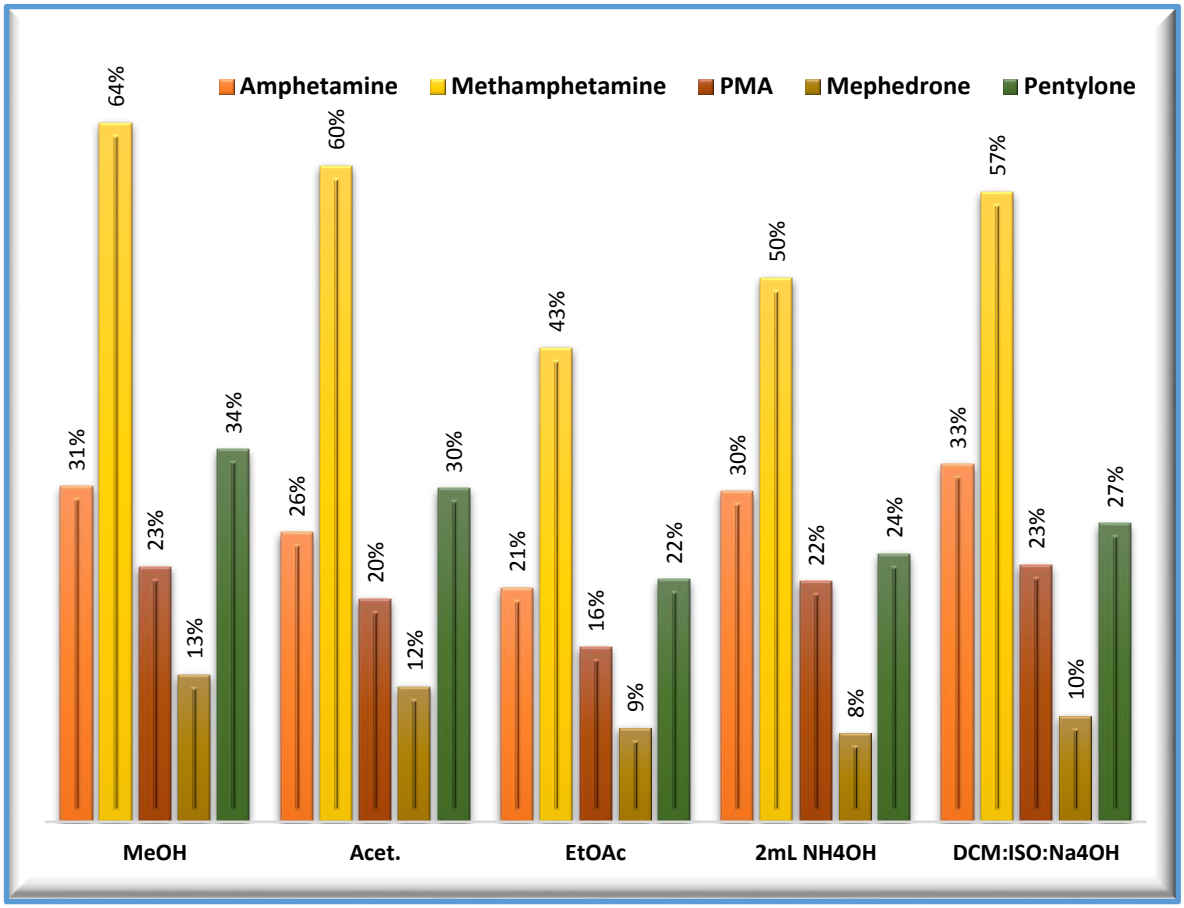


Figure 6-14: The average recovery results (%) of triplicate urine PDMS/DVB-SPME tips for the evaluation of solvent type on day two

Table 6-6: The mean of all recoveries under all solvents in each drug with SD, RSD and confidence limit 95%
The values here were calculated from the above figure.

Drug name	Amphetamine	Methamphetamine	Buphedrine	PMA	Mephedrone	Pentylone
Mean of recoveries % with Confidence limit (95%)	28 ± 5.4	55 ± 10	53 ± 29	21 ± 3.83	10 ± 2.92	28 ± 5.8
SD	4.44	8.2	23	3.08	2.35	4.71
RSD	16%	15%	**44%**	15%	**23%**	17%

Table 6-7: Single factor ANOVA applied to selected solvents and drugs on day two for study the solvent type		
ANOVA: Single factor	**P-value**	**Differences**
Compound name	**P <0.05**	**Yes/No**
Amphetamine	0.40	NO
Methamphetamine	0.17	NO
Buphedrine	**0.045**	**Yes**
PMA	0.64	NO
Mephedrone	0.15	NO
Pentylone	0.23	NO

6.3.2.12 Step 12: Extraction time and agitation speed

Extraction time required to reach equilibrium for SPME can be decreased using an appropriate agitation method. For in tip SPME, the appropriate agitation method was a shaker, which was discussed in **section (6.3.2.4)**. Once the equilibrium time reached, the error of extracted analytes decreases when repeatability is ensured [289]. Sensitivity increases when the equilibrium time point is achieved [202, 546].

The results show (see **Figure 6-15**) that on day one and at a speed of 1000 rpm, the highest recoveries were accomplished after 2 hours, however, the equilibrium time point might arise after this time. Therefore, the samples were repeated using different durations and speeds. On day two, as in **Figure 6-16** (A), similar results to day one were observed, but at a different speed (1500 rpm). The equilibrium time point was observed on day two in **Figure 6-16** (B). This point was achieved after 1 hour and when the speed was 2000 rpm. It can be concluded from the figures that a speed of 2000 rpm provided the highest recoveries for all drugs tested. The maximum speed that could be practically obtained was 2000 rpm, because when the shaker was set to 2500 rpm the tips came off of the vials. The optimum conditions in this step are 2000 rpm after 1 hour, this is based on the parameters, materials and techniques that were used in the experiments. Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

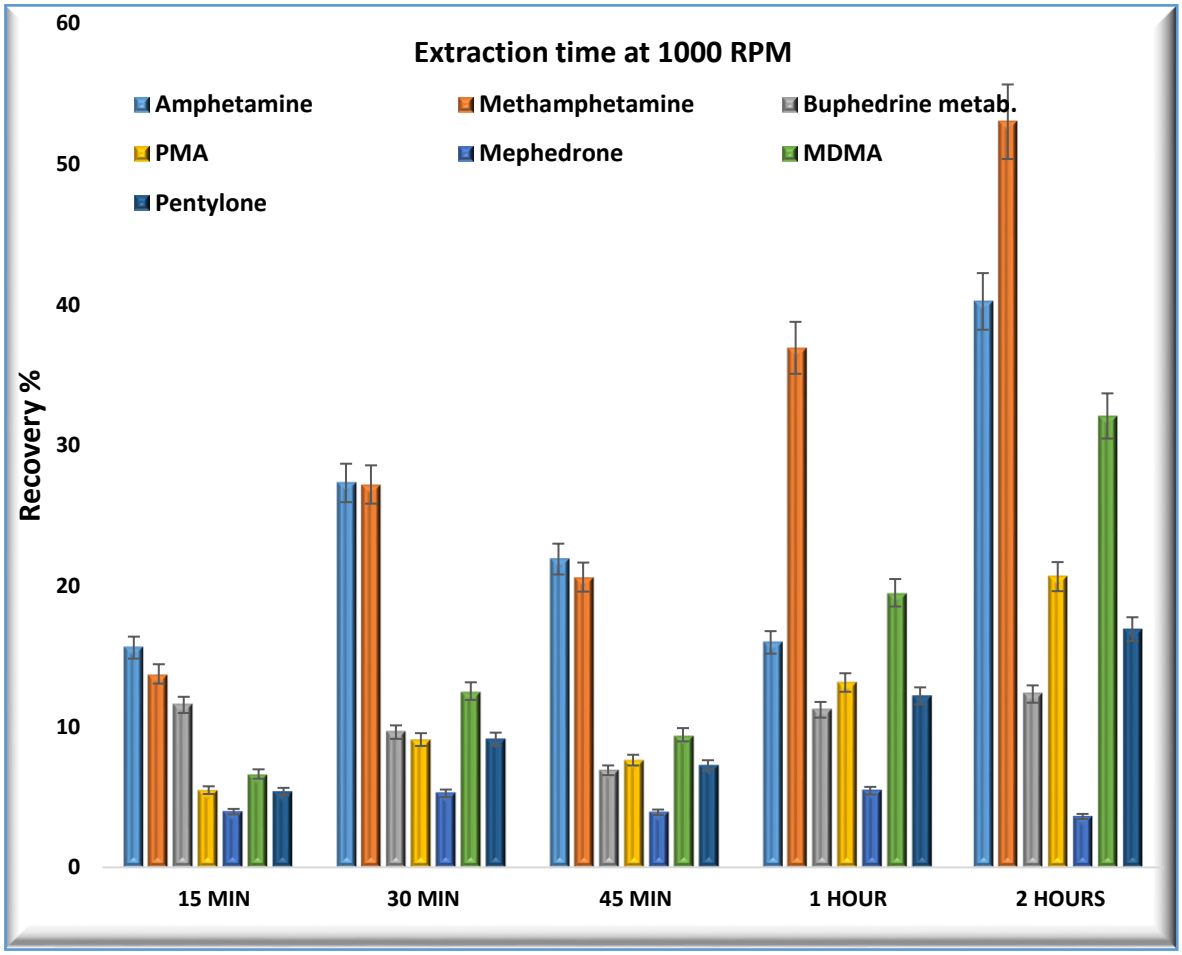
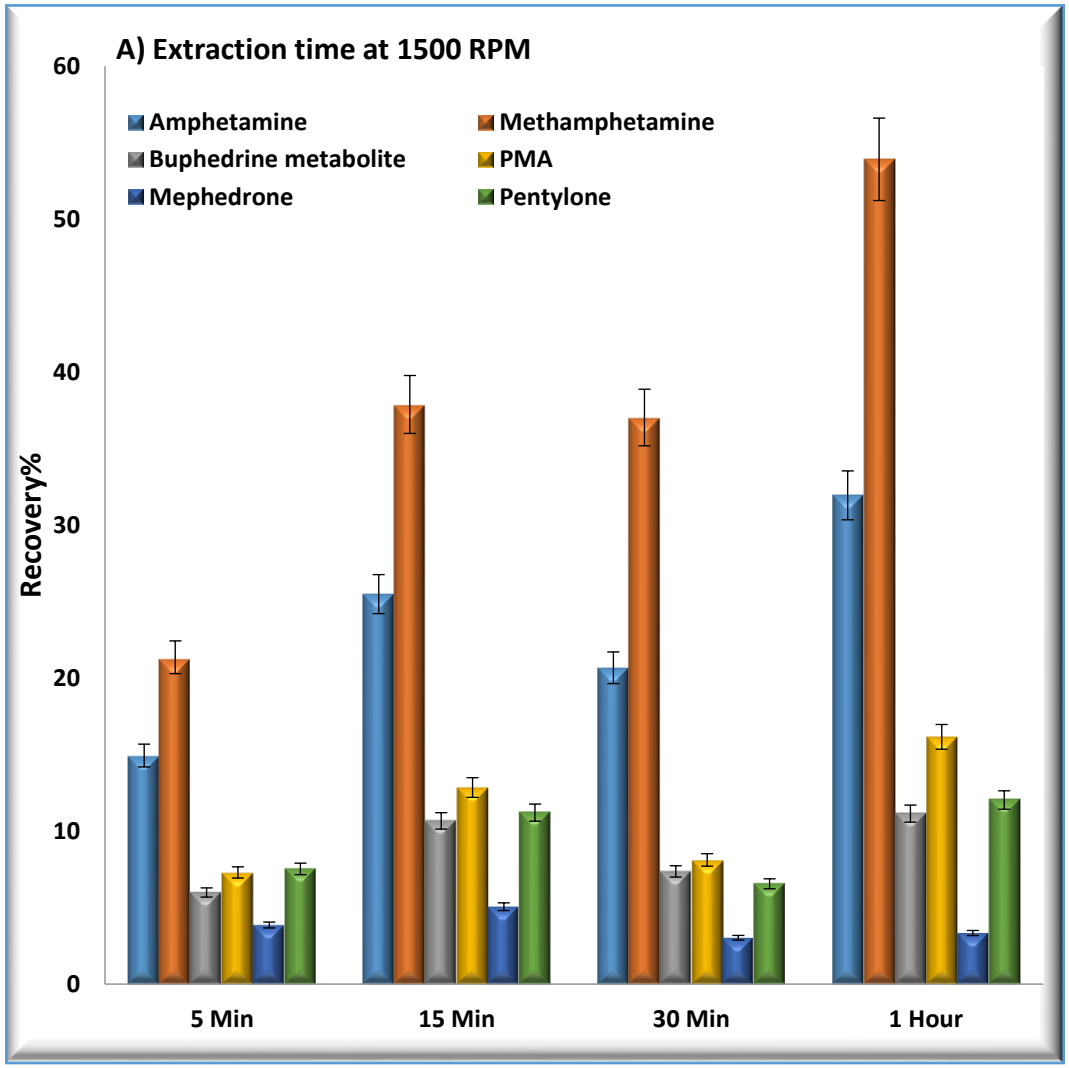


Figure 6-15: The average recovery results (%) on day one for the assessment of extraction reaction time in triplicate urine PDMS/DVB SPME tips at a speed of 100 (n= 3)



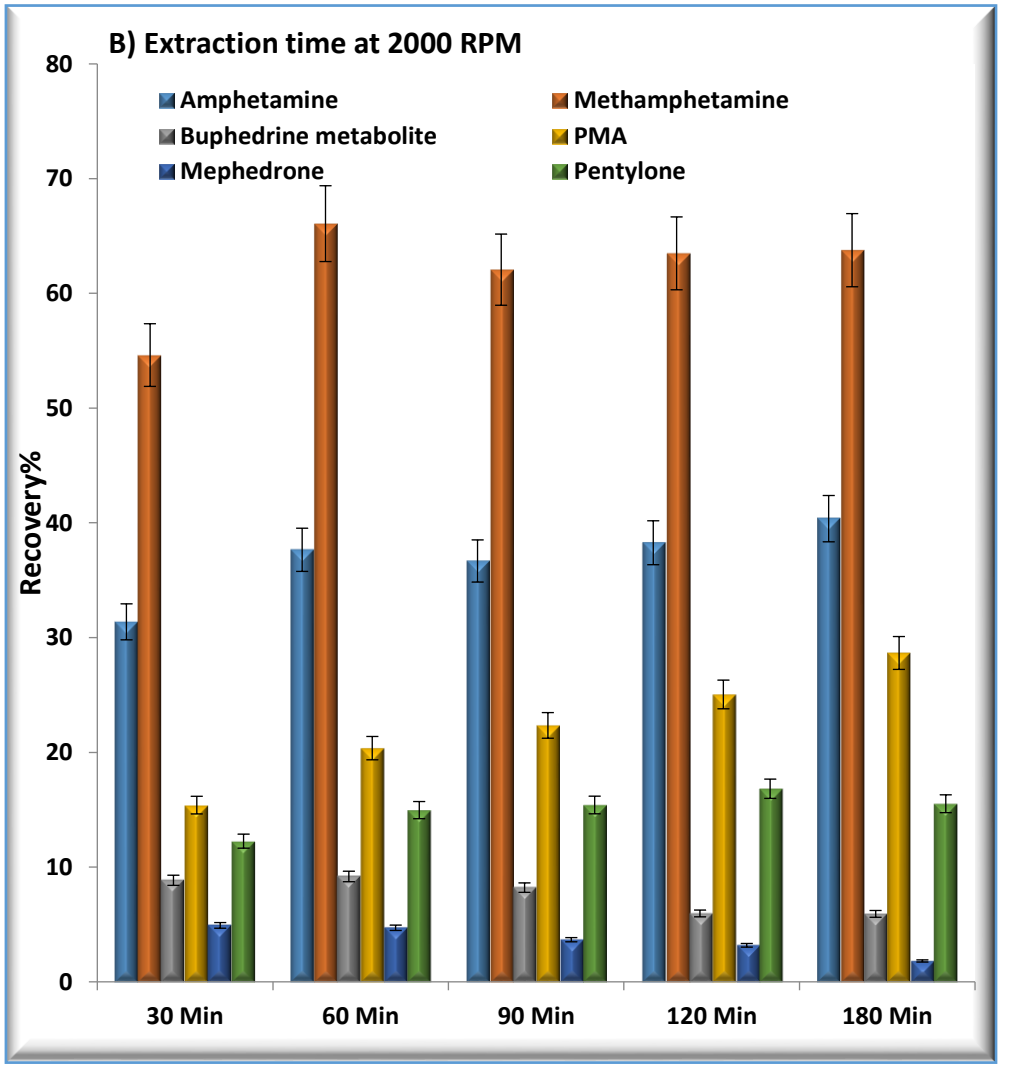


Figure 6-16: Examples of the average recovery results (%) for the estimation of extraction reaction time in triplicate urine PDMS/DVB SPME tips. A) The results at 1500 rpm on day two B) The results at 2000 rpm on day three

6.3.2.13 Step 13: Desorption time and agitation speed

To reduce the extraction time required, the desorption time and agitation speed were varied in the development protocol. The general rule was that the maximum recovery achieved is equal to the maximum analytes that desorbed at that time and speed.

On days one and two, the recovery results in **Figure 6-17** and **Figure 6-18** show that the analytes were fully desorbed during all investigated times. To confirm the above conclusion, a single factor ANOVA was performed to study the effect of agitation time on the recovery results. By observing the results in **Table 6-8**, there was no significant difference when the time was modified. This conclusion is valid based on the methods and materials that were used in the laboratory experiments. Based on the recovery results for the evaluation of agitation speed, it is clear that a speed of 1500 rpm provided higher recoveries than 1000 rpm.

On day three (see **Figure 6-19**), because the analytes were fully desorbed during all times investigated on days one and two, the following times were set for the shaker to determine when the analytes were fully desorbed: 1, 5, 10 and 20. The recovery results show that the analytes were completely desorbed, however the RSD (%) indicated that only the 10 minute duration was valid for all drugs examined. The 5 minute RSD was valid except for mephedrone. The 1 minute duration was above 20% of RSD for most drugs. The 20 minute RSD was greater than 10% for most compounds, and the error increased after 20 minutes. This may result from the effect of speed in the shaker that damaged the fibre in tips. By observing the methamphetamine recovery results in all graphs (**Figure 6-17** and **Figure 6-18**) on all experimental days, it can be concluded that the 2000 rpm speed provided the maximum recovery results. Therefore, the optimum results that were selected for PDMS SPME in tips were using a 10 minute duration and 2000 rpm.

Due to the better performance, the SPME-PDMS/DVB fibre was chosen as the best option.

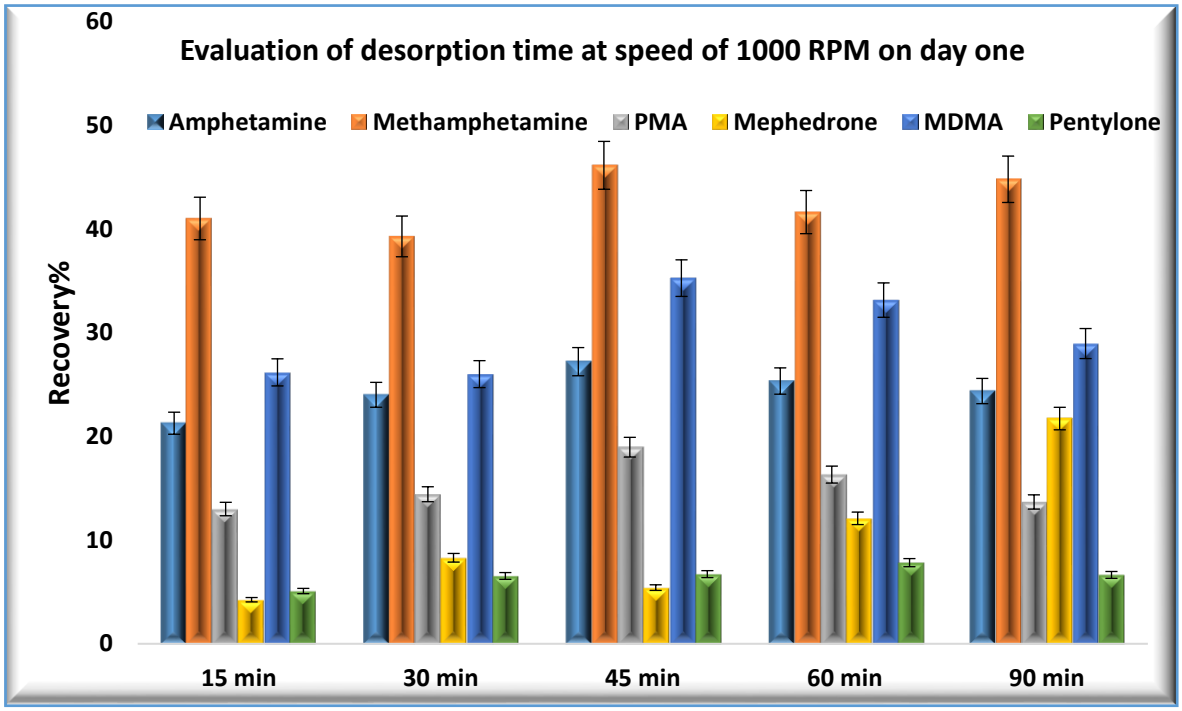


Figure 6-17: Average recovery results (%) at 1000 RPM on day one for selected ATS and SC using triplicate urine specimens and PDMS/DVB SPME tips.

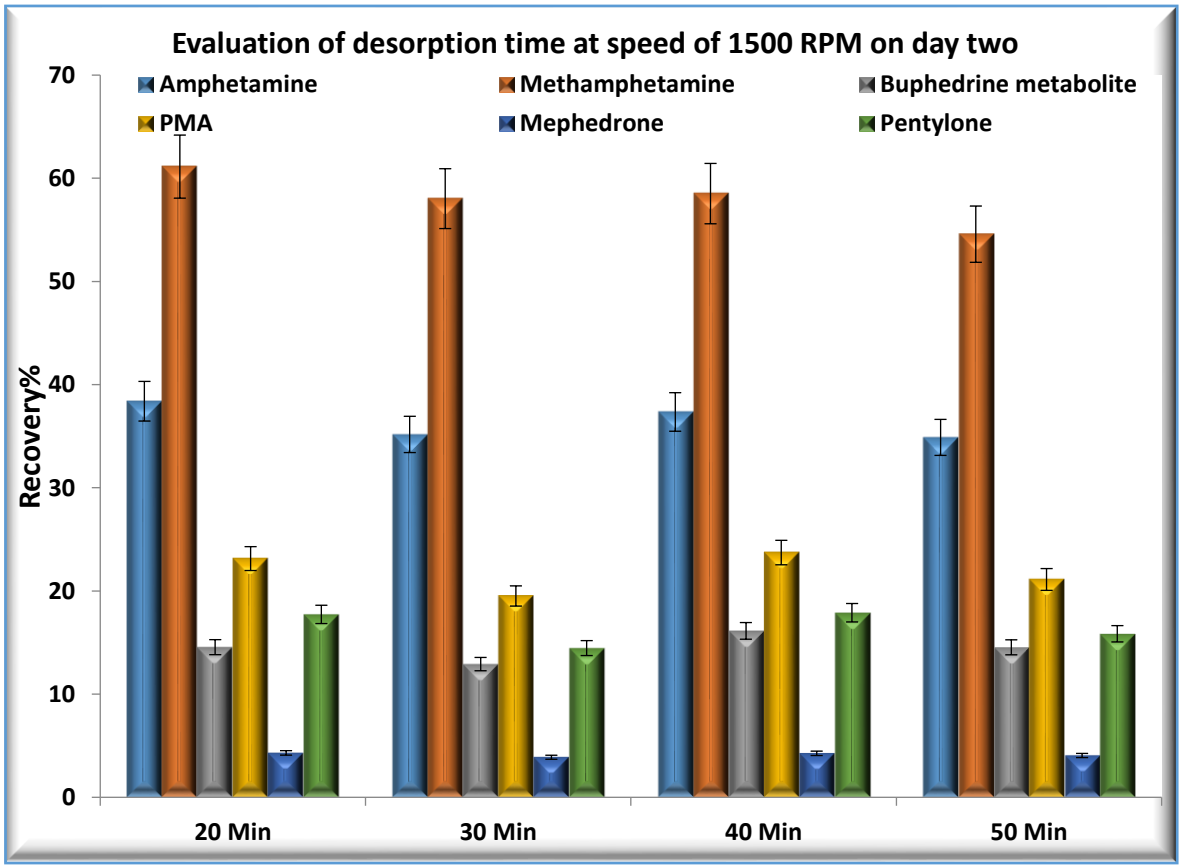


Figure 6-18: Average recovery results (%) at 1500 RPM on day two for selected ATS and SC using triplicate urine specimens and PDMS/DVB SPME tips.

Table 6-8: Single factor ANOVA applied to selected drugs on day two to study the effect of agitation time

ANOVA: Single Factor	P-value	Differences
Compound name	**P <0.05**	**Yes/No**
Amphetamine	0.85	NO
Methamphetamine	0.98	NO
Buphedrine	0.53	NO
PMA	0.54	NO
Mephedrone	0.92	NO
Pentylone	0.66	NO

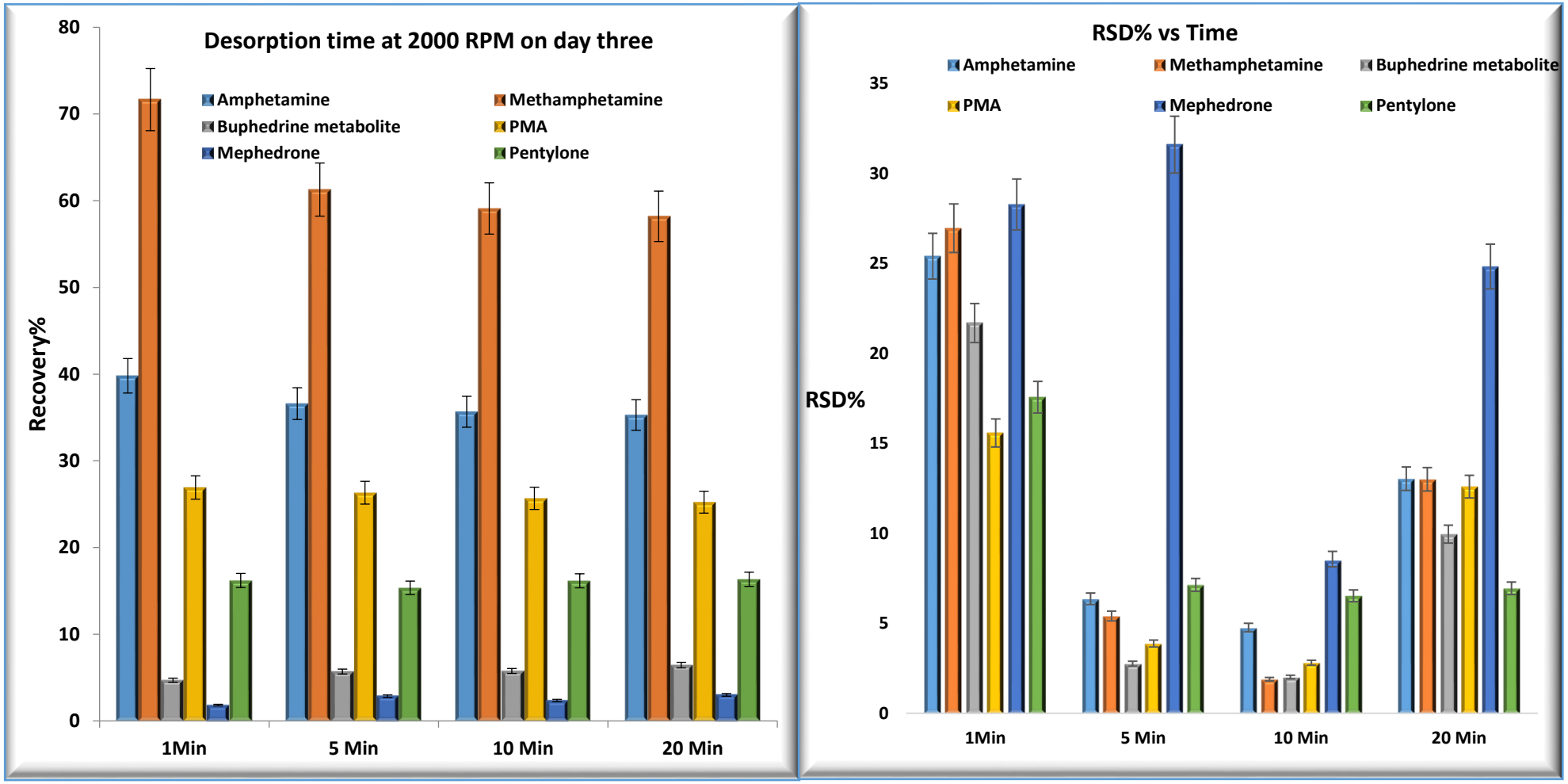


Figure 6-19: The left graph shows the time effect on day 3 for selected drugs using triplicate urine specimens and PDMS/DVB SPME tips based on the average recoveries (%), the right graph is the RSD for each length of time the drugs were tested.

6.3.2.14 The optimum procedure results

The optimised procedure was determined from the results of the previous development processing. The optimum procedure was then selected for the assessment of validation parameters.

The PDMS/DVB fibre provided the best recoveries, responses with the least error in accuracy and precision, and for that reason, only the optimum procedure of the PDMS/DVB fibre will be presented in the results and discussion sections. The procedure is detailed below.

Condition step: The SPME pipette tips coated by PDMS-DVB fibre were inserted into small glass tubes that contained MeOH: d. water (50:50 (v/v)). The fibres were conditioned for 10-20 minutes.

Sampling step: 1 mL mixtures of the eight target analytes in DFU, 100 µL ISD (amphetamine-d_{11} and pentylone-d_5), 100 µL of 10 % (w/v) NaOH and 0.5 g NaCl at a pH of 12.6 were aliquoted, added and mixed using 1.5 mL Eppendorf microcentrifuge tubes (safe-lock). The tubes were then pierced using small needle to permit the tips to enter. The tips were inserted and secured into the contents, ensuring that the tips would not escape during the shaking step.

Extraction step: The tubes with tips were put into a vortex shaker for agitation and extraction for one hour at a speed of 2000 rpm.

Desorption step: The tips were taken out of the Eppendorf tubes and transferred to 0.3 mL vials for desorption using 65 µL of MeOH. A speed of 2000 rpm for 10 minutes in a shaker was used for agitation.

Evaporation step: The samples were transferred to the hot block for evaporation, and 10 µL of 0.1 HCl with MeOH (1:9) was added to all vials. The vials were left at RT until fully dry under gentle evaporation using nitrogen gas (approximately 2 minutes).

Derivatisation step: The 0.3 mL vials were derivatised using 50 µL of PFPA: EtOAc (2:1); immediately mixed, vortexed, capped and left on the hot block for incubation at 60° for 15 minutes.

Evaporation step again: The vials were again placed on the hot block for evaporation at RT. Two to three minutes was sufficient for completion.

Reconstitution step: Vials reconstituted by adding 50 µL of EtOAc.

Analysis step: The 0.3 mL vials were placed on the GC-autosampler for analysis in GC–MS. See **Figure 6-20.**

The laboratory work was carried out to increase the recoveries (%) of the drugs and the three fibres in tips–SPME (C18, C18-SCX and PDMS/DVB) using the strategies from the method development processing stages. After the recoveries were calculated over the processing stages, it was concluded that the PDMS/DVB fibre in tips provided the greatest recovery (2-80%), followed by the fibres of C18 (0.1-10%) and C18-SCX (0.1-10%). The optimum recovery results for the developed method parameters are summarised in **Table 6-9**.

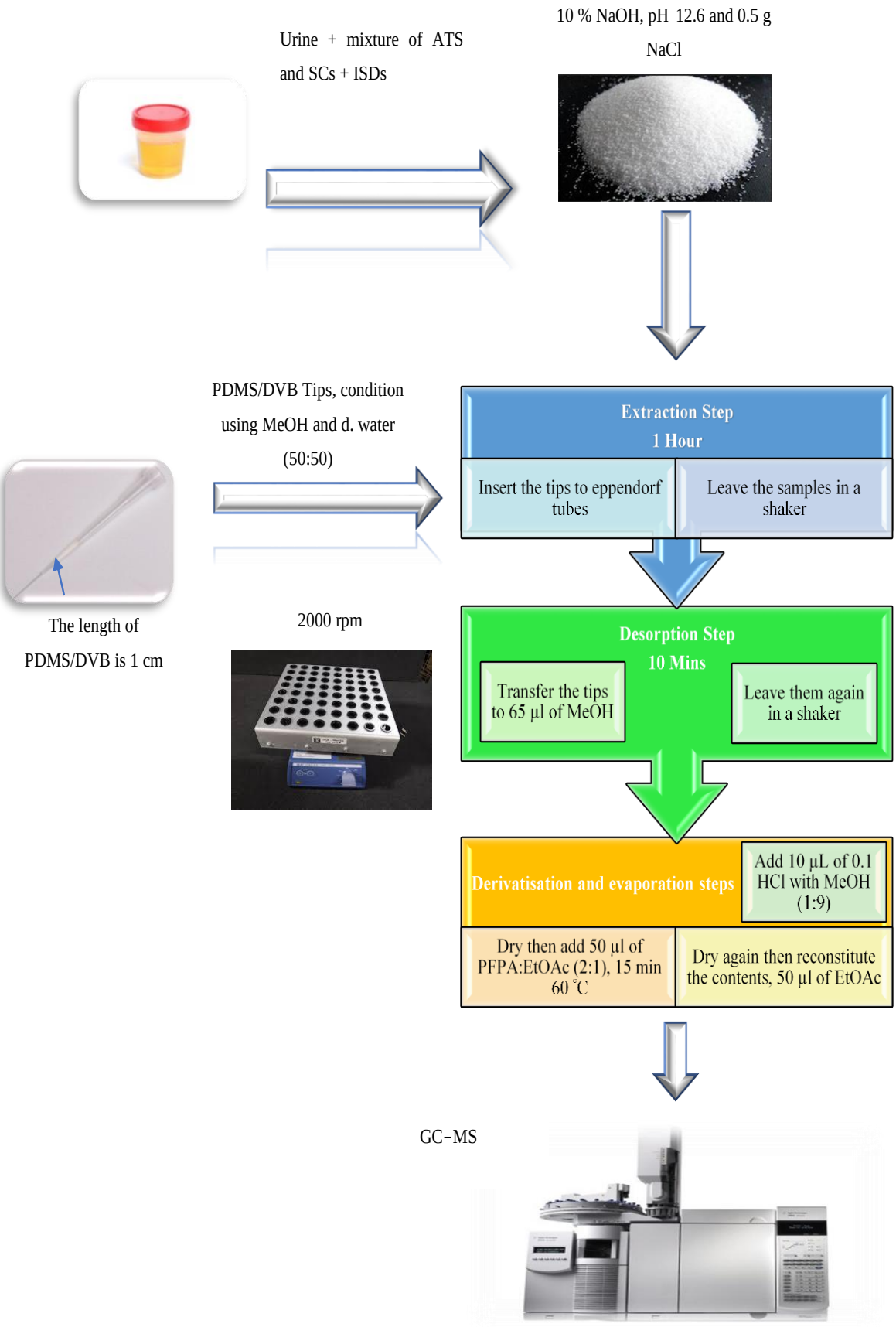


Figure 6-20 demonstrates the optimum procedure for PDMS/DVB-SPME fibre tips applied to mixtures of selected ATS and SC in DFU samples.

6.3.3 Method validation

After the method development work, it is necessary to prove the validity of this method. The new method was also evaluated through validation parameters to verify limitations, problems or disadvantages of the tips. The final optimum method development parameters were used for the assessment of the method validation in PDMS/DVB fibre only.

6.3.3.1 Linearity results

The unweighted linear calibration model was used for all drugs tested. The average R^2 was calculated from 20 calibration curves for each drug. The R^2 was greater than 0.992 for all analytes.

The concentration in each point was also calculated to study the LLOQ using RSD (%) and bias (%). Each point should be within ±20% to be considered valid. The LLOQ was ≥100 ng mL^{-1} for all target analytes. See **Table 6-10** for the linearity results with the average accuracy of the selected points as an example for the evaluation of LLOQ.

Table 6-9: The optimum conditions of the method development parameters, for the evaluation of three fibres in SPME tips

The products were decided according to the most significant recovery (%) for the eight ATS and SC substances at 1 µg mL^{-1} in urine specimen. *Invalid equates to ≥ 20% error of RSD (%) and/or accuracy (± %) in each parameter, fibre and drug alone.

Parameters vs Fibre coating type	PDMS/DVB	C18	C18/SCX
Vial type	1.2 mL kit and 1.5 mL Eppendorf vials	1.2 mL kit and 1.5 mL Eppendorf vials	1.2 mL kit and 1.5 mL Eppendorf vials
Agitation method	Orbital shaker	Orbital shaker	Orbital shaker
pH evaluation	≥11, the highest recoveries were at pH 12.60	pH 2.80	pH 3.30
Ionic strength (w/v)	10% NaOH + 0.5 g NaCl (pH 12.60)	100 µL formic acid	100 µL formic acid + 100 µL 0.1 HCl (pH3)
Salts & acid additives	NaOH + NaCl	Formic acid	Formic acid + HCl
Temperature	RT	RT	RT
Vials type validity	Both Eppendorf vials and vial kits were valid	Invalid*	Invalid
PFPA−derivatisation and analyte studies	After drying	After drying	After drying
Sample volume	1 mL	Invalid	Invalid
Solvent type	All 8 solvents were valid	Invalid	Invalid
Extraction time	≥1 hour	Invalid	Invalid
Extraction speed	2000 RPM	Invalid	Invalid
Desorption time	10 min	Invalid	Invalid
Desorption speed	2000 RPM	Invalid	Invalid
Linearity	0.920-999	Invalid	Invalid
Recovery	2-80%	0.1-10%	0.1-10%

Table 6-10: Linearity (R^2) and LLOQ studies

The average of each accuracy concentration point was used to evaluate the LLOQ.

Drug name	(R^2) n= 20	LLOQ study ng mL^{-1}	LLOQ study Grand mean of accuracy (error ± %)	Drug name	(R^2)	LLOQ study ng mL^{-1}	LLOQ study Grand mean of accuracy (error ± %)
Amphetamine	0.999	100	-4.67%	PMA	0.997	100	19%
		500	-1.49%			500	1.07%
		2000	0.54%			2000	0.27%
Methamphetamine	0.997	100	-2.10%	Mephedrone	0.994	100	13%
		500	-3.87%			500	1.46%
		2000	-1.61%			2000	-0.06%
Buphedrine (buphedrone metabolite)	0.994	100	-12%	MDMA	0.995	100	8.9%
		500	3.57%			500	-5.5%
		2000	-0.35%			2000	1.48%
4-Methylephedrine (mephedrone metabolite)	0.992	100	0.72%	Pentylone	0.999	100	14%
		500	-0.55%			500	2.00%
		2000	2.72%			2000	0.56%

6.3.3.2 Accuracy and precision results

The accuracy (%) and RSD (%) within and between run were successfully provided within an acceptable range. The error values were lower than or equal 15% for both. See **Table 6-11** below for the results.

Table 6-11: Bias and RSD results				
Drug name	Conc. (ng mL^{-1})	Intra-day RSD	Inter-day RSD	Bias
Amphetamine	250	3.78%	2.34%	1.17%
	850	6.3%	2.20%	0.91%
	1500	2.61%	1.97%	-0.66%
Methamphetamine	250	7.9%	3.40%	-0.08%
	850	10%	3.48%	2.77%
	1500	8.7%	4.16%	-0.30%
Buphedrine (buphedrone metabolite)	250	13%	5.7%	6.2%
	850	7.9%	4.30%	-1.82%
	1500	9.7%	5.8%	-2.30%
4-Methylephedrine (mephedrone metabolite)	250	13%	11%	2.95%
	850	9.9%	3.56%	-4.16%
	1500	12%	7.0%	-5.4%
PMA	250	13%	4.28%	-0.34%
	850	9.8%	1.65%	3.04%
	1500	8.3%	3.52%	-1.56%
Mephedrone	250	11%	6.1%	6.6%
	850	13%	2.62%	-3.72%
	1500	15%	2.73%	-6.9%
MDMA	250	12%	6.3%	-2.15%
	850	12%	4.30%	-1.10%
	1500	9.4%	5.6%	-0.25%
Pentylone	250	5.8%	5.5%	2.78%
	850	7.4%	2.20%	-0.24%
	1500	2.70%	1.35%	-0.99%

6.3.3.3 LOD and LLOQ

The LOD results were between 5 and 25 ng mL^{-1} for all the analytes. The LLOQ results were between 25 and 100 ng mL^{-1} for all the drugs investigated. The LOD and LLOQ for each drug is shown in **Table 6-12**. The results show the reliability of the method to quantify all selected drugs of ATS and SC. This statement is true if the concentration of each drug is truly above the value of the LLOQ quoted in the table below.

Table 6-12: Signal-to-noise ratio for LOD and LLOQ

Compound name	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})	Compound name	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})
Amphetamine	5	25	**PMA**	5	25
Methamphetamine	5	25	**Mephedrone**	25	100
Buphedrine	5	25	**MDMA**	10	50
4-Methylephedrine	10	100	**Pentylone**	5	25

6.3.3.4 Interferences and selectivity

There were no observations of peaks from an endogenous study of all blank urine samples that interfere with the $_tR$ of the eight ATS and SC substances using the SIM mode. See **Figure 6-21** for an example of the interference chromatogram peaks in blank urine with no addition of ISD.

The selectivity exogenous study of 21 drugs outlined in **section 6.2.8.4** indicated that no peak was observed that affected the interpretation of the eight drugs of interest in the SIM mode.

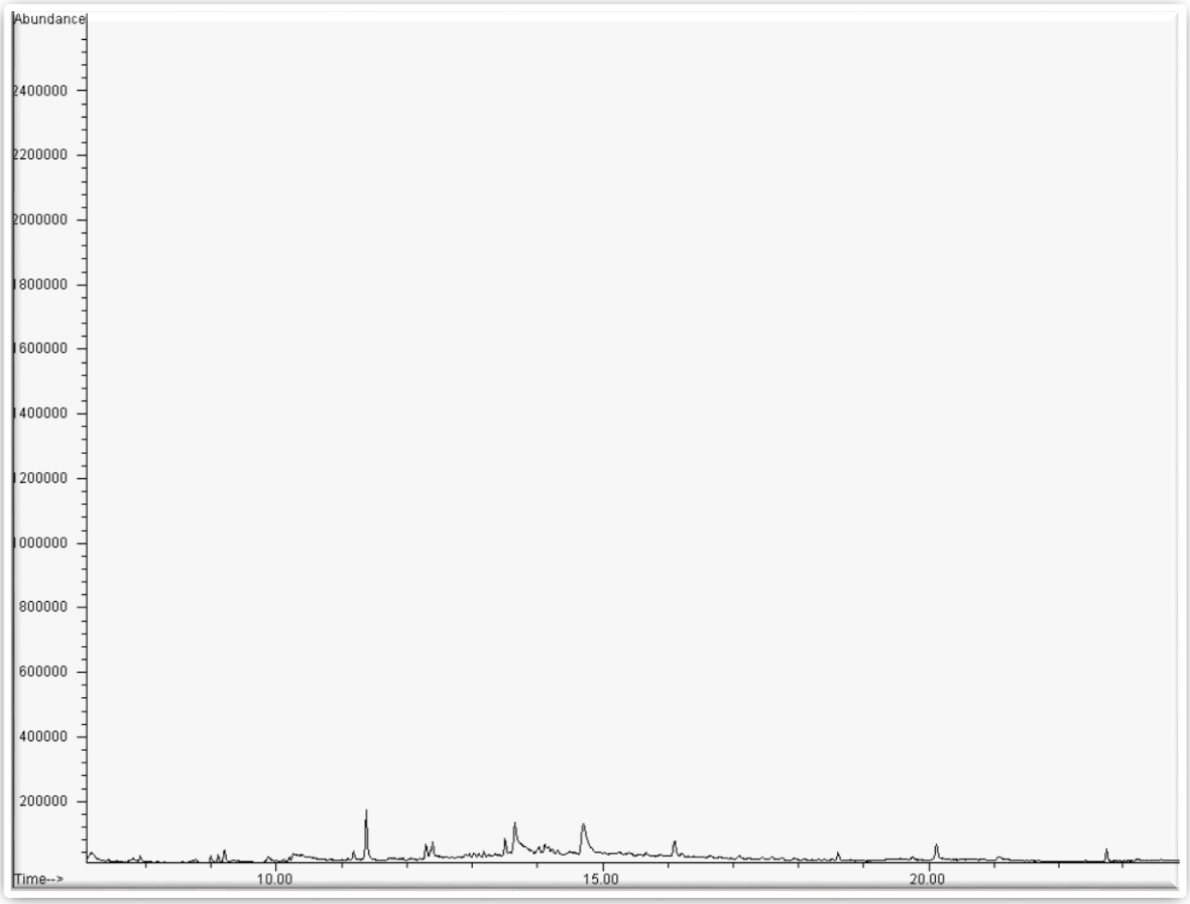


Figure 6-21: An example of a SIM Chromatogram for an endogennous interferences study using a blank urine specimen without the addition of ISD

6.3.3.5 Carryover

The stimulant analytes of interest were not detected in a sample of urine when this sample was run after the 2 μg mL^{-1} of the eight drugs. Carryover was not seen within days of the validation work. No peak had similar $_{t}R$ with ion relative ratios of the eight drugs tested (see the example of carryover result (**Figure 6-22**)).

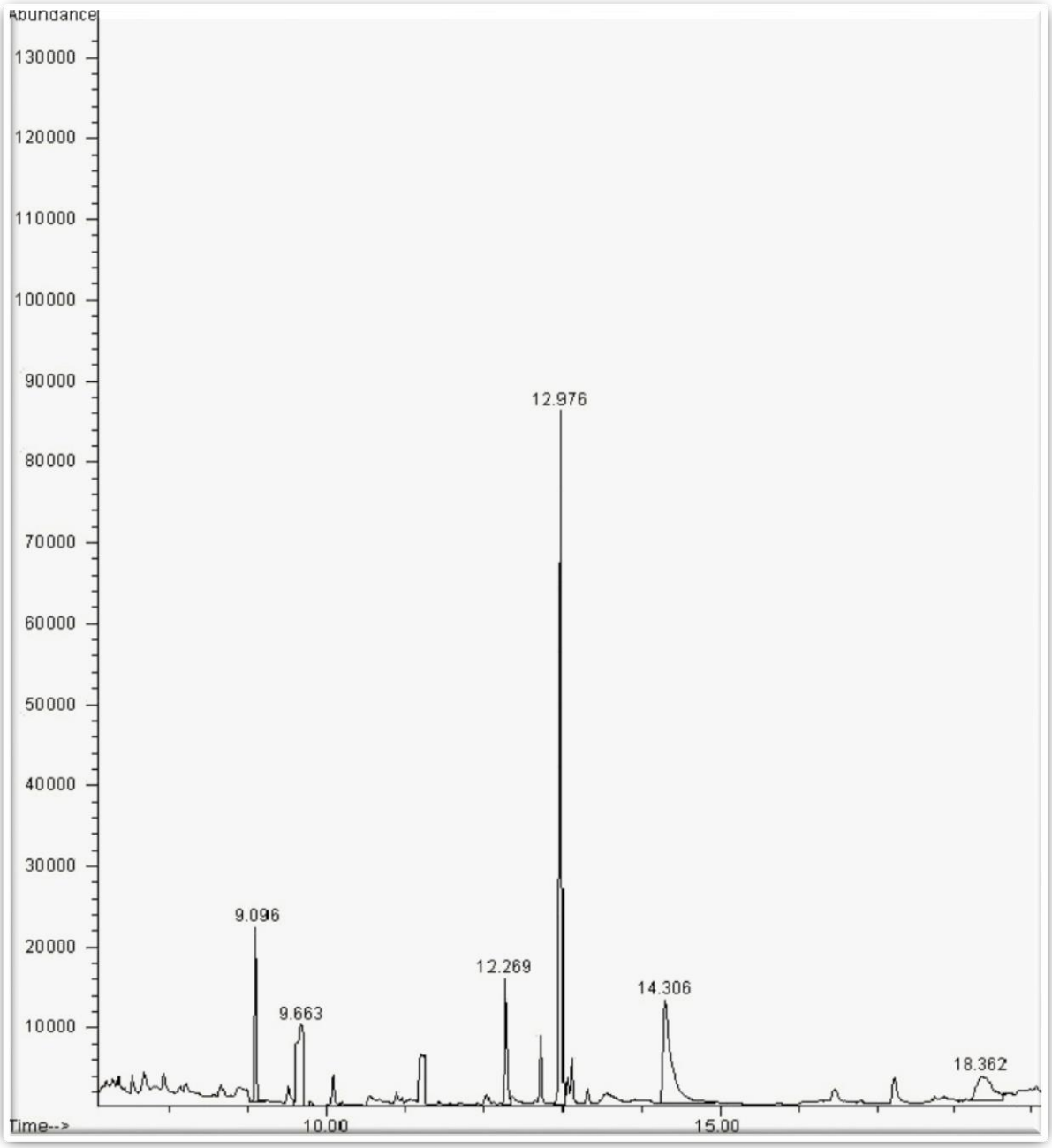


Figure 6-22: An example of a carryover study using a DFU sample

6.3.4 The comparison study between the SPME in tips and SPE

The PDMS/DVB, SPME in tips and SPE methods were applied for the confirmation of three positives of the cathinone compound. The positive confirmatory results obtained from the real human urine case samples were evidence that the SPME and SPE methods have the ability to detect and quantify similar stimulant compounds; in this instance: the cathinone drug.

Repeatability and reproducibility of the two techniques with excellent results for selectivity and sensitivity were successfully validated using the detection of real human urine specimens. See the **Table 6-13** for the comparison outcomes.

Table 6-13: The comparison study of three positives of the cathinone compound in real human urine samples

(This was applied to SPE and SPME tips using GC–MS. The samples were collected from Saudi Arabia under the acceptance approval of ethics).

Specimen vs. the results	The mean conc. of SPE with RSD% (ng mL^{-1})	The mean conc. of PDMS/DVB-SPME in tips with RSD% (ng mL^{-1})
Urine specimen 1	802 (3.99)	806 (9.4)
Urine specimen 2	1209 (3.89)	1201 (8.2)
Urine specimen 3	227 (13)	285 (18)

6.3.5 Green analytical methodology

The evaluation of green analytical methodology is complex and contains many criteria with several diversities of analytes and associated systems that must be assessed. Under certain circumstances, it is difficult to determine the ideal green analytical chemistry (GAC) for the protocol, since method validation parameters are also difficult to attain without the use of hazardous and harmful materials. In the SPME procedure, the solvents, chemicals and reagents were reduced to minimize hazards, while also fulfilling the requirements for achieving method validation.

The fundamental background of GAC for sample preparation techniques and separation chromatography methods was reviewed by Armenta et al. and Galuszka et al. [548, 575] and discussed in [576]. The results for the

comparison study of the three sample preparation methods are demonstrated in **Table 6-14**.

It can be concluded from the comparison study that the total amount of chemicals consumed per specimen using the SPME in tips procedure declined by 95% and 67% compared to SPE and LLE, respectively (95% = ((0.725 ÷ 13.6) × 100 = 5.3% – 100)), (67% = ((0.725 ÷ 2.18) × 100 = 34% – 100)).

For the energy rate study, the uncertainty values were too high to be assessed in the procedure. Therefore, the comparison study for the energy rate is invalid for all sample preparation methods.

The SPME in tips procedure decreased waste by 91% and 49% compared to SPE and LLE, respectively per sample (91% = ((1.6 ÷ 18) × 100 = 8.8% – 100)), (49% = ((1.6 ÷ 3.16) × 100 = 51% – 100). The number of wasted tubes and vials in each sample were: two (Eppendorf and vial Kit), four (culture tube, SPE cartridge, glass tube and GC vial) and three (culture tube, glass tube and GC vial) for SPME in tips, SPE and LLE, respectively. Hence, SPME in tips used less consumables by 50% and 33% compared to SPE and LLE, respectively.

For the health rate study, the extraction method of SPME in tips reduced the total health harm by 33% and 7% caused by using SPE and LLE procedures (33% = ((3 (NaOH) + 1 (NaCl) + 1 (MeOH) + 3 (HCl) + 3 (PFPA) + 2 (EtOAc)) ÷ (1 (phosphate buffer) + 3 (acetic acid) + 1 (MeOH) + 2 (DCM) + 2 (IPA) + 3 (NH_4OH) + 3 (HCl) + 3 (PFPA) + 2 (EtOAc)) × 100 = 67% – 100)), (7% = ((3 (NaOH) + 1 (NaCl) + 1 (MeOH) + 3 (HCl) + 3 (PFPA) + 2 (EtOAc)) ÷ (3 (NaOH) + 2 (DCM) + 3 (HCl) + 1 (MeOH) + 3 (PFPA) + 2 (EtOAc)) × 100 = 93% – 100). This calculation was only valid when the amount of chemicals consumed was neglected in the calculation. Accordingly, it is clear that the LLE may cause more health impacts than the SPME for labourers, because the labourer is consuming and handling larger amounts of chemicals when using the LLE procedure.

For the safety rate study, the method of SPME in tips cut the flammability to 50% and 14% of SPE and LLE levels, respectively (50% = (3 (MeOH) + 3 (EtOAc) ÷ (2 (acetic acid) + 3 (MeOH) + 1 (DCM) + 3 (IPA) + 3 (EtOAc) × 100 = 50% – 100), (14% = (3 (MeOH) + 3 (EtOAc) ÷ (1 (DCM) + 3 (MeOH) + 3 (EtOAc)) × 100 = 86% – 100). Similarly, the flammability is increased with a higher amount of chemicals consumed, which is true when using SPE or LLE procedures.

For the environmental rate study, all sample preparation methods have a similar impact on the environment. Less than 50 g of exhausted consumed chemicals are produced. Hence, no evidence was observed to confirm any differences between the methods.

Overall, according to the above results and discussion, the SPME in tips provided the lowest penalty with minimal impact based on the criteria of assessment as compared to SPE and LLE.

Table 6-14: The comparison study for the assessment of green analytical chemistry applied to three procedures of SPME in tips, SPE and LLE

a) **Energy ranking:** 1= ≤ 0.1 kWh, for example, wet chemistry and very tiny solvents used in evaporation stage; 2= ≤1.5 and >0. 1 kWh, moderate solvents used in evaporation stage and GC was applied. 3= >1.5 kWh, high volume of solvents used in evaporation stage and GC–MS were applied.

b) **Waste ranking:** 1= full waste per sample ≤50 g. 2= full waste ≤250 and >50 g. 3= full waste >250 g.

c) **Health ranking:** NFPA (National Fire Protection Association) score is 0 or 1= slightly toxic and irritant; NFPA 2 or 3= moderately toxic and temporary incapacitation; NFPA= 4 serious injury and exposure.

d) **Safety ranking:** NFPA score 0 or 1= instability score, no special hazards, flammable; 2 or 3= instability score, a special hazard is used, flammable; 4= instability score and flammable.

e) **Environmental ranking:** 1 = <50 g; 2= ≥50 g and ≤250 g; 3 = >250 g.

Method & criteria	Chemicals used per sample				Energy rate[a] (kWh)	Waste rate[b]	Health rate[c]	Safety rate[d]	Environmental rate[e]
	Chemicals amount/ sample	NFBA health rating	NFBA flammability rating	NFBA reactivity rating					
SPME procedure in tips used in this chapter	• 100 µL of 10% NaOH	3	0	0	3 • The time required for evaporation was roughly 5 min and the volume was 75 µL/sample • Agitation speed was 2000 rpm and total time was 70 min • GC–MS	1 The volume of waste per sample was 1.6 mL (urine and chemicals)	3	0 (Flammable)	1
	• 0.5 g NaCl	1	0	0					
	• 65 µL of MeOH	1	3	0					
	• 10 µL of acidified methanol (1:9)	3:1	0:3	0:0					
	• 50 µL of PFPA and EtOAc (2:1)	3:2	0:3	0:0					
	Total amount of the chemicals consumed = 725 µL/sample.								
SPE (see chapter 4 for the procedure)	• 4 mL of 0.10 M phosphate buffer- pH 6	1	0	0	3 • The time required for evaporation was (30-40	1 The volume of waste per sample was 18	3	0 (Flammable)	1

	• 1 mL of 100 mM acetic acid • 5 mL of MeOH • 3 mL of DCM: IPA: NH4OH (78: 20: 2) • 10 µL of acidified methanol (1:9) • 50 µL of PFPA and EtOAc (2:1) **Total amount of the chemicals consumed = 13.6 mL/sample.**	3 1 2: 2: 3 3:1 3:2	2 3 1: 3: 0 0:3 0:3	0 0 0: 0: 0 0:0 0:0	min) and the volume was 2.6 mL/sample • SPE Vacuum was used for 5 min • Centrifuged for 10 min at 3000 rpm • GC–MS	mL (urine and chemicals)			
LLE (see chapter 3 for the procedure)	• 0.1 mL of 25% NaOH • 2mL DCM • 25 µL of acidified methanol (1:9) • 50 µL of PFPA and EtOAc (2:1) **Total amount of the chemicals consumed = 2.18 mL/sample.**	3 2 3:1 3:2	0 1 0:3 0:3	0 0 0:0 0:0	3 • The time required for evaporation was roughly 35 min and the volume was about 2 mL/sample • Centrifuged for 10 min at 2500 rpm • GC–MS	1 The volume of waste per sample was 3.16 mL (urine and chemicals)	3	0 (Flammable)	1

6.4 Conclusion

SPME in tips provided an efficient, clean, convenient, simple, reliable, robust and inexpensive method for the extraction of ATS and SC compounds in urine samples using GC–MS. The technique was developed manually through multiple steps, followed by validation work using the selected parameters. The results of the method development showed that the detection and determination of ATS and SC achieved excellent repeatability and reproducibility using only 60 µL of MeOH. No other chemicals were used except the additive of salts. Moreover, the advantage of using PDMS/DVB-SPME in tips is that the equilibrium between the analytes and the stationary phase arose in one step. One other step was desorption, and no further steps were applied. This increases safety and decreases handling for the technicians. Further benefits of this system include improvements in the economy and environment with less spending on vials, solvents and chemicals.

This procedure used the least solvents and chemicals to meet the requirements of GAC. It had minimal effects with the lowest negative impact on health, waste and safety in relation to LLE and SPE. This method is desirable in forensic toxicology laboratories for the investigation of stimulant drugs in biological samples, such as urine. The optimum procedure involving PDMS/DVB-SPME in tips followed by PFPA derivative presented an effective extraction protocol, followed by GC–MS analysis. The extraction efficiency with a full validation of the technique was completed for the extraction and quantification of four compounds of ATS and four compounds of SC. The tips coupled with GC–MS were able to quantify the analytes even at low specimen volume and concentrations. The time required for the sample preparation procedure was 2.5 hours (this is as an average of 25 tube samples).

The validation parameters provided excellent repeatability and reproducibility with lower than 15% error in accordance to RSD and bias studies. Similar results were observed for the linearity, sensitivity and

selectivity. The LLOQ was sufficient to quantify the following drugs: amphetamine, methamphetamine, PMA, MDMA, mephedrone, buphedrine, 4-methylephedrine and pentylone at concentrations of at least 100 ng mL^{-1}.

The real human urine specimens confirmed a positive of three cathinone substances by using these tips and SPE for a comparison study. Both extraction methods confirmed the results within the acceptance range of errors lower than 20% RSD. The collected samples from Saudi Arabia demonstrated the validity and the suitability of the SPME in tips method for routine analysis of toxicology forensic specimens for the screening and confirmation of the eight drugs tested.

The SPME in tips was developed and validated to enable this procedure to be used worldwide to increase the availability of green analytical methodologies. The GC–MS provided an excellent peak shape and responses for the eight stimulant drugs in 25 minutes using the PFPA derivatives. Additionally, the GC conditions of the chromatogram allowed the separation of two metabolites in human urine samples named buphedrone ephedrine metabolite and mephedrone metabolite. The GC–MS was desirable and preferable for investigating routine specimens in many forensic toxicology laboratories.

7. Drug of abuse and synthetic cathinones in Saudi Arabia

7.1 Introduction

The information obtained from the data collected in prevalence studies were very useful to the medical and forensic communities. The data aided improvements in the quality of patient service, which helped physicians in Emergency Departments to investigate when dangerous levels of toxins were present. The data also assisted in recognising SC misuse trends and addressing sale, trade and supply of them. Moreover, the data may direct the precedencies for national care programmes regarding this subject. There are many ways of collecting the data for the prevalence study. For instance, collecting the biological samples to detect a drug of abuse is the one of most commonly used methods for studying prevalence in a defined population and region. Accordingly, the prevalence studies using the analysis of biological samples for the detection of illicit drugs of abuse and NPS have increased over the last few years, taking the place of questionnaires, interviews and supplements [406, 577-580].

These data, however, can be affected by many factors. One of the greatest limitations is collecting data samples from small sample population sizes. Other factors include self-data reporting, type of matrix and drugs, time of drug consumption, stability of drugs and drug concentration. Usage of self-reporting decreases the trustworthiness of a prevalence study. Furthermore, the information attained from the analysis of a survey is frequently insufficient to accomplish all objectives. For instance, within a survey it is very challenging to investigate all SC that may have been consumed. However, the one tool regularly used in a prevalence study is collecting biological specimens to investigate the types and concentrations of SC in the body [578]. Therefore, examination of biological matrices is an essential practice in forensic toxicology when determining whether a person has taken a substance during a specific time period. This will influence a person's behavior, which is relevant, for example, in medico-legal cases. In this project, urine specimens were used to estimate the prevalence of SC use in Saudi Arabia.

In spite of the proposals from several sources, [31] suggested that the misuse of NPS involving SC was prevalent around the globe, though no data currently exists on internet or literature that provides information on SC use in Saudi Arabia. In many countries, SC substances have been legally controlled in order to reduce the flow. In Saudi Arabia, the General Directorate of Narcotics Control (GDNC) of the Saudi Interior Ministry in cooperation with the Saudi Food and Drug Authority (SFDA) regulate and implement the legislation on narcotic drugs, medical devices, chemicals and biological substances. The GDNC (105 branches) with the help of the National Committee for Combating Drugs (Nebras) launch several educational (lectures, workshops, and training) and prevention programs to raise awareness on the harmful effects of illegal substances. One program also purported to reveal the substance traffickers' techniques and was entitled "The serious scourge and keeping society safe" [581].

181 million Captagon fenethylline tablets, 2.206 tons of cathinone, 61 tons of cannabis and 222 kg of heroin have been detained with total value of over £3 billion in Saudi Arabia between 2010 and 2012. This represents 10% of the entire quantity of illegal drugs smuggled into the kingdom. It is estimated that roughly 60% of crimes were drug-related, resulting in the arrest of 119 people for drug offences. The most common drugs were Captagon tablets, khat, heroin and hashish. Saudi Arabia accounts for 30% of all worldwide amphetamine seizures. Captagon is very widespread amongst scholars, particularly before exams, as it is thought to boost performance. It is used by staff that have strenuous jobs, such as drivers and labourers [582].

However, the appearance of NPS involving SC was not considered by the government of Saudi Arabia in the same way as traditional illicit substances.

The narcotic substances in Saudi Arabia (SA) are controlled based on the United Nations Conventions on drugs (1961, 1971, 1972 and 1988) and the International Narcotics Control Board [583-585]. Schedules of the Convention on Psychotropic Substances of 1971, updated on 18 October 2017 list the following controlled drugs of SC (these are the only SC drugs included in this thesis): cathinone, methcathinone, pentedrone, 4-MEC, mephedrone, ethylone, methylone, MDPV,

pyrovalerone and α-PVP. Other parent cathinone drugs that are still not specifically listed in SA include flephedrone, buphedrone, N-EC, methedrone, butylone, 4-EMC, pentylone, MDPPP and naphyrone. However, SA has covered any narcotic and psychotropic drugs that are not taken under medical supervision [586].

It is important to comprehend the prevalence of illegal substance use globally. The actual prevalence of illegal substance use in Saudi Arabia is unidentified because of the lack of epidemiological population reports or studies [587-589]. Nonetheless, it can be estimated by using authorised statistics and data of drugs, crime, death and seizures.

For many years, amphetamines dominated synthetic drug markets in the Middle East [590]. More than 56% of global seizures of amphetamines were in the Middle East and South-West Asia, consisting of 12 tons in 2012 alone [578]. Fenethylline (7-(2-a-methylphenyl-aminoethyl)-theophylline), well known as Captagon ('Abu Hilalain') is a hugely abused substance in Saudi Arabia, making up 40% of the total of illegally consumed substances in the kingdom. It is primarily used by juvenile and young people between 12 and 22 years old, as well as in militant groups [591]. Fenethylline is mixture of amphetamine and theophylline, with a formal name of amphetaminoethyltheophylline. It was synthesized in 1961 and was used for the treatment of hyperactivity disorders, depression and narcolepsy under medical supervision until 1986 [591, 592].

Drug use amongst Saudi adolescents is a rising concern, and drug smuggling remains a problem along the security border areas. On a weekly basis the newspapers and Saudi Press Agency [245] announce large drug seizures, which constantly contain fenethylline, cannabis or alcohol. For instance, five million Captagon tablets and 350 grams of cannabis was found at the Halet Ammar customs checkpoint on 12 March 2018 [593, 594]. Principal punishment for narcotics smuggling is commonly imposed, and according to media broadcasting, 63 individuals were executed for drug smuggling in 2015 [595]. In the same year, methamphetamine was being injected rather than smoked and first appeared in youth at a hospital in Jeddah city in the western region. ATS was found to be used

on a daily basis in the eastern provinces and resulted in 1000 confirmed problem drug users which is more than double the number of opiate users (450 cases). The number of people treated for ATS drug abuse was greater than 50% of total number of people treated for all types of illegal drug use in the country. 5200 people visited the emergency-room due to drug abuse, and the highest mortality rates were due to the use of opiates in 2015 [596]. Greater than 60% of all crime in SA is associated directly or indirectly with the use of illicit drugs between 2010-2012 [582, 597].

In spite of illicit drugs being strongly prohibited under the Islam religion and the social stigma, many Saudi people are addicted to illegal substances and alcohol. There are five cities in five different regions of Saudi Arabia where drugs are more accessible: central (the capital Riyadh), south (Jizan), western (Jeddah), east (Dammam), northwestern (Tabuk). Alcohol can be easily smuggled through the bridge between Bahrain and Dammam. Dammam and Jeddah have ports that increase the availability of drugs. Jizan is a well-known hub for the use of khat (prevalence = 21.4% [598]). No prevalence studies of the epidemiological population have estimated the prevalence of substance abuse in Saudi Arabia. However, there were several studies investigating the prevalence of drug abuse patients in treatment.

All these are indicators of the size of the illegal drug abuse problem within the population of Saudi Arabia.

Drug abuse has been recognised as a public health issue. The Saudi government constructed three specialised hospitals for drug abuse treatment in three different regions of Saudi Arabia: Riyadh, Jeddah and Dammam, treating both male and female patients.

In central Saudi Arabia, Al-Nahedh determined that 29.5 years was the average patient age in Al Amal hospital (Al-Amal means “hope” in Arabic) in Riyadh. This hospital has treated drug abuse patients since 1998, with an average of abuse duration of 9.5 years. The mean age of first exposure to substance use was 19 years. The sample size was 160, using a questionnaire survey. Alcohol was used by 23.75% of patients, with other drugs including sedatives (23.12%), heroin (18.75%),

cannabis (10.63%), glue sniffing (9.38%) and 14.38% of patients used a combination of two or more drugs [599]. 423 patients (based on clinic epidemiological information within the Al-Qassim region) reported that alcohol was the most used substance at 52%, followed by amphetamines (25%), heroin (7%) and cannabis (4%) in 2000 [600].

In western Saudi Arabia, 799 patients were surveyed in a voluntary detoxification unit within drug treatment services of Al-Amal hospital, Jeddah. This study was carried out in 1996 and 1997 with 68% of participants under 35 years of age. 97% of participants were tobacco smokers and 55% commenced smoking prior the age of 15. 64% started taking drugs prior to the age of 25, and 34% had been on the drugs for less than five years. 87% abused alcohol or heroin, with 33% and 66% of patients initiating use prior the age of 20 and 25, respectively [601]. At the same hospital, another study was conducted by questionnaire including 101 patients between July and August, 2002. The average age of the patients was 29.6 years, with 65% having used two substances or more. 2% patients had used cocaine, 8% benzodiazepines, 25% heroin, 61% cannabis, 72% amphetamine and 89% had smoked tobacco [602]. Osman reported in same region of Jeddah that heroin (43%) and alcohol (16%) were the most common drugs abused over the course of one year. This study was undertaken in a psychiatric hospital with 485 patients in 1992 [603]. Heroin and alcohol were also found to be the most commonly abused drugs in 2000 (799 patients) and 2001 (302 patients) in Jeddah, at 70% and 68% for heroin and 18% and 21% for alcohol, respectively. 116 patients in Al-Amal were investigated by the psychiatric team in 1995. The findings showed that 84% of the patients abused heroin, 31% alcohol, 26% cannabis and 10% used stimulants [604].

The high percentage of heroin abuse may reflect the highly addictive nature of the substance. Osman in 2003 documented 67 serious medical complications that resulted from heroin addiction in Jeddah, with two deaths during the treatment of 48 patients [605]. Therefore, the percentage of use in these studies does not reflect the actual prevalence of drug use in Saudi Arabia, but instead is only a measure of the prevalence of Saudi patients already in addiction treatment settings.

In the south and south west regions, a study was aimed at measuring the prevalence of chewing Khat (cathinone, cathine, cathidine, eduline and ephedrine [73, 606]) amongst 10,000 students (15 and 25 years old) at college and secondary school in May 2006 in the Jazan region using a self-administered questionnaire. The prevalence of khat was 21%. The findings also showed that the Khat use was significantly different depending on gender, age and education [598].

A study of prohibited compounds (doping) was assessed by Al Ghobain et al. The prevalence was 4.3% among 1142 male sport players, using systematic random sampling techniques in 18 cities across all regions in Saudi Arabia in 2016 [607].

A study was conducted with 143,833 patients that visited the Emergency department in the University hospital in Dammam. 5574 were admitted and 253 (4.5%) were drug-related cases. From the 253 admissions, 19.8% patients suffered from overdose toxicity, 11.5% were drug-interactions, 10.3% were accidental or suicidal drug ingestions, 7.1% were drug abuse (opiates (morphine and heroin), cannabis, benzodiazepines, clozapine, amphetamine and alcohol) and 3.2% were allergic reactions. 26.5% patients stayed in the hospital for 7-102 days and 4% died [608].

In Saudi Arabia, new employees in selective positions and sectors must undergo laboratory tests, such as drug abuse testing. This workplace testing is similarly carried out for military staff when a promotion is received. Job applicants in military, transport and medicine sectors are also required to comply with workplace drug abuse testing. Random workplace drug abuse testing is also carried out during selection of military staff in certain situations. Forensic toxicology laboratories are used to investigate these drugs. Forensic toxicologists must detect and report the results of examined abused drugs using screening and confirmatory methods of human specimens, such as urine, following specific guidelines; i.e. Substance Abuse and Mental Health Service Administration [195] or European Workplace Drug Testing Society (EWDTS) guidelines [609, 610]. There are hundreds of laboratories for drug testing distributed across the country for the detection and screening of amphetamines, cannabinoids, opiates, barbiturates, cocaine, benzodiazepines, alcohol and methadone. Thousands of urine samples are

investigated every day. Ministries of Interior and Health select specific laboratories to carry out the examination of positive samples for confirmation. However, no prevalence or statistical information were found from all these examined samples; this may be due to ethical or confidential considerations.

To estimate the prevalence of the selected SC within workplace drug abuse testing in Saudi Arabia, a study was performed in participation with the Security Forces Hospital (SFH) in Riyadh City, Saudi Arabia. This study is exclusive, as no previous publications have investigated SC amongst Saudi citizen to assess the incidence. Urine specimens were collected from workers or random people that visited SFH over a period of a month. The urine matrix was preferred because it is easy to collect with less contaminants than other matrices. It can also deliver evidence of parent drugs and metabolites even in low concentrations. Certain substances remain in the body for several days after the drugs are taken and can still be detected in urine.

7.1.1 Aim

It is important to determine the range of substances that have an effect on a specific population. The purpose of the study was to estimate the prevalence rate of SC use in specific population in Riyadh City in Saudi people within a month period by examining urine specimens collected in July 2017. This study correspondingly provided exclusive information on SC, which could help to better understand the estimation prevalence rate of SC in the new region of Saudi Arabia. The prevalence study was conducted in Riyadh City at Security Forces Hospital (SFH), which is one of the largest health care providers in Saudi Arabia.

7.1.2 Ethical approval

Procedures in this project complied with the guidelines written by the College of MVLS Ethics Committee for Non-Clinical Research Involving Human Subjects at the University of Glasgow (See Appendix 2). All procedures were approved by the Research Committee at SFH in Riyadh City in Saudi Arabia (See Appendix 3). The

committees individually reviewed the proposed research study and agreed that there is no objection on ethical grounds.

For the ethical approval, three forms were submitted consisting of the application form, the participant information sheet (Appendix 4) and the consent form (Appendix 5).

7.1.3 Study design

Participants were both male and female Saudi citizens. Urine collection was undertaken by the toxicology department in SFH in order to provide urine specimens for drug abuse testing. The urine samples were collected for the study was over a month-long period (every official work day in July 2017 (10-12 am). Participation in the study was voluntary. If the donor accepted, the information sheet was provided. Most volunteer participants were submitted to regular urine drug abuse screening to complete requirements for specific jobs or promotions. Participants were completely informed concerning the objective of the study and were permitted to withdraw from the study at any time without objection. After a volunteer read the information sheet and approved donation, the consent form was read and signed. Any individual below the age of 18 was rejected from this study. An empty plastic container was given to the donor to collect a urine specimen. Urine collection was performed by the researcher (author) under the supervision of a urine collection employee from the SFH administration.

Consent and the urine specimens were collected at the same time. Each urine specimen had a distinct number and the consent form was signed by the participant at the time of the urine collection. The consent form was separated from the urine specimen to ensure anonymity; i.e. there was no labelled number on the consent forms to link to the urine sample. This procedure yielded completely anonymous urine specimens, which at no point could be associated to any person. 273 individuals agreed to participate and provided urine samples. Therefore, each urine container had a unique number from 1 to 273. Each participant provided a single urine sample on a single occasion. Each urine specimen contained approximately 10-20 mL. The participant was given 5 minutes

to donate the specimen. The location was appropriate for the collection of urine samples and the system respected SAMHSA guidelines. The study design within the stage of urine collection is summarised in **Figure 7-1**.

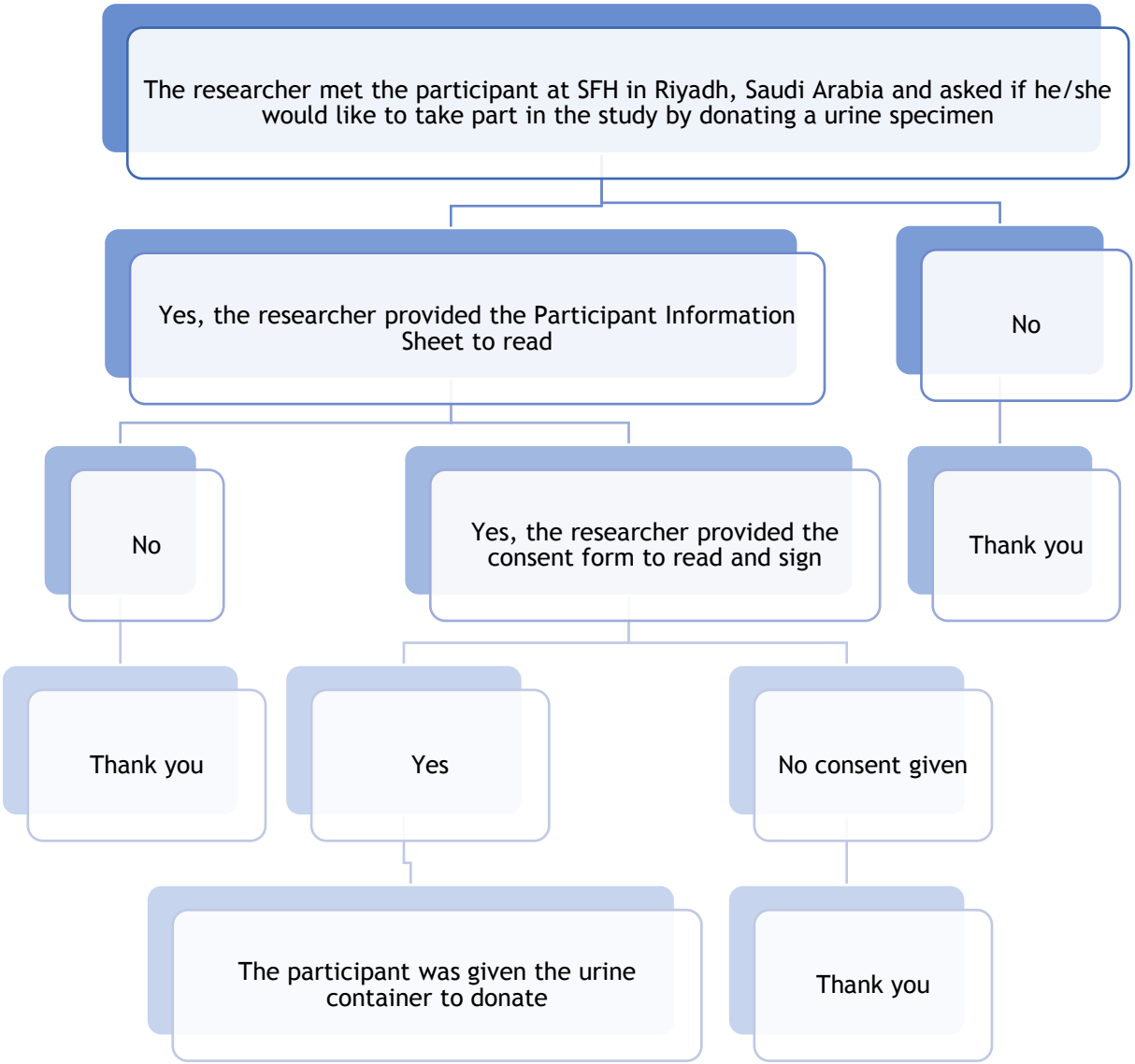


Figure 7-1: The study design in the urine collection stage

In the toxicology laboratory, a total of 273 urine specimens were collected, securely saved in the toxicology laboratory and stored at −20°C in the freezer while awaiting shipment. The toxicology laboratory at SFH had accreditation with the Standards Council of Canada, which indicated capability, robustness and competence of the laboratory. Human urine specimens were chilled during transportation and were delivered to the Forensic Medicine and Science (FMS) department at the University of Glasgow by FedEx within two days. The condition

of the specimens was confirmed directly by the author after receipt and the specimens were kept at −20°C, pending analysis.

7.2 Method and materials

Since the profile of using synthetic cathinones in Saudi Arabia is unknown, the selected drugs were those that were prevalent in other populations, such as in the UK and the USA. The chemical structures of the selected substances are illustrated in **Table 2-2**. The policy and procedure of this work were completed according to those used in the toxicology department of SFH. Each sample was analysed after few days from the completion of urine collection using GC−MS in the Forensic Medicine and Science (FMS) laboratory at the University of Glasgow to detect SC compounds. In the forensic toxicology laboratory, all 273 samples were prepared and analysed by aliquoting 1 mL urine sample then using the procedure of SPE, evaporation, PFPA, evaporation again and reconstitution, following the method and materials of chapter 4. A calibrator, three QCs, a DFU and 39 specimens were analysed each day, over seven working laboratory days. There was a one-day gap in between each working day, because each batch required more than 45 hours of run time. In each sample, the SIM mode was used to investigate the target cathinones (cathinone, flephedrone, buphedrone-ephedrine (metabolite), methcathinone, 4-methyl ephedrine (metabolite), 4-methyl-N-ethyl-norephedrine, buphedrone, N-ethyle cathinone, mephedrone, pentedrone, 4-MEC, 4-EMC, methedrone, α-PVP, methylone, butylone, ethylone, pyrovalerone, pentylone, MDPPP, MDPV and naphyrone) , while general cathinones with metabolites were investigated using the scan mode. See Table 7-1 for the drug selection ions with their ISD.

Table 7-1: Drugs analysed with their ions during the analysis of Saudi samples. Quantification ions in bold. The other related ions were selected as qualifier ions with their ratios (%).

Drug name	$_tR$	m/z	Ratio (%)	Drug name	$_tR$	m/z	Ratio (%)
AMPHETAMINE-d_{11}	8.422	**194**	100	PMMA	12.408	**121**	100
		128	72			204	159
						148	102
		98	33				
						160	42
CATHINONE	9.937	**105**	100	4-EMC	12.472	**133**	100
		77	79			204	20
		51	36			160	10
		190	9.2				
FLEPHEDRONE	10.024	204	100	METHEDRONE	13.330	**135**	100
		123	59			77	8.0
		95	19			160	6.1
		91	14			204	9.1
BUPHEDRINE	10.030	**218**	100	PVP	14.384	**126**	100
		119	12			77	6.1
		308	2.6			105	3.2
METHCATHINONE	10.316	**105**	100	METHYLONE	14.716	**149**	100
		204	102			204	19
		160	36			160	11
4-METHYLEPHEDRINE	10.403	**204**	100	BUTYLONE	15.667	**218**	100
		119	13			149	480
		160	18			160	35
		308	2.60			367	20
4-M-N-E-NOREPHEDRINE	10.706	**218**	100	ETHYLONE	15.923	**190**	100
		119	20			149	655
		190	22			218	198
		322	2.50			367	22
BUPHEDRONE	10.905	**218**	100	PYROVALERONE	16.564	**126**	100
		105	51			91	4
		77	21				
		160	23				
N-EC	11.091	218	100	PENTYLONE-d_3	16.941	**193**	100
		105	45			235	86
		190	40			149	380
		77	23				
PMA	11.110	**121**	100	PENTYLONE	16.946	**149**	100
		148	42			190	22
		190	5			232	19
		311	7.4			381	5
MEPHEDRONE	11.482	**119**	100	MDPPP	18.703	**98**	100
		204	25			149	10
		91	20				
		160	14				
PENTEDRONE	11.668	**232**	100	MDPV	20.539	**126**	100
		190	59				
		105	45				
		337	3.90				
4-MEC	12.220	**119**	100	NAPHYRONE	22.667	**126**	100
		218	37				
		190	30				
		91	17				

7.3 Results and Discussion

The 273 collected urine specimens were investigated using GC–MS. The compound of cathinone was positive in three urine samples (1.01%). The comparison concentration study was discussed in chapter 6 for the three positive samples using two different sample preparation methods. No other cathinone substances or metabolites were found for all examined samples, particularly the following drugs and metabolites: cathinone, flephedrone, buphedrone-ephedrine (metabolite), methcathinone, 4-methyl ephedrine (metabolite), 4-methyl-N-ethyl-norephedrine, buphedrone, N-ethyle cathinone, mephedrone, pentedrone, 4-MEC, 4-EMC, methedrone, α-PVP, methylone, butylone, ethylone, pyrovalerone, pentylone, MDPPP, MDPV and naphyrone. However, other SC or metabolites that are not listed above may have been present in the specimens at low concentrations. Other exogenous or endogenous substances in the matrix could have detected and interfered with the SC in scan mode, therefore the method cannot identify all SC. The method was only robust for investigating the selected 22 SC drugs, as other SC lacked reference standards. Hence, the data were interpreted with caution.

Even though the result of the prevalence study for the cathinone compound was 1.01%, this study had several limitations. The first one was the small sample size of only 273 samples. The plan was to collect as many urine samples as possible within one month, and though more samples were sought, some volunteers refused to participate. The people that may take SC or other substances could refuse to donate, due to the sensitivity of the subject. As a result of ethical practices, it was not possible to include routine workplace testing without the agreement of organisations and participants. These large samples could provide much better understanding of SC prevalence. No information was ethically accepted to identify age, sex or other positive results other than the drugs related to SC compounds.

Furthermore, the study was completed within a single society in Riyadh City that may restrict the demonstrative. The stability issue was another concern, because transportation of urine specimens overseas may have affected the findings even though samples were kept in the freezer condition throughout. Therefore, the

study might not reflect the SC prevalence a in large population such as Saudi Arabia. The government in cooperation with institutions are able to investigate thousands of biological samples in many cities across Saudi Arabia and could determine the actual figure for SC prevalence. Even though the results show low prevalence of SC use in Saudi Arabia, this study was the first attempt to investigate the issue. A prospective population-based study is recommended in order to fully investigate and understand the progression of drug involvement in Saudi Arabia. The documentation of factors that lead to substance is necessary to aid in treatment and rehabilitative measurements.

7.4 Conclusion

273 human urine specimens were collected in July 2017 and investigated for the purpose of studying the prevalence of SC in Riyadh City, Saudi Arabia. The 22 SC with metabolites that were analysed using the SIM mode in GC–MS were: cathinone, methcathinone, buphedrone metabolite, flephedrone, mephedrone metabolite, 4–MEC metabolite, buphedrone, N–ethylcathinone, mephedrone, pentedrone, methedrone, methylone, butylone, ethylone, pyrovalerone, 4–EMC, 4-MEC, α–PVP, pentylone, MDPPP, naphyrone and MDPV. The cathinone compound was confirmed positive in three cases with a prevalence of 1.01%. No other SC drugs or metabolites were observed, even under scan mode.

Since no information or previous study described the presence of SC in the Kingdom of Saudi Arabia, this study was carried out. The finding provides an estimation of the prevalence of SC. Even though the study has several limitations, such as a small population size within one province, this is the first study investigating the issue.

Legislators, government and forensic scientist communities must monitor the global illicit drug marketplace in order to ascertain that the appropriate laws, schemes, prevention programs and forensic laboratories are in place. As new substances emerge in the drug-user market, toxicologists must act rapidly and identify the biomarkers through in vitro examinations. Standard suppliers must

synthesise certified reference standards to detect NPS. At present, forensic laboratories have expanded regarding these substances.

It is suggested that the “Early Warning System” applied in the Europe Union should follow in Saudi Arabia. This can enhance measures to fight criminal drugs, expressly NPS, beyond simply sharing information on the appearance of NPS.

8. Conclusions

In chapter 3, the project was conducted to investigate illicit drugs, such as ATS and ALS, in urine samples using GC–MS. GC–MS was initially developed to increase the sensitivity and selectivity of the method using nine new recreational cathinones (butylone, ethylone, flephedrone, MDPV, mephedrone, methedrone, methylone, pentedrone and pyrovalerone) and six derivatisation reagents (PFPA, TFA, CLF_2AA, HFBA, AA and PA). The technique was optimised by modifying the temperatures of the injector port and the oven until the necessary separation and detection were observed with excellent peak shape. Next, the derivatisation was optimised using incubation time and temperature. The optimum procedure was selected based on the maximum peak area values. The optimum time was 20–25 minutes for most derivative SC under all reagents. The temperature that provided a better response in cathinones was 70°C for the high M.W and BP reagents. The comparison of the acylation agents inspected a number of parameters, including ion number, relative ion ratios, quantity of unique ions, peak area, recovery, LOD, linearity, precision, accuracy and interferences. All reagents provided good fragmentation with high sensitivity and repeatability, and PFPA was found to be the best. PFPA was then further studied in whole blood using validation parameters. The sensitivity of GC–MS was also elevated by using acidified methanol. One internal standard was adequate to cover all derivative SC. LLE and SPE had good recoveries for the analysed substances. Butyric anhydride was unsuitable to be used for SC. The tertiary amines were underivatised. The fragmentation patterns of derivative CLF_2AA cathinones were presented for the first time.

In chapter 4, after the development work was completed, the method was validated using SWGTOX guidelines for urine samples. 20 out of 29 stimulant drugs were successfully validated. The nine remaining substances were valid at high concentrations. The SPE drug recoveries were between 80-120% for most substances. No carryover or interferences were observed that affected the interpretation of the results. The LOD and LLOQ were between 0.5–10 and 5–50 ng mL^{-1}, respectively using the S/N technique. The LLOQ was between 50–500 ng mL^{-1} for all studied substances using the accuracy parameter in the linearity

calibration points. The bias and precision were lower than 20% for all valid drugs. The novelty of the work included the validation of the selected PFPA (ATS and ALS) derivatives using GC–MS in a single procedure. Therefore, it is recommended that this method is used in forensic toxicology laboratories to decrease the time, cost and efforts for stimulant drug testing.

In chapter 5, after the methods were validated, the stability work was accomplished. no study had previously been published documenting the stability of SC for longer than six months, therefore this study was conducted to determine the stability of ATS and SC for more than a year in urine samples. This chapter was also conducting for the importance of the stability for the overseas samples that were collected in SA. All stimulant substances for ATS and ALS were stable over the entire period of study at iced temperature (−20°C). Samples at refrigerator temperature (4°C) were stable for ATS and SC substances until day 349 and 14, respectively. The valid SC substances were undetected between day 77 and 349 at the refrigerator condition. Specimens at RT were unstable after 349 days and after 24–48 hours for ATS and SC, respectively. All valid ALS compounds were undetected in less than a month. All stimulants were stable within three days in an autosampler. No concentration-dependence was observed for all analysed drugs at concentrations of 1 and 0.5 μg mL^{-1}, but the stability differences between and within SC classes were diverse. The half-lives of 14 SC were briefly estimated and discussed. The recommendation is to keep all urine samples immediately after collection in freezer conditions, otherwise, the cathinones promptly start degrading.

In chapter 6, the automation of SPME in tips coupled with GC–MS was developed manually to provide an effective, clean, easy, inexpensive and fast method to quantify new stimulants in urine samples. After the completion of development work using several parameters such as pH, sample volume, solvent type, extraction time, etc., the optimum procedure was determined. The validation parameters were used on the selected compund cathinones using SWGTOX guidelines. The RSD and accuracy were less than 15% error for all analysed compounds. The technique quantified all examined substances at ≥100 ng mL^{-1} with LOD between 5–25 ng mL^{-1}. The R^2 was greater than 0.992 for all analytes.

Three fibre tips were evaluated, and the PDMS/DVB fibre was favoured based on recovery results. All chemicals that were added in the extraction stage used 60 µL of MeOH and salts. The SPME in tips offered minimum impacts on health, waste and safety as compared with LLE and SPE procedures. The real positive human urine samples were investigated, and the results were compared by applying the two extraction methods. The results showed that by using both SPME and SPE, the cathinone compound was identified and quantified in all tested specimens in GC–MS.

In chapter 7, the prevalence of cathinones was studied using 273 urine samples collected in Riyadh City, Saudi Arabia in July 2017. This was the first prevalence study that attempted to investigate SC using biological samples in specific population in Riyadh City in Saud Arabia. The cathinone compound prevalence was 1.01%. No other cathinones were identified. Consideration of the prevalence of NPS, such as SC, can deliver important information to the government, the justice system and the forensic community. More prevalence studies should be introduced using greater populations and other drugs in Saudi Arabia.

9. Future work

Proposals for future work:

- Although GC–MS is dominant in most forensic toxicology laboratories, the work here for investigating SC and SPME in tips could be also carried out using more modern and sensitive instrumentation, such as LC–MS-MS.

- Screening instrumentation methods such as UPLC–quadrupole time of flight (UHPLC-TOF) could be used to detect numerous new recreational drugs.

- The work here was completed using a single procedure in urine samples, but the mixtures of SC and ATS could be also determined using a single procedure in other matrices, such as saliva and hair.

- More metabolite SC drugs could be evaluated and investigated in urine.

- More stimulants drugs could be evaluated and validated to cover wider range using GC–MS.

- Despite the fact the SPME in tips was proven as a powerful extraction method, the technique could be developed using other materials and methods to extract more drug mixtures.

- Research could be focused on developing microextraction methods to meet the GAC.

- Three SPME fibre tips were evaluated here, but other material fibres could be industrialised for the purpose.

- Six derivatisation reagents were researched to increase the sensitivity and selectivity of GC–MS, but more agents could be evaluated and investigated.

- These techniques could also be applied to more case specimens to ensure the applicability of the methods to “real-life” circumstances.

- The stability of SC could be carried out using various matrices and conditions by experimenting with each drug alone rather than a mixture. This will be beneficial for studying the breakdown products in each SC compound using the scan method.

- The prevalence study could be carried out on regular basis (such as each year) to determine the trends of illicit drug use, such as SC.

- Further parameters could be collected for the purpose of the prevalence study, such a higher sample size, various regions and populations, data concerning sex and age or dissimilar biological samples in order to produce a thorough understanding of SC users.

- A combination of several tools and surveys could be used to improve the understanding of NPS use, such as SC using data from questionnaires, seizures, dark website market, deaths and drug abuse testing of selected populations (prisons, schools, sports) and general populations (countries, cities, ethics, religious), organisations and provinces.

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Appendices

Appendix 1: Ethical approval for collection blank urine samples

Dear Dr Hilary Hamnett

MVLS College Ethics Committee

Project Title: *Collection of blank biological samples*
Project No: *200160020*

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. We are happy therefore to approve the project, subject to the following conditions:

- Project end date: End January 2018.
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Dr Terry Quinn

Terry Quinn
MD, FRCP, BSc (hons), MBChB (hons)
Senior Lecturer / Honorary Consultant

College of Medicine, Veterinary & Life Sciences
Institute of Cardiovascular and Medical Sciences
New Lister Building, Glasgow Royal Infirmary
Glasgow
G31 2ER
terry.quinn@glasgow.gla.ac.uk
Tel – 0141 201 8519

The University of Glasgow, charity number SC004401

Appendix 2: Ethical approval to collect urine specimens from Saudi individuals (university of Glasgow)

University of Glasgow

21/3/17

MVLS College Ethics Committee

Project Title: Khat and Synthetic Cathinones in Saudi Arabia
Project No: 200160055

Dear Dr Morrison,

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project.

- Project end date: End January 2018.
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely,

Jesse Dawson
MD, BSc (Hons), FRCP, FESO
Clinical Reader / Honorary Consultant
Clinical Lead Scottish Stroke Research Network / NRS Stroke Research Champion
Chair MVLS Research Ethics Committee

Institute of Cardiovascular and Medical Sciences
College of Medical, Veterinary & Life Sciences
University of Glasgow
Room M0.05
Office Block
Queen Elizabeth University Hospital
Glasgow
G51 4TF

jesse.dawson@glasgow.ac.uk

Appendix 3: Ethical approval to collect urine specimens from Saudi individuals (Security Forces Hospital)

KINGDOM OF SAUDI ARABIA
MINISTRY OF INTERIOR
General Administration for Medical Services
SECURITY FORCES HOSPITAL PROGRAM

المملكة العربية السعودية
وزارة الداخلية
الإدارة العامة للخدمات الطبية
برنامج مستشفى قوى الأمن

MEMORANDUM

Date: 3 August 2016

RN: 16 - 190 - 24

To (Researcher): **Khalid Alsenedi**
Senior Medical Technologist, Lab Toxicology

From: **Dr. Marzoug Al Badi**
Chairman, Research Committee

Subject: ***Research Approval***

Reference: RS-AA/16

Following the meeting of the Research Committee on Thursday the 20th of June 2016, I am pleased to inform you that your research proposal titled *"Khat and Synthetic Cathinones in Saudi Arabia"* is approved with one condition; that the researcher will collect the samples with filling the consent form. Those samples are not known if they positive or negative for Khat and Synthetic cathinones but samples are negative for drug of abuse testing in screening methods only. As evidence of continuing compliance, the research committee in Security Forces Hospital requires that the researcher immediately report proposed changes to the protocol including changes to the investigators involved.

With the Committee's best wishes for the success of this project.

Thank you and best regards.

Cc:
- Research File
- Director of Laboratory & Blood Bank

Academic Affairs - Riyadh

Appendix 4: Participant information sheet for collection of urine specimens in Saudi Arabia

University of Glasgow | College of Medical, Veterinary & Life Sciences

PARTICIPANT INFORMATION SHEET

Khat and Synthetic Cathinones in Saudi Arabia

You are being invited to participate in a research project organised by the Unit of Forensic Medicine and Science at the University of Glasgow. Before you decide it is important for you to understand why this research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others if you wish. Please ask us if anything is not clear or if you would like more information.

What is the purpose of the Study?

In recent years, the using of new psychoactive substances (NPS) including synthetic cathinones has been increasing around the world. Synthetic cathinones has been sold as cheap substitutes for other stimulants such as amphetamine and cocaine and has become widely available on the internet. Some people may think that these substances are safe. In fact, these drugs provide a wide range of harmful effects on the body ranging from psychoactive effects, altered mood, lowered inhibition, anxiety, and depression, tachycardia and hypertension [1].

For this reason, the purpose of the project is to develop a method to detect these drugs in urine and to give us an idea of the prevalence of synthetic cathinones use in Saudi Arabia.

Why have I been chosen?

People who have to undergo regular urine drug screenings offer ideal human samples (urine) to be used in this project. The analysis of these samples is an important step in the method validation process.

Do I have to take part?

It is up to you to decide whether or not to participate in this project. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form.

What will happen to me if I take part?

To participate in the study, you are asked to consent freely to providing urine sample. You will be given plastic container to donate a urine sample. Your urine sample will be anonymised and there will be no way of connecting the urine sample with you once it has been donated.

The urine sample will be tested firstly for drug of abuse testing and synthetic cathinones. You will not be given any personal results from the tests.

What I have to do?

To participate in the study, you are asked to consent freely to donate a urine sample.

What are the possible disadvantages and risks of taking part?

No disadvantage will be found. Urine sample collection is very simple and non-invasive.

What are the possible benefits of taking part?

This research will provide an extremely important tool to assess the popularity of synthetic cathinones and in turn can help in assessing the importance of providing education on the use of synthetic cathinones.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.
I confirm to you that the results of testing your urine sample will never be linked to you as an individual and will be anonymised. No individual results will be communicated to any government officials or to any other person including the hospital. Your anonymised results along with all other participants will be published in peer-reviewed papers and presented at international conferences.

What will happen to the results of the research study?

The results of the tests using these samples will be kept anonymous from the moment you donate your sample and any publications resulting from them would contain no information from which a donor could be identified. You will not receive any results from these tests. It is only the combined results from all the participants that is of interest and will be published.

Who is organising and funding the research study?

This research is funded by Saudi Arabia Government.

Who has reviewed the Study?

This research has been assessed by an independent group of people; the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow, United Kingdom. To protect your safety, rights, wellbeing and dignity.

Contact for Further Information

For any further information concerning this project please contact Khalid Abdulaziz F Alsenedi at the khalidsenedi@gmail.com

Thank you for participating in the project.

References

1. Prosser, J.M. and L.S. Nelson, *The toxicology of bath salts: a review of synthetic cathinones.* Journal of Medical Toxicology, 2012. **8**(1): p. 33-42.

Appendix 5: Consent form for collection urine specimens in Saudi Arabia

University of Glasgow | College of Medical, Veterinary & Life Sciences

Subject Identification Number for this research:

CONSENT FORM

Title of Project: Khat and Synthetic Cathinones in Saudi Arabia

Name of volunteer :

Date of birth :

Name of City :

Please initial box

I confirm that I have read and understand the information sheet dated 28 Jan 2015 (version 1.4.1) for the above study and have had the opportunity to ask questions and I have had these answered satisfactorily.	☐
I understand that the participation is voluntary and that I am free to give the sample at any time, without giving any reason, without my legal rights being affected.	☐
I agree that additional biological samples may be retained and used for ethically approved medical research	☐
I agree that data may be collected and stored on a secure database	☐
I authorise that the urine sample may be kept indefinitely for medical research and they will be disposed of lawfully when the research is completed or they are no longer usable.	☐
I understand that any information collected will be treated as confidential and made available to researcher only in a form which preserves anonymity.	☐
I agree to take part in the above study.	☐

Name of volunteer	Date	Signature

Researcher Name	Date	Signature

Name of Researcher(s):

Khalid Alsenedi, Dr Calum Morrison

(1 copy for subject; 1 copy for researcher)

Appendix 6: Articles, Oral presentations and posters published in support of this thesis.

Articles:

1. Alsenedi, K. A. and Morrison, C. (2017) *Comparison of six derivatizing agents for the determination of nine synthetic cathinones using gas chromatography-mass spectrometry*. Analytical Methods, 9(18), pp. 2732-2743. (doi:10.1039/C7AY00597K).

2. Alsenedi, K. A. and Morrison, C. (2018) *Determination and long-term stability of twenty-nine cathinones and amphetamine-type stimulants (ATS) in urine using gas chromatography-mass spectrometry*. *Journal of Chromatography B*, 1076, pp. 91-102.(doi:10.1016/j.jchromb.2018.01.027) (PMID:29406033).

3. Alsenedi, K. A. and Morrison, C. (2018) *Determination of amphetamine-type stimulants (ATSs) and synthetic cathinones in urine using solid phase micro-extraction fibre tips and gas chromatography-mass spectrometry*. *Analytical Methods*, 10(12), pp. 1431-1440. (doi:10.1039/c8ay00041g).

Oral presentations:

4. Alsenedi, K. A. (2015), *Solid Phase Microextraction Applied to Synthetic Cathinones*. Forensic Medicine and Science, University of Glasgow on 11 May.

5. Alsenedi, K. A. (2016), *Comparison of Six Derivatizing Agents for the Determination of Nine Synthetic Cathinones by Acylation and Gas Chromatography-Mass Spectrometry*. Forensic Medicine and Science, University of Glasgow on 01 June.

6. Alsenedi, K. A. (2017), *New Trends in Solid Phase Microextraction*. Forensic Medicine and Science, University of Glasgow on 01 June.

7. Alsenedi, K. A. (2017), *Determination of Twenty-Nine Cathinones and Amphetamine-type stimulants (ATS) in Urine Using Gas Chromatography-*

Mass Spectrometry. Forensic Medicine and Science, University of Glasgow on 01 June.

8. Alsenedi, K. A. (2018), *Determination of Amphetamine-Type Stimulants (ATS) and Synthetic Cathinones in Urine Using Solid Phase Micro Extraction Fibre Tips and Gas Chromatography-Mass Spectrometry*. Emerging Analytical Professional, Knutsford, UK, 11-13th May.

Posters:

9. Alsenedi, K. A. and Morrison, C. (2016), *Comparison of Six Derivatising Agents for the Determination of Nine Synthetic Cathinones by Acylation and Gas Chromatography–Mass Spectrometry*. The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT), Manchester, UK, 18th-19th August 2016.

10. Alsenedi, K. A. and Morrison, C. (2017), *Determination and Long-Term Stability of Twenty-Nine Cathinones and Amphetamine-Type Stimulants (ATS) in Urine Using Gas Chromatography-Mass Spectrometry*. The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT), Oxford, UK, 31st August – 1st September.

Attendance:

11. Alsenedi, K. A. (2015), UKIAFT Annual Meeting and Conference. Bournemouth University, UK, 10th & 11th September 2015.