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Chromatographic Analysis and Survey Studies to Evaluate the Emerging Drugs of Synthetic Cannabinoids in Scotland and Saudi Arabia

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Abstract

Synthetic cannabinoid receptor agonists or more commonly known as synthetic cannabinoids (SCs) were originally created to obtain the medicinal value of THC but they are an emerging social problem. SCs are mostly produced coated on herbal materials or in powder form and marketed under a variety of brand names, e.g. "Spice", "K2". Despite many SCs becoming controlled under drug legislation, many of them remain legal in some countries around the world. In Scotland, SCs are controlled under the Misuse of Drugs Act 1971 and Psychoactive Substances Act 2016 that only cover a few early SCs. In Saudi Arabia, even fewer are controlled. The picture of the SCs-problem in Scotland is vague due to insufficient prevalence data, particularly that using biological samples. Whilst there is evidence of increasing use of SCs throughout the world, in Saudi Arabia, there is currently no data regarding the use of products containing SCs among Saudi people. Several studies indicate that SCs may cause serious toxicity and impairment to health therefore it is important to understand the scale of use within society.

A simple and sensitive method was developed for the simultaneous analysis of 10 parent SCs (JWH-018, JWH-073, JWH-250, JWH-200, AM-1248, UR-144, A-796260, AB-FUBINACA, 5F-AKB-48 and 5F-PB-22) in whole blood and 8 corresponding metabolites (JWH-018 4-OH pentyl, JWH-073 3-OH butyl, JWH-250 4-OH pentyl, AM-2201 4-OH pentyl, JWH-122 5-OH pentyl, JWH-210 5-OH pentyl, 5F-AKB-48 (*N*-4 OH pentyl), 5F-PB-22 3-carboxyindole)in urine using LLE and LC-MS/MS. The method was validated according to the standard practices for method validation in forensic toxicology (SWGTOX, May 2013). All analytes gave acceptable precision, linearity and recovery for analysing blood and urine samples. The method was applied to 1,496 biological samples, a mixture of whole blood and urine.

Blood and/or urine samples were analysed from 114 patients presenting at Accident and Emergency in Glasgow Royal Infirmary, in spring 2014 and June-December 2015. 5F-AKB-48, 5F-PB-22 and MDMB-CHMICA were detected in 9, 7 and 9 cases respectively. 904 urine samples from individuals admitted to/liberated from Scottish prisons over November 2013 were tested for the presence of SCs. 5F-AKB-48 (N-4 OH pentyl) was detected in 10 cases and 5F-PB-

22 3-carboxyindole in 3 cases. Blood and urine samples from two post-mortem cases in Scotland with suspected ingestion of SCs were analysed. Both cases were confirmed positive for 5F-AKB-48. A total of 463 urine samples were collected from personnel who presented to the Security Forces Hospital in Ryiadh for workplace drug testing as a requirement for their job during July 2014. The results of the analysis found 2 samples to be positive for 5F-PB-22 3-carboxyindole.

A further study in Saudi Arabia using a questionnaire was carried out among 3 subpopulations: medical professionals, members of the public in and around smoking cafes and known drug users. With regards to general awareness of Spice products, 16%, 11% and 22% of those participants of medical professionals, members of the public in and around smoking cafes and known drug users, respectively, were aware of the existence of SCs or Spice products. The respondents had an overall average of 4.5% who had a friend who used these Spice products.

It is clear from the results obtained in both blood and urine testing and surveys that SCs are being used in both Scotland and Saudi Arabia. The extent of their use is not clear and the data presented here is an initial look into their prevalence. Blood and urine findings suggest changing trends in SC use, moving away from JWH and AM SCs to the newer 5F-AKB-48, 5-F-PB-22 and MDMB-CHMICA compounds worldwide. In both countries 5F-PB-22 was detected. These findings clarify how the SCs phenomenon is a worldwide problem and how the information of every country regarding what SCs are seized can help and is not specific for that country. The analytes included in the method were selected due to their apparent availability in both countries, however it is possible that some newer analytes have been used and these would not have been detected. For this reason it is important that methods for testing SCs are updated regularly and evolve with the ever-changing availability of these drugs worldwide. In addition, there is little published literature regarding the concentrations of these drugs found in blood and urine samples and this work goes some way towards understanding these.

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Acknowledgement

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 Definitions/Abbreviations

A&E — Accident and Emergency

A&E MT — Accident and Emergency Medical Treatment

AALs — Aminoalkylindoles

AAPCC — American Association of Poison Control Centres

AB-001 — Adamantan-1-yl(1-pentyl-1*H*-indol-3-yl)methanone

AB-CHMINACA — *N*-[(1S)-1-(Aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide

AB-FUBINACA — *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide

AB-PINACA — *N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide

ACMD — Advisory Council on the Misuse of Drugs

ACN — Acetonitrile

ADB-CHMINACA — (*N*-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide)

ADB-FUBINACA — *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide

ADBICA — *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indole-3-carboxamine

ADB-PINACA — *N*-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide

AKB-48 — *N*-(1-Adamantyl)-1-pentyl-1*H*-indazole-3-carboxamide

AM-679 — (2-Iodophenyl)(1-pentyl-1*H*-indol-3-yl)methanone

AM-694	1-[(5-Fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone
AM-1220	[1-[(1-Methylpiperidin-2-yl)methyl]-1*H*-indol-3-yl]-(naphthalen-1-yl)methanone
AM-1241	(2-Iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]methanone
AM-1248	Adamantan-1-yl[1-[(1-methyl-2-piperidinyl)methyl]-1*H*-indol-3-yl]methanone
AM-2201	[1-(5-Fluoropentyl)-1*H*-indol-3-yl]-1-naphthalenylmethanone
AM-2233	(2-Iodophenyl)[1-[(1-methyl-2-piperidinyl)methyl]-1*H*-indol-3-yl]-methanone
amu	Atomic Mass Units
APCI	Atmospheric Pressure Chemical Ionisation
APICA	*N*-(1-Adamantyl)-1-pentyl-1*H*-indole-3-carboxamide
APT	Addiction Prevalence Testing
CE	Collision Energy
CI	Chemical Ionisation
CP	Cyclohexylphenol
CP47, 497	(2-[(1R,3S)-3-*H*ydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol)
CP-55,940	((-)-*cis*-3-[2-h y d roxy-4.(1,1-dimethyl-heptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol)
%CV	Coefficient of Variation percentage
DEA	Drug Enforcement Administration
DCM	Dichloromethane

DRD	Drug-related death
DUID	Driving Under the Influence of Drugs
EI	Electron Impact Ionisation
ELISA	Enzyme Linked Immunosorbent Assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electrospray Ionisation
EU	European Union
EWDTS	European Workplace Drug Testing Society
EWS	Early Warning System
5F-AKB-48	N-(1-Adamantyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
5F-PB-22	1-(5-Fluoropentyl)-8-quinolinyl ester-1H-indole-3-carboxylic acid
FID	Flame Ionisation Detector
FMS	Forensic Medicine and Science
g	Gram(s)
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GDNC	General Directorate for Narcotic Control
GDS	Global Drug Survey
h	Hour(s)
HEIA	Homogeneous Enzyme Immunoassay

HPLC	High Performance Liquid Chromatography
HU-210	3-(1,10-Dimethylheptyl)- 6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol
IPs	Identification Points
ISDs	Internal Standards
JWH-015	1-Naphthalenyl(2-methyl-1-propyl-1*H*-indol-3-yl)methanone
JWH-018	1-Naphthalenyl(1-pentyl-1*H*-indol-3-yl)methanone
JWH-019	1-Naphthalenyl(1-hexyl-1*H*-indol-3-yl)methanone
JWH-020	1-Heptyl-1*H*-indol-3-yl-1-naphthalenyl-methanone
JWH-073	1-Naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone
JWH-081	4-Methoxynaphthalen-1-yl-(1-pentylindol- 3-yl)methanone
JWH-122	4-Methylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-200	1-Naphthalenyl[1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl]methanone
JWH-203	2-(2-Chlorophenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone
JWH-210	4-Ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-250	2-(2-Methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone
JWH-251	2-(2-Methylphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone
JWH-307	[5-(2-Fluorophenyl)-1-pentyl-1*H*-pyrrol-3-yl](naphthalene-1-yl)methanone
JWH-387	(4-Bromo-1-naphthalenyl)(1-pentyl-1*H*-indol-3-yl)-methanone
JWH-398	1-Pentyl-3-(4-chloro-1-naphthoyl)indole

JWH-412	(4-Fluoro-1-naphthalenyl)(1-pentyl-1*H*-indol-3-yl)-methanone
L	Litre(s)
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC–MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LC–HR/MS	Liquid Chromatography–High Resolution Mass Spectrometry
LLE	Liquid–Liquid Extraction
LOD	Limit of Detection
LLOQ	Lower Limit of Quantification
MAM-2201	[1-(5-Fluoropentyl)-1*H*-indol-3-yl](4-methyl-1-naphthalenyl)methanone
MeOH	Methanol
μL	Microlitre(s)
min	Minute(s)
mL	Millilitre(s)
MOH	Ministry of Health
MOI	Ministry of Interior
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSPs	Members of the Scottish Parliament

MTBE	Methyl-*Tert*-Butyl-Ether
m/z	Mass to charge ratio
NCCD	National Committee for Combating Drugs
NHS	National Health Service
NHS GG&C	National Health Service Greater Glasgow & Clyde
ng	Nanogram(s)
NPD	Nitrogen Phosphorus Detector
NPS	New Psychoactive Substances
NRS	National Records of Scotland
PB-22	1-Pentyl-1*H*-indole-3-carboxylic acid 8-quinolinyl ester
PPT	Protein precipitation
QC	Quality control
QUCHIC	Quinolin-8-yl 1-(cyclohexylmethyl)-1*H*-indole-3-carboxylate
R^2	Correlation Coefficient
RCS-4	(4-Methoxyphenyl)(1-pentyl-1*H*-indol-3-yl)methanone
RCS-8	1-(2-cyclohexylethyl)-3-(2-methoxyphenylacetyl)indole
rpm	Revolutions per minute
SALLE	Salting-out Liquid–Liquid Extraction
SALSUS	Scottish Schools Adolescent Lifestyle and Substance Use Survey
SAMHSA	Substance Abuse and Mental Health Services Administration

SCJS	Scottish Crime and Justice Survey
SCs	Synthetic Cannabinoids
SFDA	Saudi Food and Drug Authority
SFH	Security Forces Hospital
SIM	Selected Ion Monitoring
SLE	Supported Liquid Extraction
SoHT	Society of Hair Testing
SPE	Solid-Phase Extraction
SPS	Scottish Prison Service
STS-135	N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indole-3-carboxamide
THC	Δ^9-Tetrahydrocannabinol
TOF/MS	Time-Of-Flight Mass Spectrometry
UK	United Kingdom
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
UR-144	(1-Pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone
V/V	Volume to volume
WADA	World Anti-Doping Agency
WDT	Workplace Drug Testing
WEDINOS	Welsh Emerging Drugs and Identification of Novel Substances

WIN48,098   (4-methoxyphenyl)-[2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]methanone

WIN55 212-2   *R*-(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1 naphthalenyl)methanone mesylate

WoSRES   West of Scotland Research Ethics Service

XLR-11   [1-(5-Fluoropentyl)-1*H*-indol-3-yl](2,2,3,3-tetramethylcyclopropyl)methanone

Chapter 1 – Introduction and Aims

1.1 Rationale and Aims

Recently the use of new psychoactive substances (NPS) especially synthetic cannabinoids (SCs) has increased at an unprecedented pace around the world [1]. SCs typically act as agonists at cannabinoid receptors and are intended to mimic the effects of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive compound of the C*annabis sativa* plant [2]. Some of these drugs seem to display more toxicity than THC due to their increased potency and uncontrolled dose that might cause overdose and serious clinical consequences [3]. They represent an emerging drug problem in many countries [4]. The response to this phenomenon varies from one country to another depending on the apparent extent of the problem, the availability of resources and information, and the awareness of their risks [5]. To study the extent of a drug problem in a particular society requires evaluation of their prevalence. Prevalence studies can help to outline the scale of the problem which is critical to prioritising resources around harm treatment and prevention. There is little evidence in terms of prevalence estimates for the use of synthetic cannabinoids internationally. The picture of the SCs-problem in Scotland is incomplete due to insufficient prevalence data, particularly that using biological samples to confirm the presence of specific compounds.

The picture in Saudi Arabia is almost completely unclear. The government of Saudi Arabia exercises efforts in controlling drug addiction in Saudi society. Large amounts of illicit drugs enter Saudi Arabia through its borders. According to the Saudi General Directorate for Narcotic Control (GDNC), 181 million Captagon tablets (fenethylline, combination of amphetamine and theophylline), 61 tons of hashish, 2.206 tons of khat and 222 kilos of heroin have been seized between 2010 and 2012 [6]. Despite the suggestion from different sources that use of SCs is widespread around the world, there is no data to determine their use in Saudi Arabia. However, the United Nations Office on Drugs and Crime (UNODC) reported that in 2011, 16 countries (including Saudi Arabia) reported seizures of SCs [7]. In addition, there are some indicators leading to assume the probability of the presence and use of these products in Saudi Arabia.

Saudi Arabia is considered the Islamic country which follows the Islamic traditions most strictly. The use of alcohol and illicit drugs is forbidden in Islam [8]. Therefore, the possession, sale, or consumption of illicit drugs including alcohol is strictly forbidden in Saudi Arabia [9]. The country's religious beliefs and legislation have made the use of illicit drugs to be socially unacceptable, which may lead users to experiment with new drugs which are undetectable in common drug-screening tests. Other indicators come from neighbouring countries of Saudi Arabia where thousands of Saudi people travel to daily; nine individual SCs were identified in seizures in the United Arab Emirates [10], the Kuwait Ministry of Interior announced the seizure of "crazy monkey" which is a new "Spice" product, and Turkey has reported a 22-fold increase in the trafficking of SCs between 2011 and 2013 and that these products are smuggled into Turkey from China, United States (US) and European countries [11]. Moreover, there are several online stores selling SC products targeting Saudi individuals.

The fight against drugs of abuse has to continue at various levels such as developing new analytical methods that can identify the new drugs. Hence, the work to monitor the world drug market can be more effective to ensure that clinical and forensic laboratories are keeping up with the illegal drug suppliers. In addition, the clinical challenge in the Accident and Emergency (A&E) is to treat patients who have taken these new drugs and therefore the analytical methods for the detection and determination of these drugs in biological samples has become a crucial issue. Prevalence studies to estimate the scale of use of these new drugs can lead to a better understanding of the scale of this issue in Scotland and Saudi Arabia.

The purpose of the questionnaire study in this project was to explore the knowledge and usage of Spice among Saudi people. Questionnaire survey allows the collection of information relatively inexpensively and quickly. It also helps to obtain valuable information from a large sample of individuals and from all parts of Saudi Arabia, particularly for online survey. In addition, this can help to raise the awareness regarding these drugs and their risk to individuals, through information involved in a participant information sheet.

1.2 Project Objectives

The main aim of this project was to assess the prevalence of SCs in Scotland and Saudi Arabia as well as the awareness of these new drugs within Saudi Arabia.

The objectives to meet the aim were to:

1) Develop and validate a sensitive method with low limits of detection for the identification of SCs in blood and urine samples using chromatographic analysis techniques.

2) Test blood and urine samples for SCs from 3 different subpopulations in Scotland (A&E patients, prisoners and fatalities).

3) Test urine samples for prevalence of SCs in Saudi individuals involved in workplace drug testing.

4) Prepare a questionnaire and collect data among 3 subpopulations of Saudi Arabia (known drug users, medical professionals and members of the public in and around smoking cafes).

Chapter 2 – Background and Literature Review

2.1 Forensic Toxicology

Toxicology can be defined as the study of the toxic or adverse effects of chemicals on living organisms [12]. Forensic toxicology is a specialised area of toxicology concerned with the medicolegal (relating to both medicine and law) characteristics of the harmful effects of alcohol, drugs or poisons on humans and animals [12]. It has developed in recent years and includes theoretical considerations and methods from several sciences such as pharmacology, biochemistry, analytical chemistry, physiology, epidemiology and pathology. Forensic toxicology is widely used to assist in workplace, in criminal matters involving drug use, in death investigation, in road safety, in sports doping as well as in matters involving environmental pollution [13]. It is aimed at assisting in identification, determination and interpretation of drugs and chemical compounds in human biological matrices through toxicological analysis for medicolegal purposes [13].

2.2 Drugs of Abuse and 'Legal Highs'

A drug can be described as a "Drug of Abuse" when it is inappropriately used in a maladaptive pattern or for recreational purposes, which does not necessarily include dependency [14]. Drug abuse is not limited to the use of drugs for recreational purposes but they include any drug used inappropriately such as the use of anabolic steroids, β blockers and diuretics in sports [13]. Drugs of abuse can be classified according to their actions and effects as narcotics, depressants, stimulants and hallucinogens. Drugs of abuse have been controlled by legislation and UN Conventions and scheduled to specify the degree of control and level of penalties. Forensic communities often describe the drugs of abuse which have been used for a long time (e.g. amphetamine, cannabis, cocaine, and common benzodiazepines) as "traditional drugs" or "classical drugs" [15].

UNODC [16] estimates that a quarter of a billion people of the world population (1 out of 20 adults) aged between 15 and 64 years, used at least one drug in 2014, and over 29 million of them were problem drug users, suffering from drug use disorders. Figure 2.1 shows annual prevalence of illicit drug use amongst the

world population aged between 15 and 64 years. Although with varying legislation around the world the data is limited because only "illegal" drugs are counted. Cannabis is the most frequently used illicit drug (183 million), followed by amphetamines (33 million) [16].
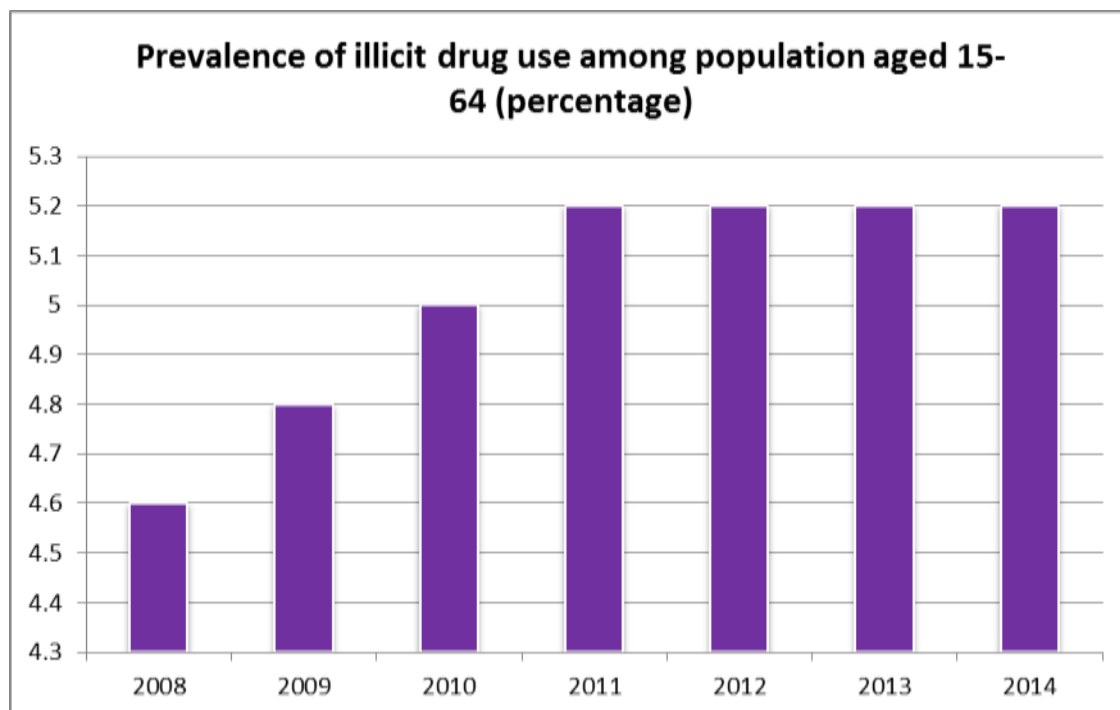
Figure 2.1: Global Trends in the Estimated Prevalence of Illicit Drugs, 2008-13 (Source: UNODC, 2015)

Recently, the phenomenon of new drugs related to adverse effects and/or death has attracted much attention. The terms "legal highs", "club drugs" and New Psychoactive Substances (NPS) are often used interchangeably to describe these new substances or drugs that mimic the effects of traditional recreational drugs [17]. The term "legal highs" is misleading for two reasons. Firstly, some of the drugs which appear on the drug market are already controlled under national legislation and international drug conventions. Secondly, not all these drugs are "highs" and have desired effects along the lines of euphoria: some may cause psychosis, anxiety or paranoia which can be caused by ATS, as well as some of these drugs are sedative in nature e.g. benzodiazepines. "Club drugs" are drugs associated with use by young adults and teenagers at parties, concerts and nightclubs. The term "club drugs" is also confusing because it includes long established drugs such as methamphetamine and MDMA. In general, NPS are defined as psychoactive drugs which are not prohibited by international drug conventions or national legislation created to implement the first UN international convention such as the Misuse of Drugs Act 1971 in the United

Kingdom (UK), but which can cause a threat for human health [18]. However, it is not always useful to consider the legal status of a drug when discussing NPS, as it suggests the moment it is controlled it is not an NPS. NPS may contain legal drugs, illegal drugs, or a mixture of both [18]. They can also contain a wide range of different kinds of drugs including prescribed medication e.g. gabapentin, historically controlled drugs e.g. Para-Methoxyamphetamine (PMA) and newly developed drugs which mimic the effects of controlled drugs such as synthetic cannabinoids [18]. These drugs can either be abused in a new way or are newly developed therefore the term "new".

2.3   Synthetic Cannabinoid Receptor Agonists

Cannabinoids are a group of chemical compounds which act on specific receptors (CB_1 and CB_2) in the brain. Cannabinoids can be divided into three groups: phytocannabinoids (plant cannabinoids), endogenous cannabinoids and synthetic cannabinoids [19].

Phytocannabinoids are cannabinoids produced by plants and were first used around 3000 years ago in India for therapeutic and religious purposes [20]. The interest in synthesising cannabinoids increased after the complete syntheses of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive compound of *Cannabis sativa* L plant, in 1964 by Raphael Mechoulam [21]. THC acts as a partial agonist at both CB_1 and CB_2 cannabinoid receptors, exhibiting lower CB_2 than CB_1 efficacy. These receptors are G protein coupled receptors. The activation of CB_1 receptors by THC leads to an inhibition of cellular cyclic adenosine monophosphate (cAMP) levels and modulation of the ion channel activity leading to inhibition of sodium, potassium and N-, and P/Q- type-calcium channels [22]. After smoking cannabis, THC is rapidly distributed throughout the body. Phase-I oxidation reactions of THC include oxidation of alcohols (the 11-methyl groups oxidised to 11-hydroxy-THC and then to 11-carboxy-THC), allylic and aliphatic hydroxylations, *β*-oxidation, and degradation of the pentyl side chain [19]. The common Phase-II reaction is conjugation with glucuronic acid [23].

Many people use cannabis for pain relief and there is a large body of evidence showing the health benefits for conditions such as acquired immune deficiency syndrome (AIDS) [24]. Doctors also could prescribe medical cannabis to treat

muscle spasticity, tremor and dystonia, nausea caused by anticancer drugs and poor appetite caused by chronic illness [25]. However, its use for medical purposes is controversial due to its addictive potential and threat to public health. For all these reasons the use of cannabis has been restricted in most countries. In the USA, there are critical differences in cannabis laws from one state to another. 24 states have passed laws allowing smoked cannabis to be used for medical purposes [26]. However, the use of cannabis continues to be an offence under Federal law [26].

Endogenous cannabinoids such as anandamide and 2-arachidonoylglycerol are produced naturally in the body [27]. Anandamide (*N*-arachidonoylethanolamine) was the first endogenous ligand of cannabinoid receptors isolated in 1992 [27]. Anandamide is structurally different from THC of plant origin but they share critical pharmacophores that allow for binding to common targets [28].

Synthetic cannabinoid receptor agonists, also known as synthetic cannabinoids (SCs), are a large group of structurally dissimilar compounds that were originally created during the 1990s to investigate the human endogenous cannabinoid system and their potential as therapeutic agents but they emerged as a problem in the early 2000s [4]. Although, SCs were created as pharmaceutical agents, it was found that the separation of the desired effects from unwanted psychoactive effects is difficult. They typically act as agonists at cannabinoid receptors (CB_1 and/or CB_2) and mimic the effects of THC [29].

2.3.1 The Emergence of Synthetic Cannabinoids in Herbal Products

From 2004, illicit drug manufacturers began to produce herbal smoking materials under a variety of brand names, e.g. "Spice" [4, 30]. Google Insights web searches reports suggest that the availability of Spice products started in the beginning of 2004 [18, 31]. Figure 2.2 shows a comparison of the popularity of 4 terms; "legal cannabis", "synthetic cannabinoids", "synthetic cannabis" and legal highs on Google over the period 2004-2016. The graph also shows the rapidly increasing popularity of these terms since 2009-10.

Herbal products have been sold in European countries such as Switzerland, Austria and Germany since at least 2006 [32]. They have been easy to buy for several years over the internet, from physical shops (such as petrol stations and "head shops") or from traditional 'street dealers' without age restriction [33, 34]
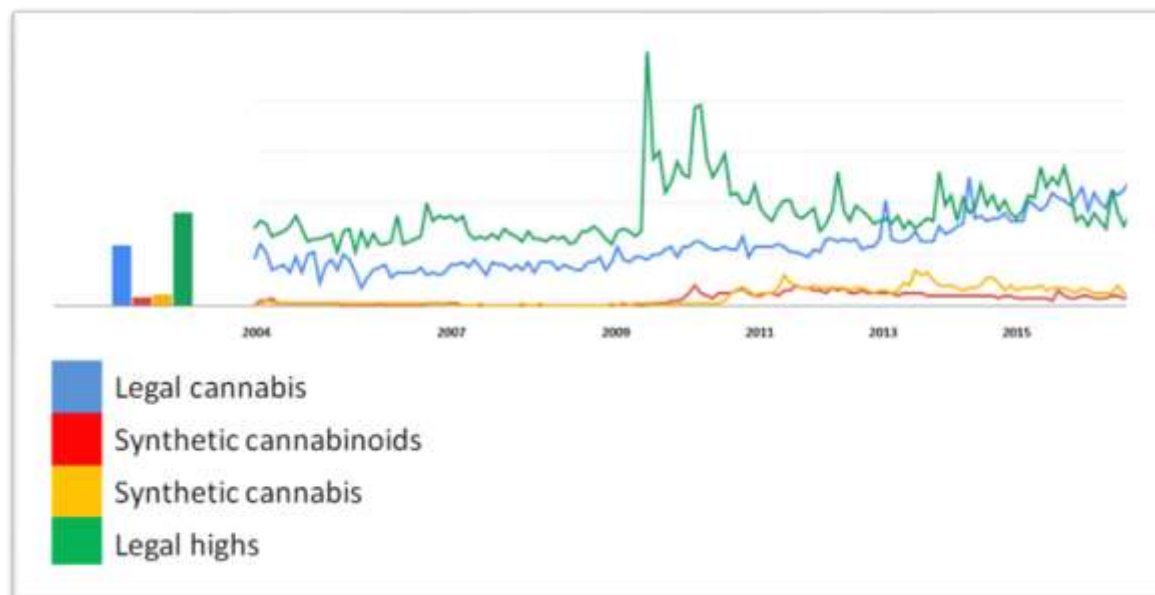
Figure 2.2: Google Trends of "legal cannabis", "synthetic cannabinoids", "synthetic cannabis" and "legal highs", 2004-16 (Source of Information: Google Trends) [31].

To produce these herbal smoking mixtures, SCs are dissolved in a solvent, usually one with a low boiling point e.g. acetone, then sprayed onto dried plant materials [35]. They have also been packaged in other forms including solids (resins) and powders [36]. Lately, liquid products containing SCs have been introduced on to the marketplace for use with electronic cigarettes. Some of these products come without declaration of ingredients and others may come with an ingredients list, however these do not always contain the true contents [37]. In addition, they do not often come with consumer information about the use and the effects.

A study in 2009 of the available legal high products offered by UK-based Internet retailers found 1308 products; 91.9% of these products failed to list side effects, 86.3% failed to list drug interactions, 81.9% failed to list contraindications and 40.1% failed to list ingredients [38]. The language used on labels often makes users believe the effects come from the plant materials used. However, studies on the botanical materials in 62 Spice products prove that most of the plant species were free of psychoactive properties [39]. The authors identified the

origins of each plant species in these Spice products and found that *Turnera diffusa* (common name is Damiana) and Labiatae or Lamiaceae (the mint or deadnettle family) were the plants most frequently detected in the products. The authors also observed that most plant species identified in these products were different from the plants indicated on the ingredients list labels. Similar findings have been reported by Piggee [40] who noted that the ingredients list labelled on Spice products did not appear to cause psychoactive effects.

For marketing, these herbal mixture products are typically sold as "incense", "herbal blends" or legal highs and, being sold under the generic brand name Spice, these include, but are not limited to: "Spice Diamond", "Spice Gold", "Spice Silver", "Spice Egypt", "Spice Tropical Synergy", "Spice Arctic Synergy", "etc." [32]. In the novel by Frank Herbert, Spice was the name of a drug used by the people on the planet Dune, and that might be where the term comes from [29]. These drugs have also been branded as "K2" in the USA [41]. Illicit manufacturers of Spice products continue evolving brand names.

Some brand names of SCs have been chosen to help the marketing such as AKB-48 which refers to a popular Japanese girl band [42]. Another example is XLR-11 which has probably been named after the first developed liquid rocket fuel in the USA [42]. STS-135 potentially refers to the 135th and final mission of the American Space Shuttle program [43]. They are sometimes branded with names which are euphemistic and misrepresentative.

Clandestine illicit laboratories may change active ingredients of the same product over time and location [44]. For instance, the forensic examinations of a product labelled as "MOCARZ" have confirmed the presence of high concentrations of the following SCs: XLR-144, UR-144, BB-22, 5F-PB-22, AB-CHMINACA, and in other seizures the same product contained mixtures of the following SCs: JWH-203, JWH-019, JWH-081, AM-2201 [44]. Another example, "Black Mamba" initially contained natural mixtures called "Damiana" but latterly has contained various SCs when analysed [45]. The concentrations of SCs present in Spice products might also be variable within the same brand. For instance, samples of "Psyclone" have been tested and gave considerable variation in the ratio of 5F-AKB-48 and 5F-PB-22 [46]. This variation in different

concentrations of different active ingredients makes it very difficult to estimate the optimum dose, which can lead very easily to acute intoxications.

Driving this changing market is complex legislation across countries and illicit drugs manufacturers trying to bypass the law by moving on to newer compounds. Most NPS including SCs follow a cycle of supply and prohibition as shown below in Figure 2.3.
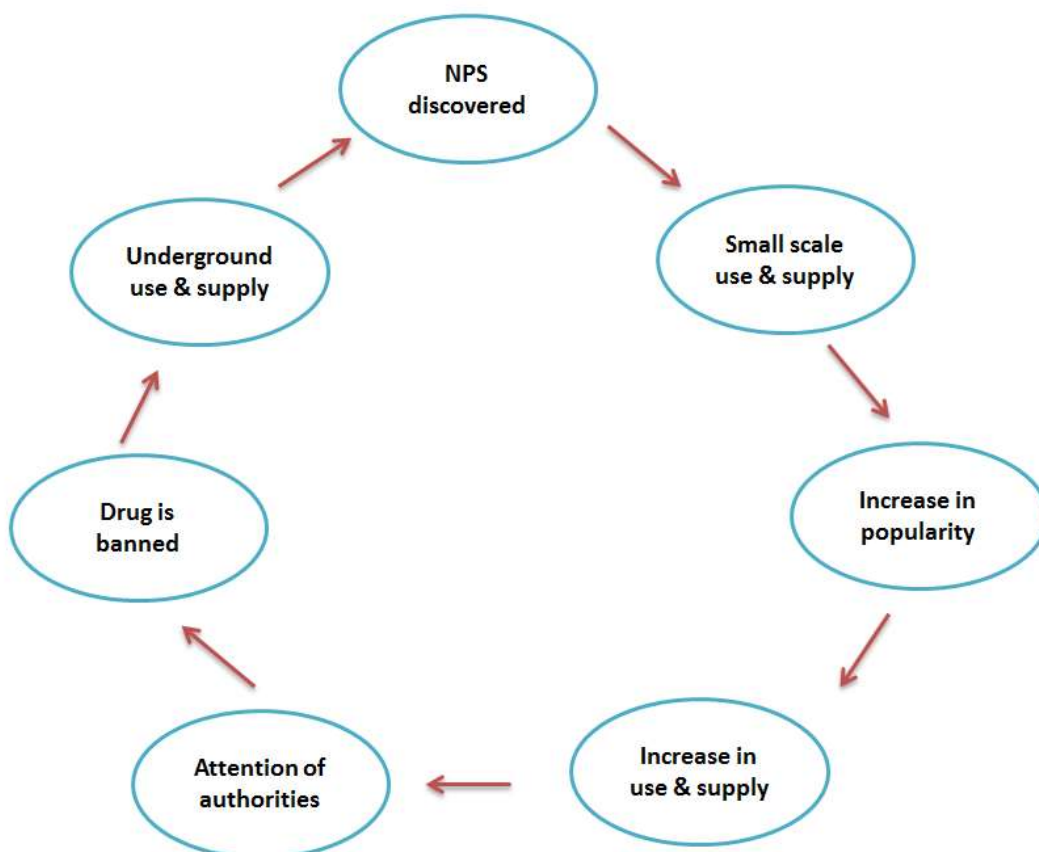
Figure 2.3: The Cycle of Supply and Prohibition of NPS Compounds [47].

Prices can vary from one product to another depending on the source and purity [48]. Generally, the prices of these herbal mixtures are affordable by young people (£6.3–12 per gram) making these products highly attractive [49]. Moreover, internet shops offer discounts of the products on individual or bulk purchases to help the marketing [34].

It is still unclear where the production of SC actually takes place. However, some national systems suggest that some of these products have been manufactured in China and distributed around the world [32].

Other herbal preparations introduced on the marketplace claimed that they have a similar make-up to "Spice": e.g. "Aroma", "Dream", "Scope", "Earth Impact", "Genie", "Yucatan Fire", "Blue Lotus", "Smoke", "Galaxy Gold", "Skunk", "Black Mamba", "Kronic", "Exodus Damnation", "Annihilation", "Clockwork Orange", "Diesel", "Psyclone (clown)", "Spunout", etc. [32, 50-52]. Despite various attempts to stem the flow of Spice products, it would appear that they continue to be available containing new derivatives of SCs making several waves or generations of products, most likely to continue circumventing legal actions.

It was not really until August 2008 that these new products became popular, coinciding with the time that media in Germany claimed that they were a "legal" alternative to marijuana but could not be detected by common drug-screening tests [40, 53, 54].

By the end of 2008, forensic researchers in the German pharmaceutical company THC-Pharm first detected JWH-018, a synthetic cannabinoid, in 3 Spice products [54]. The compound was also detected at the same period in at least three versions of Spice (Spice Diamond, Gold and Silver) in Austria by the Austrian Agency for Health and Food Safety (AGES) PharmMed [32]. In the beginning of 2009, researchers from University of Freiburg (Germany) identified and characterised another cannabinoid, the C8 homologue of CP47,497 in Spice products [33]. In March 2009, the United States Drug Enforcement Administration (DEA) reported the detection of SCs including HU-210 in five small foil packets containing plant materials [55]. In June 2009, the substance had also been identified in three Spice products in the UK [32]. In the first three months of 2009, another synthetic cannabinoid, JWH-073 was detected in a herbal product called "Scope" and other products in Germany, and it was also found in powder seizures in Denmark and the Netherlands [32]. In the three months of 2009, two new substances from the JWH family, the phenylacetylindole JWH-250 and the napthoylindole JWH-398 were reported for the first time by Germany and the UK respectively [32].

Since 2009, the number and type of Spice products has increased, and the list of SCs seems to grow almost daily [35]. In 2012, AKB-48 was identified in herbal smoking mixtures in Japan [56, 57]. In 2013, a new wave of compounds that involved a large change in chemical structure emerged such as 5F-AKB-48 and

5F-PB-22. 5F-AKB-48 had been identified in four products including both plant material and powder seized by Danish customs authorities [58], and 5F-PB-22 was identified by a Japanese research group [56]. The new substance MDMB-CHMICA was reported by Danish authorities in December 2014 [59]. Table 2.1 lists the first identification of the most common SC compounds in herbal products from different generations.

Table 2.1: List of Some SCs Identified in Herbal Products with the Date and Place of their First Appearance

SCs	Date and Place of First Detection	Ref
JWH-018	December 2008 (Germany, Austrian)	[33, 35]
CP-47,497	December 2008 (Germany, Japan)	[33, 35]
HU-210	January 2009 (USA) / June 2009 (UK)	[32, 55]
JWH-073	January 2009 (Germany)	[32, 35]
JWH-250	October 2009 (Germany)	[32, 35]
JWH-398	October 2009 (UK)	[32, 35]
JWH-200	December 2009 (Russia)	[35]
JWH-122	July 2010 (Latvia)	[35]
JWH-210	September 2010 (Germany)	[35]
AKB-48	2012 (Japan)	[56]
5F-AKB-48	2013 (Denmark)	[58]
5F-PB-22	2013 (Japan)	[56]
MDMB-CHMICA	December 2014 (Denmark)	[59]

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), a survey of the European Union countries in 2009 showed that Spice products were identified in 21 countries. EMCDDA reported that during 2014, a total of 101 NPS were notified for the first time through the organisation's early warning system, up from 81 substances in 2013, 73 in 2012 and 49 in 2011, however, this increase in numbers may partly reflect increasing capability to detect NPS [60]. EMCDDA data shows that number of SCs detected continues to grow (Figure 2.4) with a total of 160 SCs having been monitored by the European Union (EU) Early Warning System (EWS) as of December 2015 [61]. In 2013, EMCDDA confirmed that 21,495 seizures of SCs were reported across Europe which represented 40 percent of the total number of seizures of NPS [60].

The same trend has been noted by UNODC: SCs are the largest drug group monitored in 2014 [4]. According to UNODC, a total of 541 NPS had been reported to UNODC Early Warning Advisory (EWA) by 95 member states and

territories by December 2014. The majority of countries that reported the emergence of NPS were from Europe, Asia, Africa, the Americas and Oceania respectively, and the majority of NPS reported in 2014 were SCs (39 per cent), followed by phenethylamines (18 per cent) (Figure 2.5) [4].
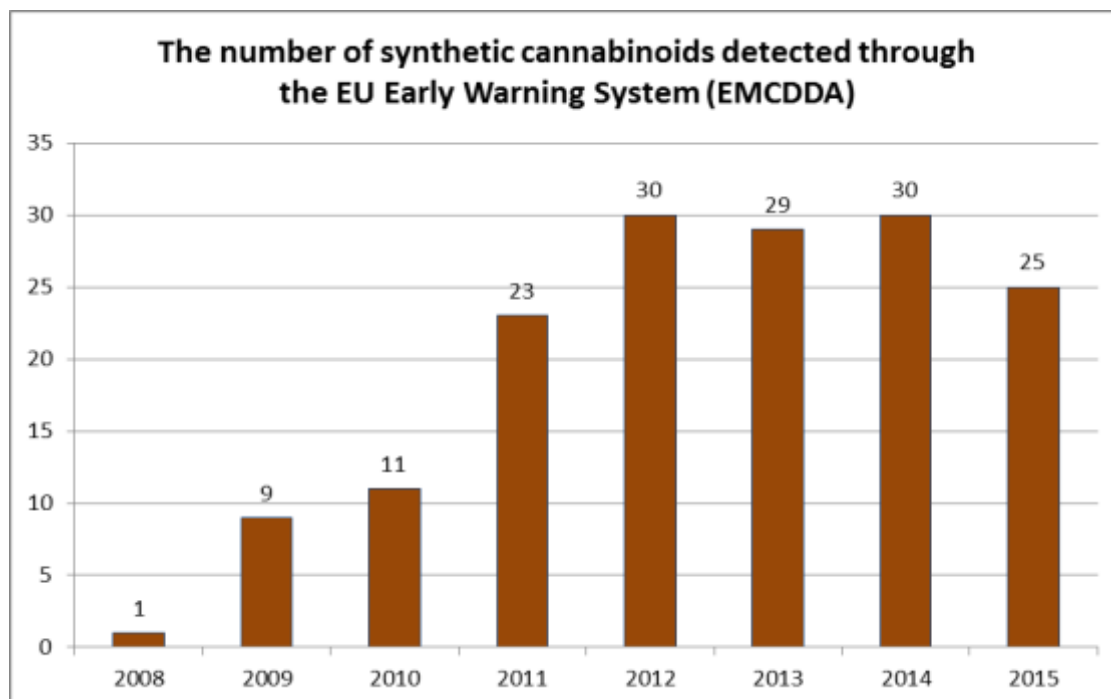
Figure 2.4: Number of SCs detected through the EU Early Warning System, 2008-15
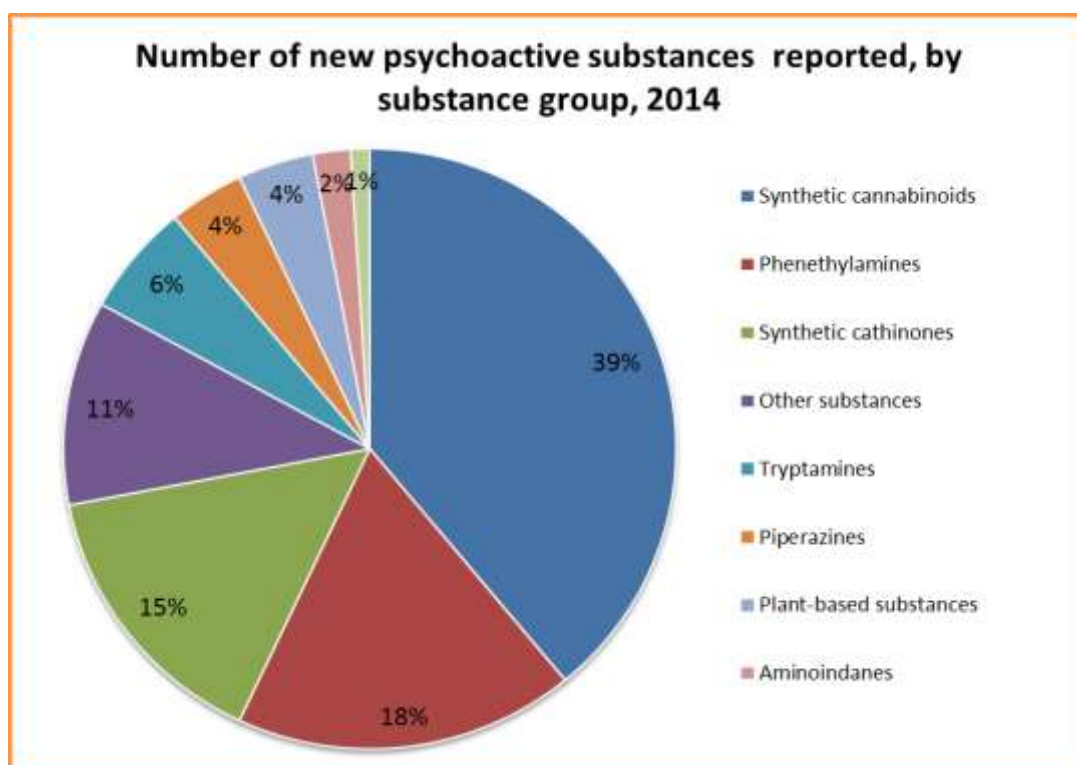
Figure 2.5: Number of NPS Reported, By Substance Group, 2014. (Source: UNODC, Early Warning Advisory on NPS, 2014)

2.3.2 Structural Classification of Synthetic Cannabinoids

After the completion of THC synthesis in 1964 [21] and the identification of CB_1 [62] and CB_2 cannabinoid receptors in rat brain and characterisation of these in the early 1990s [63], the interest in synthesising cannabinoids increased to yield a total number of 134 substances by the end of 2014 [64].

The names of SCs often contain abbreviations and numbering. The abbreviations can refer to who had developed a substance or to the place where a compound was synthesised for the first time. Some SCs might have attractive names to help the marketing such as AKB-48 (See Chapter 2.3.1). More details of the reasons for the nomenclature of each compound will be explained later.

SCs contain a great variety of structurally dissimilar compounds. Despite slight differences in the chemical structures, all SCs are non-polar, lipid-soluble, small molecules, containing 20 to 26 carbon atoms and are fairly volatile compounds [65]. Due to the wide chemical structure modifications of SCs and their ongoing development, the classification of SCs is not fully systematised. The classification of SCs, based on the chemical structures, was published by Howlett *et al.* [66] and Thakur *et al.* [67], and includes classical cannabinoids, nonclassical cannabinoids, eicosanoids, aminoalkylindoles (AAls) and others (Figure 2.6). However, the classifications can vary, depending on the source. The following paragraphs represent the main classification groups and principal representatives of each class of SCs with a special focus on the most commonly used.

Classical cannabinoids (e.g. HU-210) are structurally similar to Δ^9-THC, based on a dibenzopyran ring. Nonclassical cannabinoids, the cyclohexylphenol (CP) series (e.g. CP47,497), are structurally unrelated to THC. Eicosanoids are synthetic analogues of endocannabinoids such as methanandamide (also known as AM-356) which is a synthetic analogue of anandamide. Aminoalkylindoles (AAls) can be categorised into sub-groups which include naphthoylindoles, "phenylacetylindoles" and "benzoylindoles" [66, 67]. AAls are arguably the dominant components of several herbal products as they are relatively easy to synthesise [68]. The naphthoylindoles class includes JWH-018, JWH-073, JWH-122, JWH-210, JWH-200 and AM-2201. The JWH-series (ranging from JWH-

001 to JWH-450) is named after John William Huffman, a professor at Clemson University, who first synthesised them in the laboratory during the 1990s while investigating the human endogenous cannabinoid system and their potential as therapeutic agents [69]. Phenylacetylindoles include JWH-250 and JWH-251. Benzoylindoles include AM-694 and RCS-4. The last group of SC classes is "other" which encompasses other structural types such as indazole carboxamides e.g. APINACA (AKB48).

Analogues of SCs can be synthesised or modified by changing the length and arrangement of the alkyl chain [68]. Changes can also be made by the addition of an alkyl, alkoxy, halogen, or other substituents to the aromatic ring [68]. Examples of the addition of a halogen are AM-2201, F5-PB-22 and 5F-AKB-48.

SC compounds included in the analytical method in this project were selected due to their apparent availability in Scotland and Saudi Arabia. The Scottish Police Authority kindly provided very useful information about the most significant SCs that are circulating and they are seeing in seizures in Scotland which helped target the analysis. According to the Scottish Police Authority, 5F-AKB-48 and 5F-PB-22 were the most popular in 2014. The other selected drugs were the most prevalent SCs from different generations because the picture of use of SCs in Saudi Arabia is unclear. These included HU-210, CP47,497, JWH-018, the major metabolite of AM-2201, JWH-073, JWH-250, JWH-200, UR-144, AM-1248, AB-FUBINACA and A-796260. The next paragraphs provide a review of each of the SCs that have been studied.

JWH-018 and the major metabolite *N*-(4-hydroxypentyl)

JWH-018 (Figure 2.7) has high pharmacological activity and is easily synthesised, and this may be why it was selected to be one of the first SCs abused [69, 70]. Several studies reported that typical metabolites of JWH-018 are formed in humans after ingestion of AM-2201 [71]. However, Hutter *et al.* [71] found that JWH-018 4-OH pentyl can serve as a good marker to distinguish consume of Spice containing AM-2201 from JWH-018 use.
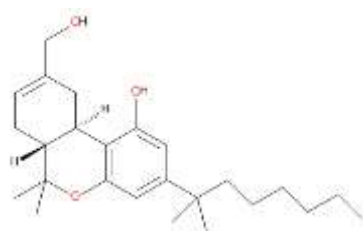
HU-210
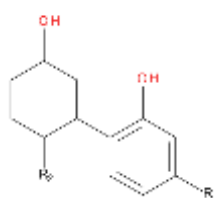
CP 47,497: R$_1$= 1,1-dimethylheptyl
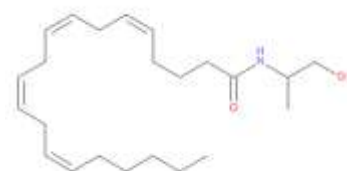
AM-356

1. Classical cannabinoids

2. Nonclassical cannabinoids

3. Eicosanoids
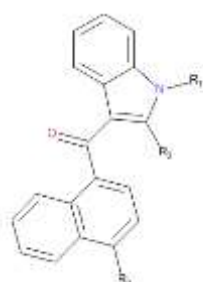
JWH-018: R$_1$= pentyl
JWH-073: R$_1$= butyl
JWH-122: R$_1$= pentyl, R$_3$= methyl
JWH-210: R$_1$= pentyl, R$_3$= ethyl
AM-2201: R$_1$=fluoropentyl
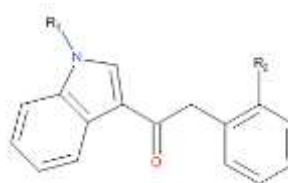
JWH-250: R$_1$= pentyl, R$_2$=methoxy
JWH-251: R$_1$= pentyl, R$_2$= methyl
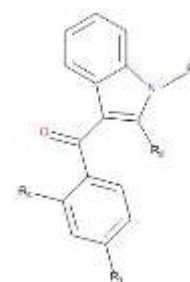
AM-694: R$_1$= 5-fluoropentyl, R$_4$=I
RSC-4 : R$_1$= pentyl, R$_3$= methoxy

4.1 Naphthoylindoles

4.2 Phenylacetylindoles

4.3 Benzoylindoles
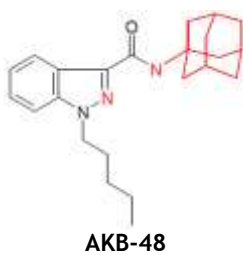
AKB-48
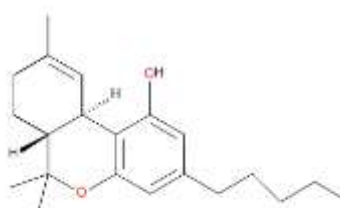
THC

5. Others

Figure 2.6: Structural Classes of SCs.

* The structure of THC is given for comparison

CP 47,497

CP 47,497 (Figure 2.7), which was developed by the biopharmaceutical company (Pfizer) in 1970 during the study of the potential value of these products [72], lacks the dihydropyran ring of THC. Auwarter and co-workers found that CP 47,497 was the active compound in many kinds of herbal mixtures in Germany in 2008 [33]. The popularity of CP 47,497 may be because it has high affinity for the CB_1 receptor and it is more potent than Δ^9-THC [73, 74], like its derivative CP 55,940 [75].

HU-210

HU-210 (Figure 2.7) was first synthesized by a team led by Dr Raphael Mechoulam to advance medical treatments at the Hebrew University in 1988 [76]. The letters HU followed by three numbers refer to the Hebrew University. HU-210 is a full agonist at CB_1 and is reported to increase the duration of the effects compared with natural THC [77]. However, it is difficult to synthesise [75].

JWH-073 and one of the major metabolites JWH-073 3-OH butyl

JWH-073 (Figure 2.7) is an alkyl homologue of JWH-018. Like JWH-018, JWH-073 is poorly soluble in water and produces no hydrophilic conjugates [78]. The main metabolic pathways of JWH-073 and JWH-018 compounds might be hydroxylation and *N*-dealkylation of the parent compound [79].

JWH-250 and one of the major metabolites JWH-250 4-OH pentyl

JWH-250 (Figure 2.7) is potential AAls "phenylacetylindoles" contained in Spice products [80]. They are categorised in a different class because, unlike other JWH series, they do not have a naphthalene ring (fusion of a pair of benzene rings) [81].

AM-2201 4-OH pentyl

The AM-series is designated by the initials of Dr Alexandros Makriyannis from Northeastern University who synthesised them, followed by a series of numbers [82]. AM-2201 (Table 2.3) differs from JWH-018 in the presence of a fluorine atom on the pentyl chain [83]. AM-2201 has been detected in many herbal smoking mixtures [65, 82]. 4-hydroxpentyl (Figure 2.7) is one of the major metabolites of AM-2201 [83].

AM-1248

AM-1248 (Figure 2.7) has an amide and an adamantyl group [57]. AM-1248 was found in several illegal products [57].

JWH-122 5-OH pentyl

JWH-122 5-OH pentyl (Figure 2.7) has been detected in many acute intoxication cases [84, 85]. JWH-122 and its respective fluorinated analogue, MAM-2201 produce common metabolites, *N*-4-hydroxylated, *N*-5-hydroxylated and carboxylated JWH-122 metabolites. The key factor to prove JWH-122 or MAM-2201 abuse is the relative concentrations of JWH-122 *N*-4-OH M and JWH-122 *N*-5-OH M [86]. *N*-4-hydroxylated JWH-122 metabolite is the predominant in JWH-122 metabolism, whereas *N*-5-hydroxylated JWH-122 metabolite was the primary metabolite of MAM-2201 [86].

JWH-210 5-OH pentyl

JWH-210 5-OH pentyl is one of the major metabolites of JWH-210 which is one of numerous potential AAls ("naphthoylindoles") contained in Spice products. JWH-210 5-OH pentyl (Figure 2.7) has been detected in many acute intoxication cases [85, 87].

5F-PB-22 and one of the major metabolites 5F-PB-22 3-carboxyindole

5F-PB-22 (Figure 2.7) is a quinolinyl carboxylate derivative that differs from AM-2201, by replacing the naphthalene group with an 8-hydroxyquinoline moiety. It

has a fluorine atom on the pentyl chain that would offer the possibility of oxidative defluorination [88]. 5F-PB-22 and PB-22 were the first marketed SCs with an ester group [88]. 5F-PB-22 3-carboxyindole (Figure 2.7) is a potential marker for 5F-PB-22 [88].

5F-AKB-48 and one of the major metabolites 5F-AKB-48 (*N*-4 OH pentyl)

5F-AKB-48 (Figure 2.7) is based on an indazole ring substituted at R_1 with a fluoropentyl chain and connected to an adamantyl group through a carboxamide linkage [58]. 5F-AKB-48 (*N*-4 OH pentyl) (Figure 2.7) is a good target metabolite for 5F-AKB-48 [58].

JWH-200

JWH-200 (Figure 2.7) appeared on the market around 2009 [89]. JWH-200 has been detected in whole blood from some cases of severe intoxication [90].

AB-FUBINACA

AB-FUBINACA (Figure 2.7) appeared on the market around 2013 and has been detected in many severe toxicity cases [91].

UR-144

UR-144 (Figure 2.7) is originally designated by Abbot Laboratories [92]. It contains a cyclopropane ring, which is thermally unstable [93]. UR-144 has also been detected in many acute intoxication cases [87, 90].

A-796260

A-796260 (Figure 2.7) has been identified in a seizure of the German Customs [94].

Figure 2.7 shows chemical structures of all analytes included in this project.
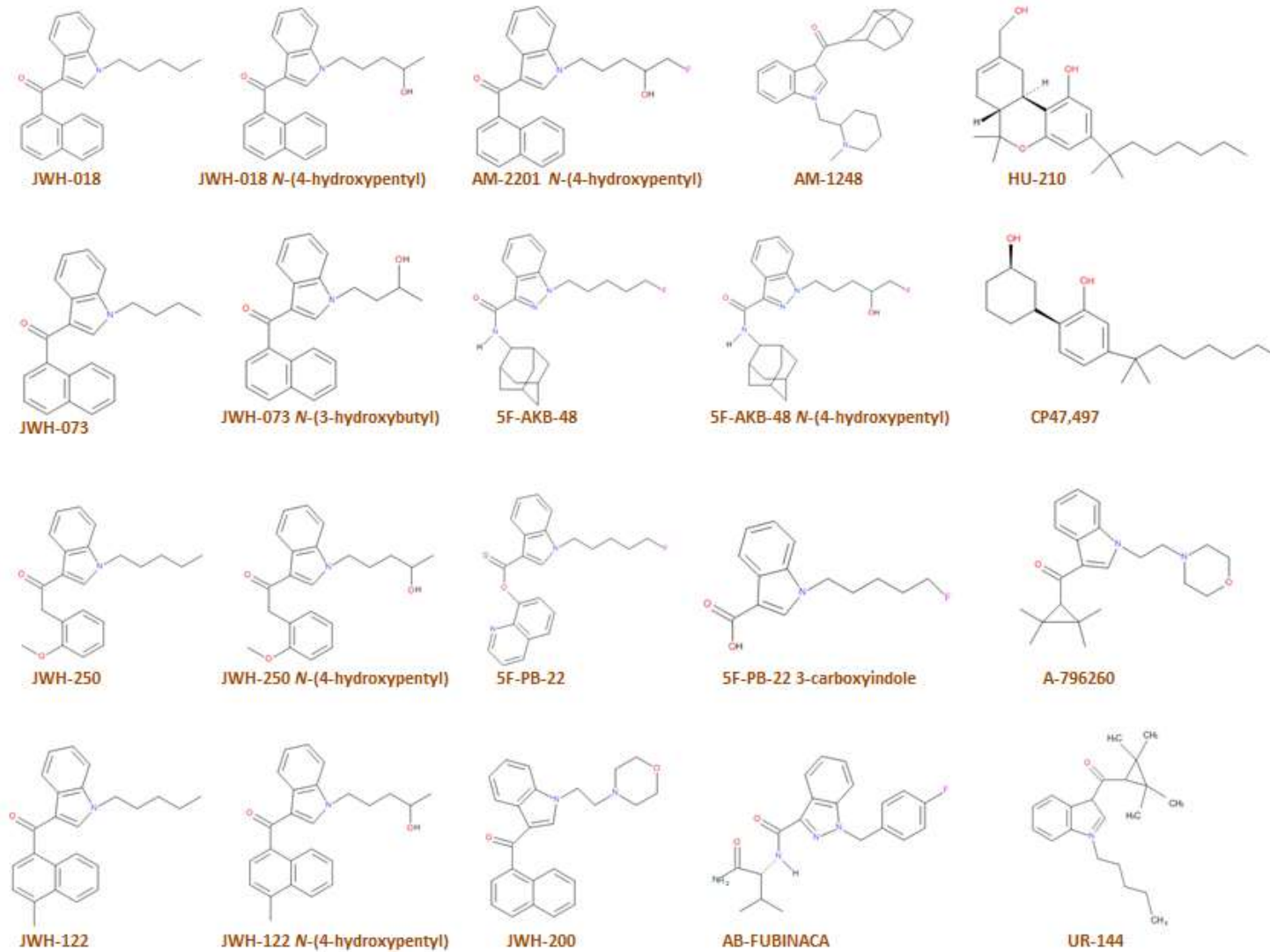
Figure 2.7: Chemical Structures of All SCs Included in this Project

2.3.3 Pharmacokinetics of Synthetic Cannabinoids

SCs have structural features that allow binding to cannabinoid receptors CB_1 or CB_2 or to both of them, in the brain [95]. CB_1 receptors are found in the central and peripheral nervous system, reproductive system, vascular endothelium, lungs, liver, bone and heart, and are responsible for the psychoactive or euphoric effects of cannabinoids, while CB_2 receptors are located in immune system cells and may mediate immune modulatory effects and possibly play a minor role in pain control [96]. CB_2 receptors are also found in the central nervous system but in smaller numbers relative to CB_1 receptors [97].

SC substances bind mostly to CB_1 receptors as full agonists, rather than as partial agonists, as with THC, and then trigger a biological response [98]. CB_1 receptors are present everywhere in the brain and when SC compounds bind in a strong-binding and long-lasting way, they can cause an enormous variety of side effects. However, the picture of the pharmacology and toxicology of many SCs in humans is not clear.

Different methods have been used in order to measure the binding affinity of a substance for a specific receptor. For SCs, most published studies have measured the affinity of binding according to the competitive binding assay illustrated by Compton *et al*. [99]. In *in vitro* studies, it was found that some SCs bind to cannabinoid receptors (mostly CB_1) more avidly than THC as measured by the affinity constant K_i and the effect of this is to produce a stronger response, depending on the dose [2, 85]. Binding affinity (K_i) might provide a useful indicator for assessing the abuse potential of SCs. The K_i values of many SCs have been measured experimentally and they are available in published papers [100]. However, it can vary depending on the experimental set-up used. A low affinity for a receptor means a higher concentration of the compound is needed for binding. THC binds almost equally to CB_1 with a receptor affinity of 40 nM and CB_2 with an affinity of 36 nM. As an example of SCs, JWH-018 binds to CB_1 and CB_2 with receptor affinities of 9 nM and 2.9 nM, respectively. Therefore, most SCs may be 4-5 times more potent than THC [101]. SC substances with a lower affinity for the CB_1 receptor than THC such as JWH-015 are not likely to be used in herbal products.

There are an increasing number of SCs featuring a terminal fluorine atom on substituted *N*-pentylindoles such as 5F-PB-22 and 5F-AKB-48. Banister *et al*. [102] studied the *in vitro* functional activities of JWH-018, PB-22, APICA and UR-144, and their respective fluorinated analogues AM-2201, 5F-PB-22, STS-135 and XLR-11 at CB_1 and CB_2 receptors. They found that the fluorinated analogues generally have higher potency at the CB_1 receptor by 2-5 times. 5F-PB-22 binds to CB_1 with a receptor affinity of 0.13 nM [103] which is more than 69 times lower than that of JWH-018. This increased potency of 5F-PB-22 subsequently increases the risk of toxicity. Table 2.2 shows the published receptor affinities K_i for a selection of SCs of current interest or concern.

Table 2.2: Affinity of Some Synthetic Cannabinoid Agonists for CB_1 and CB_2 Receptors

Compound	CB_1 Ki (nM)[a]	CB_2 Ki (nM)[a]	Ref
JWH-015	336±36.0	13.8±4.60	[104]
JWH-200	42±5	-	[104]
THC	40.7±1.7	36.4±10	[104]
UR-144	29±(0.9)	4.5±(1.7)	[105, 106]
JWH-250	11±2	33±2	[2, 106]
JWH-018	9.0±5.0	2.9±2.7	[104, 106]
JWH-073	8.9±1.8	38.0±24.0	[104]
AM-2201	1	2.6	[104, 107]
5F-AKB-48	0.87±0.14	-	[103]
JWH-122	0.69±0.5	1.2±1.2	[2, 105]
JWH-210	0.46±0.03	0.69±0.01	[106, 108]
5F-PB-22	0.13±0.01	-	[103]

[a] Results are reported as mean plus/minus standard deviation or (standard error of the mean).

The pharmacokinetics of compounds includes the absorption, distribution, metabolism and excretion of compounds throughout the body. There are only a few studies on the absorption and distribution of SC compounds. SCs are lipophilic substances like THC. The usual mode of administration for the SCs is smoking via a conventional pipe, in cigarette papers or via a water pipe [35]. After inhalation, SCs are absorbed through the lungs and diffuse rapidly to fat tissue and can accumulate, leading to a quick decrease of parent drug concentration in blood after administration [109]. Saito *et al*. [110] reported the

accumulation of some SCs such as MAM-2201 in adipose tissue due to their high lipophilicity. SCs can also cross the blood brain barrier and accumulate in brain tissues [111]. At the time Spice products containing SCs were introduced on the marketplace the metabolism pathways of these compounds were generally unknown [112]. Therefore, it has been necessary to study their metabolism in order to identify probable urinary markers to document intake of these products [113]. These studies can also help in developing analytical methods and illustrate the shared metabolism pathways which make the interpretation of results easier.

There are few self-experiments or controlled administration studies for SCs intake in humans because they are ethically questionable [113]. Therefore, most investigations on the metabolism of SCs use models in *in vitro* experiments with human or rat liver microsomes, which is one of the most common approaches used to identify possible metabolites [66, 88, 89, 113-125]; however, some studies of SCs are based on *in vivo* experiments [97, 81, 89, 113, 115, 126-132]. Generally, the metabolism studies showed that SCs are easily metabolised and have extensive metabolism pathways. The most common metabolic pathway of naphthoylindole compounds was the hydroxylation at the indole, alkyl and naphthyl substructures. Figure 2.8 shows the hydroxyl metabolites of JWH-018. Carboxylation, *N*-dealkylation and dihydrodiol formation at the naphthyl group were also observed [79, 114]. 5F-PB-22 can be metabolised with a specific biotransformation (ester hydrolysis) which is the predominant metabolic pathway, yielding a variety of (5-fluoro)pentylindole-3-carboxylic acid metabolites [88]. Pentanoic acid metabolites are generally favoured by the fluorinated analogues. Phase II conjugates of most SC metabolites are glucuronides. The other conjugates include sulphate- (e.g. JWH-122 and JWH-200) and cysteine-conjugation (e.g. 5F-PB-22).

The metabolites of SCs are mainly excreted in urine as glucuronide and/or sulphate conjugates. It was found that monohydroxylated [79] and carboxylated [129] metabolites are present in urine in the highest quantities. Due to the speed of both the emergence and spread of SCs on the world market, the pharmacokinetic properties of some SCs have not been satisfactorily investigated to date. Table 2.3 lists metabolic pathways of a selection of SCs included in this study and the major metabolite for each parent compound.

JWH-018 M4 [(5-hydroxyl-1-pentyl-1*H*-indol-3-yl)(napthalen-1-yl)methanone]

JWH-018 M3 [(4-hydroxyl-1-pentyl-1*H*-indol-3-yl)(napthalen-1-yl)methanone]

JWH-018 M1 [(1-(5-hydroxypentyl)-1*H*-indol-3-yl)(napthalen-1-yl)methanone]

JWH-018

JWH-018 M5 [(6-hydroxyl-1-pentyl-1*H*-indol-3-yl)(napthalen-1-yl)methanone]

JWH-018 M6 [(7-hydroxyl-1-pentyl-1*H*-indol-3-yl)(napthalen-1-yl)methanone],

JWH-018 M2 [5-(3-(1-napthoyl)-1*H*-indol-1-yl)pentanoic acid]

Figure 2.8: Chemical Structures of Alkyl Side Chain (M1 & M2), and Indole-ring (M3-M6) Hydroxylated Metabolites of JWH-018

Table 2.3: Metabolism of SCs
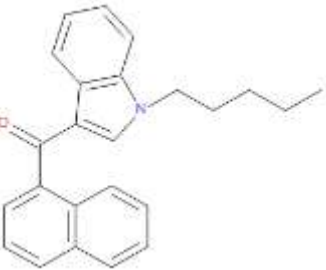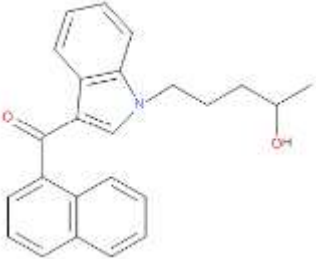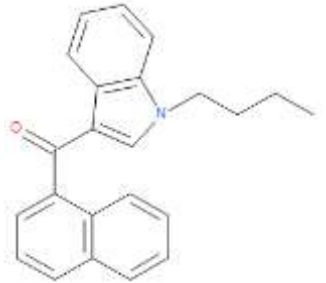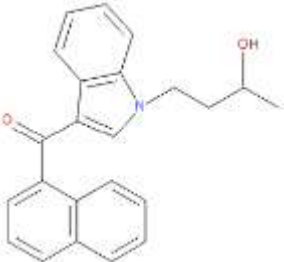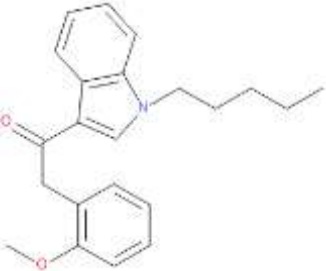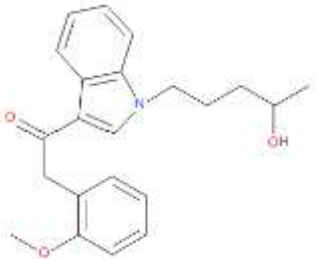
SC	Chemical Structure	Metabolic Pathways		Major Metabolite
		Phase I	Phase II	
JWH-018		Hydroxylation, Carboxylation, Dehydrogenation, *N*-Dealkylation, Dihydrodiol formation	Glucuronide conjugates	JWH-018 *N*-(4-hydroxypentyl)
JWH-073		Hydroxylation, Carboxylation	Glucuronide conjugates	JWH-073 *N*-(3-hydroxybutyl)
JWH-250		Hydroxylation, Carboxylation, Dehydrogenation, *N*-Dealkylation	Unspecified	JWH-250 *N*-(4-hydroxypentyl)

Table 2.3: Metabolism of SCs (Continued...)
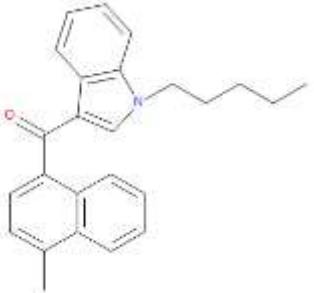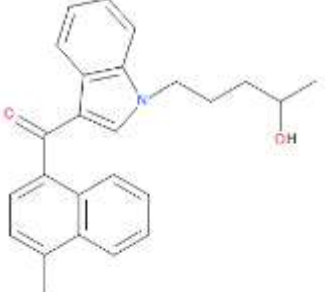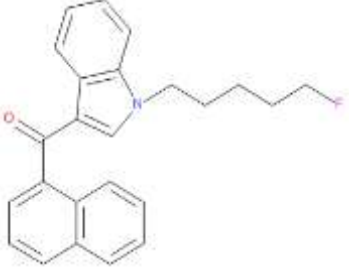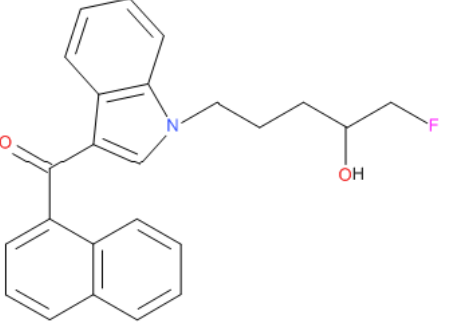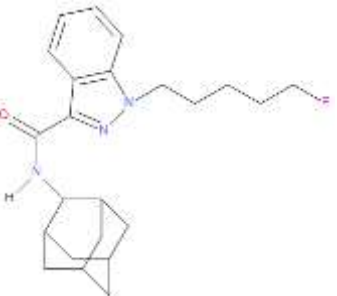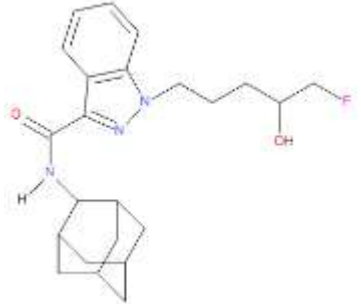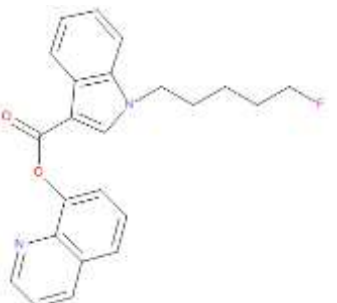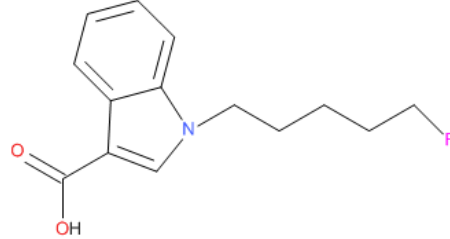
SC	Chemical Structure	Metabolic Pathways		Major Metabolite
		Phase I	Phase II	
JWH-122		Hydroxylation, Carboxylation, Dehydrogenation, N-Dealkylation, Dihydrodiol formation	Glucuronide/ Sulphates conjugates	JWH-122 N-(4-hydroxypentyl)
AM-2201		Hydroxylation, Carboxylation, N-Dealkylation, Dihydrodiol formation, Oxidative defluorination	Glucuronide conjugates	AM-2201 N-(4-hydroxypentyl)

Table 2.3: Metabolism of SCs (Continued...)

SC	Chemical Structure	Metabolic Pathways		Major Metabolite
		Phase I	Phase II	
5F-AKB-48		Hydroxylation, Carboxylation, *N*-Dealkylation, Ketone formation, Oxidative defluorination	Glucuronide conjugates	5F-AKB-48 *N*-(4-hydroxypentyl)
5F-PB-22		Hydroxylation, Carboxylation, Dihydrodiol formation, Oxidative defluorination	Glucuronide/Cysteine conjugates	5F-PB-22 3-carboxyindole

2.3.4 Pharmacological Effects of Synthetic Cannabinoids

It is difficult to predict the harmful or desired effects on the human body after smoking SCs due to the significant variability in the amount and type of SCs between herbal blends and within a package. After use of SCs, clinical symptoms can start immediately or after some minutes or hours; however, the duration of symptoms is variable and may last hours [133]. Most of the information comes from case reports in the medical literature, from Poison Control Centres and from emergency service calls.

Like THC, SC compounds bind to cannabinoid receptors [95] which results in the creation of cannabis-like effects (e.g. impaired sense of time, paranoia, sedation, hallucinations and anxiety [85, 134]. THC in high dose is a hallucinogen and can cause negative effects such as panic attacks which are reported as effects from SCs [135]. However, some users reported and/or showed additional unique effects from SCs such as agitation [85], acute kidney injury [136], stroke [137], myocardial ischaemia [138], hypokalaemia, hyperglycaemia, seizures and emesis [139, 140]. Other side effects of SCs, including tinnitus, coughing, conjunctival hyperaemia, sensitivity to light, blurred vision and mouth dryness have also been reported [29]. It has been reported that the duration of psychoactive effects exerted by fluorinated SCs (e.g. 5F-PB-22, 5-F-AK48) may be longer than that of non-fluorinated parent compounds [64]. 5F-PB-22 potently produced depressant effects lasting 120–150 minutes [141].

There are few studies of the toxicological properties of SCs at the cellular level [142-144]. Although the authors described several processes contributing to this action, like interference with protein synthesis, DNA damage, apoptosis and damage of cell membrane, molecular mechanisms of SCs-induced cell death are still not very clear [64].

Symptoms may depend on the duration and frequency of use and dose. Chronic use and overdrose of SCs may cause adverse effects including tachycardia and altered mental status [145]. Withdrawal symptoms of SCs have been documented in two case reports, and include but are not limited to nausea, vomiting, diarrhoea, headache, insomnia, palpitations, tremor, diaphoresis (sweating) and uneasy feelings [29, 139].

2.3.5 Concentration of Synthetic Cannabinoids in Fatal Cases

SCs have been reported in association with deaths in several publications. Behonick *et al*. [3] reported four deaths following use of 5F-PB-22. In one case, an autopsy revealed pulmonary oedema in both lungs, the congestion of visceral organs and necrotising granulomatous inflammation with histo-plasma microorganisms and the toxicology report confirmed the presence of 1.5 ng/mL of 5F-PB-22 in superior vena cava blood. The cause of death was suspected acute drug intoxication by 5F-PB-22. In two cases, the cause of death was attributed to 5F-PB-22 intoxication. The fourth case showed that the cause of death was fulminant liver failure caused by 5F-PB-22 and THC.

Another case associating SCs with death has been reported by Patton *et al*. [146] involving a 23-year-old man found with a stab wound to the neck, with no known history of previous psychiatric care, mental illness, or past or current use of illicit drugs or antipsychotics. Toxicological analysis of blood was negative for alcohol, THC, cocaine, amphetamines, benzodiazepines and opiates. Further testing using procedures capable of detecting many types of SCs and their metabolites proved the presence of 12 ng/mL of AM-2201 along with 2.5 ng/mL of oxidised metabolite of AM2201. The case indicated the potential mortality associated with the psychiatric complications of AM-2201 use.

Recently, ADB-CHMINACA (also known as MAB-CHMINACA) has been reported in association with two deaths. The first one was reported by the Hungarian National Focal Point [147] and it was amongst other substances. The second one was reported by Hasegawa *et al*. [148] that occurred in Japan. MAB-CHMINACA was found in nine solid tissues; the highest concentration was found in the liver (156 ng/g) followed by the kidney, pancreas and heart muscle.

2.3.6 Concentration of Synthetic Cannabinoids in Cases of Abuse

At present, there are deficiencies in the literature regarding toxic and lethal concentrations of most of the SCs. Teske *et al*. [149] reported maximum concentrations of SCs in serum at 5 minutes after smoking (administration of approximately 50 µg/kg of JWH-018) in the range of 10 ng/mL, and then decreasing rapidly to less than 10 % of the maximum concentration within 3

hours. This study is compatible with the observations of the effects of Spice by users in several cases [150].

A single self-administered dose of herbal products named "Kronic" containing JWH-073 and JWH-018 was smoked to assess the authors' analytical method [151]. Peak urine concentrations of 1 ng/mL JWH-073 *N*-butanoic acid, 10 ng/mL JWH-018 *N*-5-hydroxypentyl and 2 ng/mL JWH-018 *N*-pentanoic acid were found. In contrast, in some severe intoxications or psychiatric clinics cases, concentrations might be much higher, for example, in one case the concentration of JWH-122 was up to 230 ng/mL in urine [152].

Hermanns-Clausen *et al.* [85] studied the clinical and laboratory findings for 29 patients seeking emergency treatment after use of SCs between September 2008 and February 2011. Table 2.4 shows acute and death levels of some SCs in blood. In most of these cases, SCs were considered as a contributory factor in the death or as the cause of death. It can be observed that there is an overlap in the concentration ranges between Accident and Emergency (A&E) admissions with fatal concentrations, and this may be due to individual variation or cases of poly-drugs consumption. However, the lethal doses for many SCs are still unknown [153].

Table 2.4: Concentrations of Some SCs in Blood in Acute and Fatal Intoxication Cases

Compound	Acute Level ng/mL	Death Level ng/mL
JWH-018	0.05-11 [90]	0.1-199 [154]
JWH-073	0.1 [87]	0.1-68.3 [154]
JWH-250	0.14-0.73 [90]	-
JWH-122	0.05-21.8 [90]	-
JWH-210	0.05-8.1 [90]	-
AM-2201	0.05-7.85 [90]	12 [146]
UR-144	0.05-25.9 [90]	
5F-PB-22	-	1.1-1.5 [3]
MAM-2201	0.05-21.3 [90]	12.4 [110]
ADB-CHMINACA	-	6.05-10.6 [148]

2.3.7 Legal Status of Synthetic Cannabinoids

The emergence of NPS is clearly a global phenomenon. In response to the rising popularity of these compounds, many national systems and organisations have been established around the world to monitor and share information about NPS to restrict the supply of them.

The EMCDDA plays an important role in Europe's response to NPS. The EMCDDA is the main source of information on drug related issues in Europe. Information from the European countries on the trends and appearance of NPS, along with reported harms can be provided by the EU EWS. In the beginning of 2009, the EMCDDA convened a meeting and produced a report on the problem and situation of the emergence of SCs. The UNODC [4] launched Global Synthetics Monitoring: Analyses, Reporting and Trends (SMART) Programme to address the challenge of NPS through the review of existing legislation and to explore how countries have introduced new legislation to control synthetic drugs. It provides capacity building to research officers, law enforcement and laboratory personnel in the Pacific, Latin America, Africa, the Near and Middle East, South Asia and East and South-East Asia and regularly reviews the situation of NPS and ATS. In the UK, the Advisory Council on the Misuse of Drugs (ACMD) was established under the Misuse of Drugs Act 1971. It provides recommendations regarding to the classification of new drugs which appear to them to be abused and causing harmful effects to society.

2.3.7.1   Generic Legislation

SCs usually take several months to ban [155]. Generic legislation is a faster procedure that prohibits clusters of substances showing similarity with the chemical structure of an existing compound [155]. In the UK, generic definitions for cannabinoid receptor agonists were developed. In December 2009, a number of SCs were controlled under the Misuse of Drugs Act as Class B drugs on recommendation from the ACMD [156]. Psychoactive Substances Act 2016 [157] applies across the UK and had been into force from 26 May 2016. The act makes it an offence to produce, supply, possess, import or export psychoactive substances. Most substances were subsumed by several generic definitions (e.g. the indole-3-methanone, cyclohexylphenols and dibenzopyran nucleus) and only

a few were added as named entities. Many SCs have been successfully controlled based on these generic definitions in 2009. The generic definitions were incorporated into the national drugs legislation of several countries (e.g. Lithuania, Denmark, and Austria). Four years later, in 2013, further controls included five named substances and seven principal definitions controlled by the Misuse of Drugs Act [156]. The generic legislation allows countries to ban large numbers of SCs found in the marketplace [158]. However, the diversity of SC families makes generic control an inefficient process and there is also concern when using such a broad definition about unintentionally including a therapeutically useful drugs not yet discovered.

2.3.7.2 Analogue Legislation

The analogue approach is an alternative method for legislation and was first utilised in the USA. A new substance can be controlled based on the analogue approach when it meets specific conditions i.e. it has a chemical structure similar to a controlled substance and either has an effect on the central nervous system greater than or similar to a controlled substance. The conditions were defined by the Federal Controlled Substances Analogue Act, 1986 [159]. In 2010, only the State of Kansas and the U.S. Military had banned Spice products [160]. In 2012, the DEA classified five specific SCs (JWH-018, JWH-073, JWH-200, CP 47,497, and CP 47,497 C8 homologue) as Schedule 1 drugs under the United States Controlled Substance Act [161]. Following frequent waves of different structures, many SCs have since had to be temporarily designated into Schedule I: ADB-PINACA, AB-FUBINACA, PB-22 and 5F-PB-22 in 2014 [162] and MAB-CHMINACA in 2015 [163]. Analogue legislation presents a new set of problems as most SCs are not considered to be analogues of controlled substances because they have different chemical structures. For this reason, it does not appear to offer an efficient way of controlling SCs [164]. Although governments and national organisations make concerted efforts to address the challenge of SCs and start banning many of them gradually, the absence of an international regulation makes it difficult to control this issue. Moreover, legal confusion remains because Spice drugs shipped with certificates indicate the absence of regulated substances [75]. In addition, Spice products are still offered on the internet with manufacturers continually changing the chemical structures in an attempt to avoid regulations [75].

2.3.8 Prevalence of Synthetic Cannabinoids

The prevalence of SCs is still largely unknown and hard to ascertain. However, the picture is getting clearer because questions about the use of SCs are incorporated in national surveys in many countries [61]. Despite all the hard work around the world against the use of SCs, their use has increased at an alarming rate [61]. This may due to the late response to the emergence of these new drugs as well as the lack of focussed strategies that have led to the absence of the knowledge of the extent of the problem.

There are notable differences in the prevalence of SCs in drug markets around the world. In the UK, a national survey on Spice use showed low lifetime prevalence rates for adults aged 16 to 64 at 0.2% in 2010-2011 and 0.1% in 2011-2012 [61]. In Spain, in 2012, a global survey on drug use for students (14 to 18) with a sample of 27,503 reported low lifetime prevalence levels of use of herbal mixtures and Spice at 1.4% [61]. In France, in 2014, a survey of young people aged 17 years showed that 1.7 % of the sample population had already used SCs [61]. In Scotland, the Scottish Schools Adolescent Lifestyle and Substance Use Survey (SALSUS) conducted in 2013 found 4% of 15 year-olds reported having used one or more NPS at least once in their lifetime; the most commonly used NPS were mephedrone, SCs and salvia [165]. They used for the SCs question the terminologies Spice, "Black Mamba" and "Clockwork Orange", that could probably be considered the most popular brand names in Scotland at that time and reflected their attempt to avoid users misunderstanding the question. In Arabian Gulf countries, particularly Saudi Arabia, there is currently no prevalence data regarding the use of products containing SCs among Saudi people.

The selection of terminologies used is important in any questionnaire but seems especially crucial around the topic of SCs due to the mismatch between terms commonly used by users and by health professionals. Users sometimes use street names for drugs therefore it is quite important to use different terminologies to describe them. These studies have provided useful information; however, differences in their methodologies and definitions make comparisons between the data very difficult.

Prevalence studies of use of SCs are often heterogeneous and derived from a limited number of countries. Several papers were reviewed regarding the prevalence of SCs using different methods, including analytical investigation of biological samples, questionnaires/surveys, information on A&E admissions related to SCs use and calls to poison centres. Table 2.5 summarises the studies of the prevalence of use of SCs and the following paragraphs include examples studies of each method.

The first study looking at the prevalence of use of SCs using the analysis of biological specimens was conducted in Germany in 2009. 7,500 authentic specimens from doping controls were analysed for the presence of JWH-018 and metabolites. Two specimens were found positive [120]. In 2011, another study from the USA was performed. 5,956 urine samples from athletes without a suspicion of use were screened for JWH-018, JWH-073 and their metabolites. The prevalence was 4.5% for JWH-018, JWH-073, or any of their metabolites [166]. During 2011–2012, a study in Norway was conducted [167]. 762 blood samples collected from drivers suspected of driving under the influence of drugs (DUID) were analysed for 18 different SCs. The prevalence of the selected SCs in the tested material was 2.2%. A comparison cannot be made between these sets of data because the studies were conducted at different times and the biological samples were analysed for different groups of SC compounds.

For questionnaire or survey studies, the first paper was published in 2009, from the UK [168]. An online survey, with an invitation to participate, was posted on a website to the readers of the dance magazine MixMag in the UK. 2295 participates responded. The use of Spice, "Warrior" or "Magic" smoking blend was reported by 12.6% of the sample population. This high prevalence may indicate the popularity of using these products among this kind of population. Another survey, originating in the USA explored the prevalence of K2 use among college students in 2010 [169]. A mailed survey was sent to a total of 2396 college students. 8% (69 out of 852) of the sample population reported that they had been using SCs. K2 was more common among males and the high prevalence may reflect the popularity of using Spice products among college students. The response rate was 36%. There is no agreed norm as what would be received as an acceptable response rate (also termed return rate) [170], However several studies indicated the acceptiable response rate was approximately 56% [170,

171]. In 2011, another study from the UK, explored the use of NPS including SCs among gay nightclubs in South London [172]. 313 customers were surveyed over 4 nights. The prevalence of lifetime use of Spice was 9% whereas it was 0.6% for use on the same night. A global online survey was conducted in 2011 and data from 14,966 participants was collected [173]. The use of SCs was reported by 2513 (17%). This study had a good sample size; however, it was limited by the self-report methodology and the self-nominating nature of the sample.

The prevalence of SCs through the statistics of visits to hospital emergency departments or calls to poison centres can offer a vital role in prevalence studies. According to the American Association of Poison Control Centres (AAPCC), the number of calls related to SCs exposure sharply increased from 2096 calls in 2010 to 6968 in 2011, but decreased to 5230 in 2012, and dropped to 2668 in 2013; the trend of the exposures to SCs increased again with 3,679 calls in 2014 [174]. The decrease in the number of calls received in 2013 was most likely due to improving public awareness of SCs toxicity and moving some types of SCs to Schedule I. In the AAPCC data, the exposure to SCs was highest for subjects aged 13-19, comprising 48.8 % of the calls [175]. They observed that men prefer to use SCs more than women. In a sample of calls received during the time period 2010–2011 at the Texas Poison Centres [176], the prevalence of the exposure to SCs was 4.1 per 100,000 inhabitants. The rate was 4.90 in rural counties and 4.02 in urban counties. Another study [177] from the Drug Abuse Warning Network on visits to A&Es involving SCs was performed in 2010. Among the AE visits linked to SCs, a total of 8,557 visits (75%) were made by patients aged 12–29.

There is not much data regarding the types of group(s) of SCs that were present in each country, however, it is clear that this can vary from one region to another and from time to time. For example, according to the Welsh Emerging Drugs and Identification of Novel Substances (WEDINOS) project which was designed for the collection and testing of substances to identify what chemicals are in circulation in Wales, 5F-AKB-48 was the most commonly identified SC during 2013 and the beginning of 2014, and the rise in prevalence of 5F-PB-22 resulted in the displacement of 5F-AKB-48 [44].

Table 2.5: Summary of Prevalence Studies

Drugs Included	Duration Time	Number of Cases	Description of Study	Study Findings	Ref
Biological Matrices					
JWH-018	2009	7500	A selection of urine specimens from athletes in doping controls	0.03 % Positive for JWH-018	[120]
JWH-018 JWH-073	2010	25	Urine specimens from athletes who suspected of using Spice products	92% Positive for JWH-018 and/or JWH-073	[178]
JWH-018 JWH-073 JWH-250	2010	679, 50,000	679 Oral fluid 50,000 urine	15% Positive, 679 Oral fluid specimens; 18% Positive, 50,000 urine specimens	[179]
JWH-018 JWH-073	2010-11	2060	Urine specimens from users who suspected of using Spice products	29.6% Positive for at least one SC; 20.3% positive for JWH-018 metabolites; one case positive only for JWH-073; 8.9% positive for metabolites from both	[180]
JWH-018 JWH-073	2011	5956	Urine specimens from athletes	4.5% Positive for JWH-018 or JWH-073	[166]
Surveys					
Spice	2009	2295	Online survey targeting dance drug users	Lifetime use rate: 12.6%	[168]
Spice	2010	-	Survey (aged 15–18) in Frankfurt am Main	9% Lifetime; 2% last 30 days	[181]

Table 2.5: Summary of Prevalence Studies (Continued…)

Drugs Included	Duration Time	Number of Cases	Description of Study	Study Findings	Ref
Surveys					
SCs	2012	45,000-50,000	Students from three grades	11.3% (12th graders); 8.8% (10th graders); 4.4% (8th graders)	[182]
SCs	2010-11	-	Survey (aged 16–59) in England and Wales	Past year use rate: 15–24 years, 0.4% 25–59 years, 0.1%	[183]
Spice	2011	313	Questionnaire in gay nightclubs in London	9% Lifetime; 2.2% past month; 0.6% same night	[172]
Calls and Accident and Emergency Data					
SCs	2010	-	Accident and Emergency cases	Rate per 100,000: 11.1, 12–29 years; 14.9, 12–17 years; 13.9, 18–20 years; 11.8, 21–24 years; 4.1, 25–29 years	[177]
SCs	2010-11	-	Callers to the poison centre of Texas	Exposure rate per 100,000: 2.79-7.14 (11 health regions in Texas); 4.90, rural counties; 4.02, urban counties.	[176]

2.3.9 Biological Sample Preparation and Extraction Techniques for Synthetic Cannabinoids

Before introducing any samples to chromatographic techniques such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS), the analytes first need to be extracted from either the product or matrix and concentrated.

Sample preparation is an essential stage of the analytical process to convert the biological specimen into a form that is suitable for analytical investigation. The extraction step is the main part of the procedure of sample preparation, and presents two major advantages for the analysis process [184]. Firstly, it removes interfering matrix compounds (such as proteins, salts and phospholipids) which reduces background noise. Secondly, it concentrates the target drugs, increasing sensitivity and achieving lower limits of detection.

In LC-MS/MS, suitable sample preparation is important to reduce matrix effects, which is a measure of the change in signal caused by matrix components. Matrix effects may alter the MS response of the target drug or internal standard resulting in ion enhancement (gain in signal) or suppression (loss of signal) leading to effects on the precision, accuracy and robustness of the method. Matrix effects depend mainly on the sample matrix, ionisation type, mobile phase additives, quality of chromatographic separation and sample preparation procedure. Matrix components are changeable between samples (inter-individual variability) and It is not known which matrix components cause ionisation enhancement (or suppression); however, they interfere with the processes involved in the transfer of the ion into the gas phase, leading to a decrease or increase in the ionisation efficiency [185].

Simple dilution, protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE), supported liquid extraction (SLE) and salting-out LLE (SALLE) have been used for the extraction of SCs. The following paragraphs describe the main extraction methods used for SCs.

2.3.9.1   Dilute and Shoot

Dilute and shoot (also called simple dilution) is a simple sample preparation method in which one simply dilutes the sample with compatible liquid (e.g. solvent, mobile phase). The diluted sample is then injected into a chromatograph.

The main advantage of this procedure is that it is very quick. It can also reduce the matrix effect when using LC-MS [186]. However, it has many limitations such as the injected sample is usually unclean due to the interferences. These interferences might build up in the system column and inlet leading to many chromatographic problems such as ion suppression, reduced detectability and carryover [186]. Cleaning and troubleshooting the system cost the lab time and money. Few authors have used the dilute and shoot method for the sample preparation of SCs [187, 188].

2.3.9.2   Protein Precipitation

Protein precipitation (PPT) has been found to be more useful when analysing protein rich matrices, such as whole blood, serum and plasma. An organic solvent, commonly acetonitrile, is added to the specimen to reduce the solubility of the solute and precipitate the protein, and then it can be removed from the specimen by centrifugation or filtration.

Protein precipitation is a rapid and simple extraction technique; however, it does not remove many of the matrix interferences. Some authors have used protein precipitation for the extraction of SCs [146, 189].

2.3.9.3   Liquid-Liquid Extraction

LLE is applicable with most types of matrices. In SCs extraction, LLE is usually used because of their high hydrophobicity. It has been used for the extraction of SCs in the following matrices: blood [3, 90, 154, 167, 190, 191] , serum [149, 152, 192-194], plasma [195], urine [79, 82, 114, 120, 127, 129, 151, 196-200], oral fluid [201, 202] and hair [203-207].

It involves adding an immiscible organic solvent such as *tert*-butyl methyl ether [114, 198], chloroform [130] or diethyl ether [79, 132, 151] to the sample, and mixing, removing the organic solvent. The target compounds transfer from the sample layer to the organic layer. The more dense solvent will be the lower layer, while the less dense solvent will be the upper layer. The distribution of the components of the initial mixture amongst the two immiscible solvents is determined by their partition coefficients. A compound that is more soluble in the more dense solvent will preferentially reside in the lower layer. Conversely, a compound more soluble in the less dense solvent will preferentially reside in the upper layer. A sufficient amount of solvent should be used to capture all of the drugs from the original sample. The two phases are agitated by shaking the mixture, and then the organic phase is allowed to separate. Generally after extraction hydrophobic compounds (e.g. SCs) are found mainly in the organic solvents. Compounds extracted into the organic phase are simply recovered by evaporation of the solvent and then reconstituted with a small volume of an appropriate solvent (e.g. mobile phase). The Nernst distribution law (Equation 1) can help illustrate the extraction process, and states that any neutral species will distribute amongst two immiscible solvents so that the ratio of the concentration remains constant [184, 208].

$$K_D = \frac{C_o}{C_{aq}}$$

Equation 1

Where K_D is the distribution constant, C_{aq} is the concentration of the analyte in the aqueous phase and C_o is the concentration of the analyte in the organic phase.

The extraction of analyte from the aqueous phase depends on several factors including solubility of analyte in the organic solvent, pH of the aqueous phase and polarity of the organic solvent. The ideal solvent should be highly selective and allow only the active agent to be extracted, high capacity in order to reduce the amount of solvent necessary and high positive difference in density. There are also practical concerns when selecting extraction solvents [184].

LLE offers better clean up than protein precipitation because LLE removes many of the matrix interferences. In addition, it can be optimised for different compound classes. However, it is labour intensive and it is also possibly less reproducible than SPE. In addition, it sometimes requires an evaporation step to remove excess solvent prior to analysis.

2.3.9.4   Solid Phase Extraction

In recent years, SPE has become more popular as an extraction technique for the analysis of illicit drugs. Different types of SPE columns can be used depending on the cost, availability and the nature of the analytes of interest. The sample matrix is allowed to pass via the sorbent to waste, with the target analytes being retained. A series of washing steps is essential to remove matrix interferences, and then target analytes are eluted off the sorbent and collected in a clean vial [209]. Extraction of SCs using SPE has been used in blood and serum [210], urine [81, 166, 211-213] and oral fluid [126, 214, 215].

SPE will potentially remove the endogenous compounds that cause technical problems such as co-elution which may cause matrix effects. The use of a suitable sample preparation technique (e.g. SPE) and deuterated internal standards (ISDs) can help reduce matrix effects and their impact on accurate quantitation. However, Grigoryev *et al*. [78] reported nearly identical amounts of matrix elements for LLE and SPE in the extracts of SCs in urine samples. Rigdon *et al*. [216] reported SPE gave better recovery of carboxylated metabolites of JWH-018 and JWH-073 in urine samples than LLE. The disadvantages of SPE are the cost of consumables and hardware required.

2.3.10   Analytical Methods for Synthetic Cannabinoids

Immunoassays (e.g. enzyme linked immunosorbent assays 'ELISA') and separation methods such as GC/MS, LS-MS/MS and liquid chromatography–high resolution mass spectrometry (LC–HR/MS) have all been used for studying SCs and metabolites in biological samples as well as in seized products, such as herbal blends and powders.

2.3.10.1 Immunoanalysis

An immunoassay is a biochemical test which incorporates the binding reaction of an antibody with a target substance (antigen) [217]. "Immuno" refers to an immune response, and "assay" refers to a test. Immunoassay is a rapid technique used as an initial screening test to eliminate negative samples. Whilst quick, it has limitations, such as lower specificity, potentially leading to false positives. As such, it is highly recommended that all positive immunoassay findings be retested and confirmed by chromatographic analysis. Immunoassay tests have been used for the screening of SCs in oral fluid [218] and their metabolites in urine samples [198, 211, 219-221]. Different commercial kits have been available for detecting SCs and metabolites, including those made by Randox Toxicology, Neogen Corporation, Cayman Chemical, Immunalysis Corporation and National Medical Services [220]. The main limitation of immunoassay is the inability to cross-react with newer analogues of SCs as they become more widely used. It is difficult for the manufacturers to keep up within the rapidly changing analogues in use.

Rodrigues *et al*. [218] evaluated an ELISA targeting JWH-200 in oral fluid. They reported that in oral fluid ELISA targeting JWH-200 had limited cross-reactivity to JWH-018, JWH-073, JWH-015, JWH- 022, AM-2201, AM-2232 and AM-1220. Kronstrand *et al*. [222] compared the performance of an immunoassay screening for SCs with a newly-developed non-targeted qualitative LC-HR/MS confirmation method in urine. The screening method included metabolites from AM-2201, JWH-073 and JWH-018. The confirmation method included metabolites from AM-2201, JWH-073, JWH-018, JWH-398, JWH-250, JWH-210, JWH-122, JWH-081, JWH-019, MAM-2201, UR-144 and RCS-4. The authors observed no cross-reactivity with UR-144 metabolites but there was cross-reactivity with JWH-122 and MAM-2201 metabolites for the immunoassay. They also evaluated the specificity and sensitivity of the immunoassay with 87 real urine samples and found these to be 82% and 87%, respectively. They concluded that the immunoassay performed well for SCs present in the urine samples tested. Due to the rapid changing of SCs, which may cause problems for immunoassays and for confirmation methods, the authors also recommended using a time-of-flight/mass spectrometry (TOF/MS) for forensic laboratories as new SCs can be quickly included in the method and identified.

2.3.10.2 GC-MS

Gas chromatography (GC) coupled with mass spectrometry (MS) is a useful technique for analysing compounds in forensic toxicology. Gas chromatography is used for sample separation whereas mass spectrometry is for detection.

GC refers to group of analytical separation techniques utilised to separate volatile analytes in the gas phase. The term *chromatography* (derived from the Greek for colour-chroma and to write-graphein). The main components of a GC instrument are carrier gas, injector and column (Figure 2.9). The extracted drug should be injected onto the GC column via the injection port which is connected to the top of the column. The sample should be vaporised in the injection port before entering the column. The injection port contains a rubber septum through which a syringe needle is pierced to inject the sample. The sample is then transported by a gaseous mobile phase, usually helium or nitrogen through a capillary column to the detector [223].
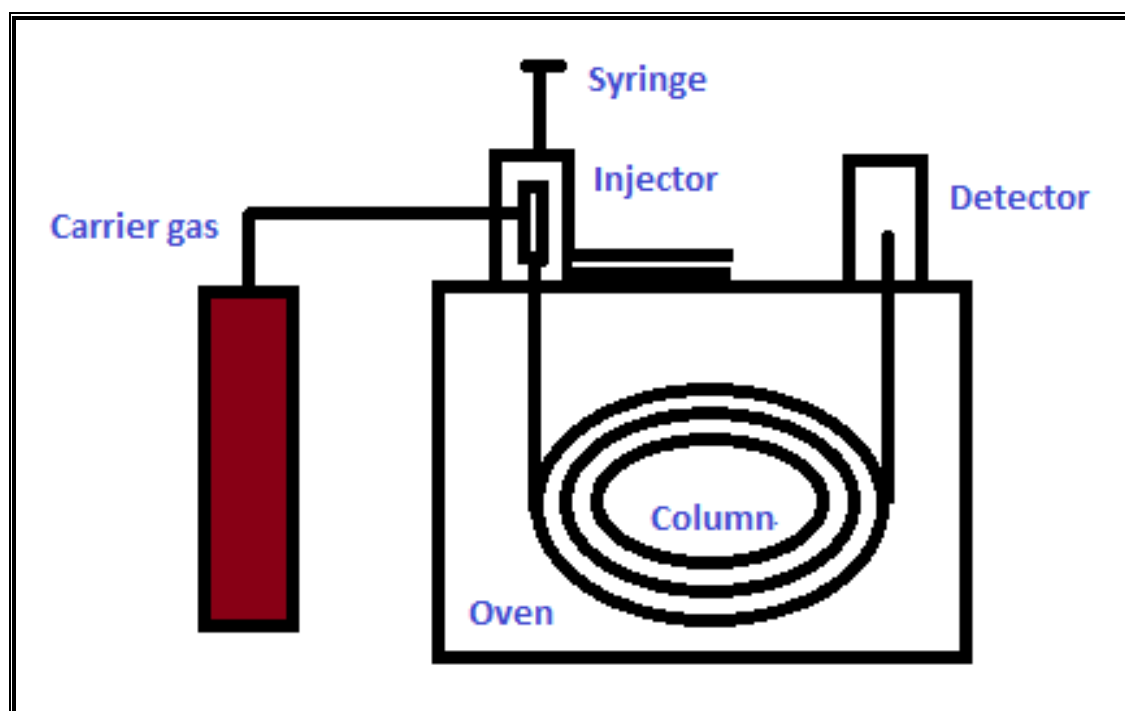
Figure 2.9: Gas Chromatography Components

Capillary columns can made from fused silica or metal, although the latter cannot be connected to the source directly. Columns vary according to stationary phase composition, from non-polar to polar and their length, from 10-100 m [223]. The separation of mixtures is based on the degree of interaction, as expressed by the partition coefficients of the components, with silicone polymer

(stationary phase), chemically bound to the silica of the column. The thickness and the properties of the stationery phase as well as length and diameter of the column influence the final extent of the interactions. Therefore, compounds travel through the column at different times (retention times). Highly volatile molecules travel via the column faster than less volatile compounds. Molecules that elute quickly have short retention times whereas analytes with higher molecular weights, many functionalities, higher boiling points, higher solubility in the stationary phase or higher polarity are retained for longer. They require higher temperatures to elute them because they are preferentially attracted to the stationary phase. The molecules are then transferred to the detector, of which there is a variety to choose from, including a flame ionisation detector (FID) and MS [224].

MS can be divided into three parts: the ion source, the mass analyser and the detector. Figure 2.10 shows the different components of the mass spectrometer in simplified form.
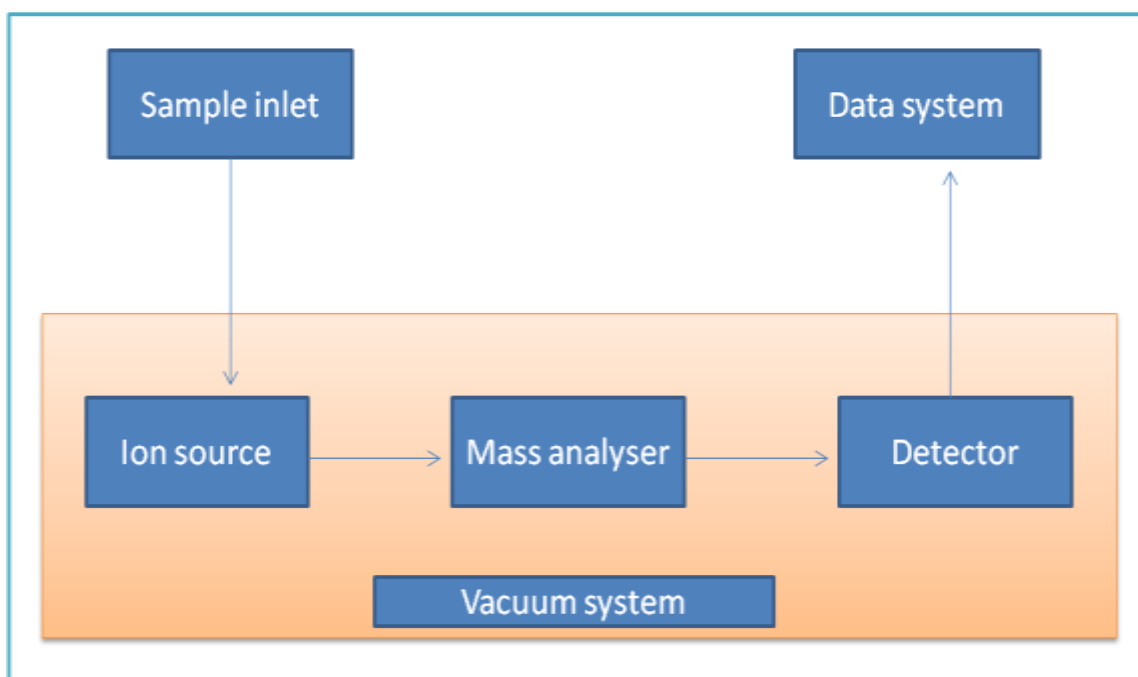
Figure 2.10: Mass Spectrometer Components

Molecules are ionised and fragmented by the ion source. In order to allow for transfer of ions through the system without collisions with molecules present in air, a vacuum is required. Data is sent to a computer which processes the huge amount of data and presents information in an accessible format. Electron impact (EI) and chemical ionisation (CI) are the most common ionisation

techniques in GC/MS. In EI, the parent molecules are impacted by a high energy electron beam in the ionisation chamber that is enough to ionise the molecules and form positive, negative, and neutral molecular fragments. When forming positive ions the reaction between gas-phase molecules and the electron beam causes the loss of an electron from the compound following the equation:

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$ **Equation 2**

The ions then pass through the mass analyser via magnetic or electric fields that separate ions depending on their mass (m) to charge (z) ratios (m/z). The mass analyser filters masses by selected ion(s) monitoring (SIM) or scanning them in scan mode (SCAN). In SIM mode, only pre-selected ions are monitored leading to greater sensitivity, whereas SCAN is advantageous in cases of identifying unknown analytes in specimens. The ions finally pass to a detector system where they are counted. The detector records the induced charge which represents the ions that hits its surface. This technique provides huge structural information. Therefore, GC-MS has become the common separation technique for identification and quantitation of drugs of abuse in most laboratories [225].

In the analysis of SCs, MS is mostly used as the detection system [226]. Other types in use are the nitrogen phosphorus detector (NPD) [227] which is suitable for most types of aminoalkylindoles, and FID [228, 229]. Most molecules fragment at 70 electron volts (eV) ionisation energy, producing several fragment ions, more than are acquired by LC–MS, that can provide useful structural information. Mass spectra under EI conditions differ significantly from one SC to another due to the great variety of their chemical structures. The fragmentation of several SC compounds can take place on both sides of the carbonyl group. The mass-spectral fragmentation pattern of SCs also involves formation of different ammonium ions at m/z = 127 and 155 (naphthyl and naphthoyl moieties) or 144 (indole moiety) [230].

Derivatisation

Although, GC-MS has become more widely used for forensic toxicology, there are some disadvantages of using GC-MS. Analytes which are thermally labile might decompose in the GC injection port. Many compounds with a polar functional

group such as an amino or hydroxyl group can cause a polar interaction between the compound and the column stationary phase leading to poor chromatography and detection of the analyte. To overcome these problems, a derivatisation step must be added after the extraction method for these compounds. Derivatisation can change the functional groups and chemical properties of the molecule, making them less reactive with the stationary phase and more stable. Derivatisation can also improve the mass spectrum, peak shape, sensitivity and specificity, reduce tailing of polar compounds and increase volatilisation and ionisation to promote chromatography. However, this additional step consumes more time and reagent, which can be toxic. Moreover, it may introduce another source of possible error.

Derivatisation can be classified into three main groups: alkylation, acylation and silylation. The combination of *N,O*-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + TMCS) is the preferred reagent for trimethylsilylation of carboxylic acids, phenols, amines, biogenic amines, alcohols, alkaloids and steroids [231] and was used in this experimental work. In the analysis of SCs using GC-MS, it seems essential to include a derivatisation step prior to GC analysis to increase detectability and stability of compounds and help to improve analytical efficiency [232]. SCs are organic compounds containing active and polar functional groups, and these react with different types of derivatisation reagents. The following derivatisations have been used for SCs: trimethylsilylation [33, 78, 81, 196], methylation [196], acetylation [78, 81] or trifluoroacetylation [78, 81]. Acetylation is preferred for routine analyses despite the slightly lower sensitivity because trifluoroacetylation can cause low thermal stability for JWH-018, JWH-073, JWH-250 and their metabolites [78].

Overall, GC-MS is the most widely used technique for analysis of SCs in Spice products in the majority of publications [30, 33, 65, 233]. GC-MS can be considered as the gold standard because it provides extensive fragmentation by EI that allows for initial identification of active ingredients [48, 65]. GC has also been used for the analyses of SCs in biological fluids [78, 83, 132, 234]; however, there are some disadvantages including the poor sensitivity, ester analogs (e.g. PB-22) decompose (or participate in ester-exchange reactions) in the injection port, and cyclopropyl compounds (e.g. UR-144) undergo thermal rearrangement (isomerisation) mainly in the injector [235, 236].

2.3.10.3 LC-MS/MS

LC-MS/MS is high performance liquid chromatography (HPLC) coupled with a tandem quadrupole mass spectrometer. HPLC utilizes a mobile phase, pump, autosampler and column, whereas the mass spectrometer contains 3 main components; the ion source, the mass analyser and the detector (Figure 2.11).
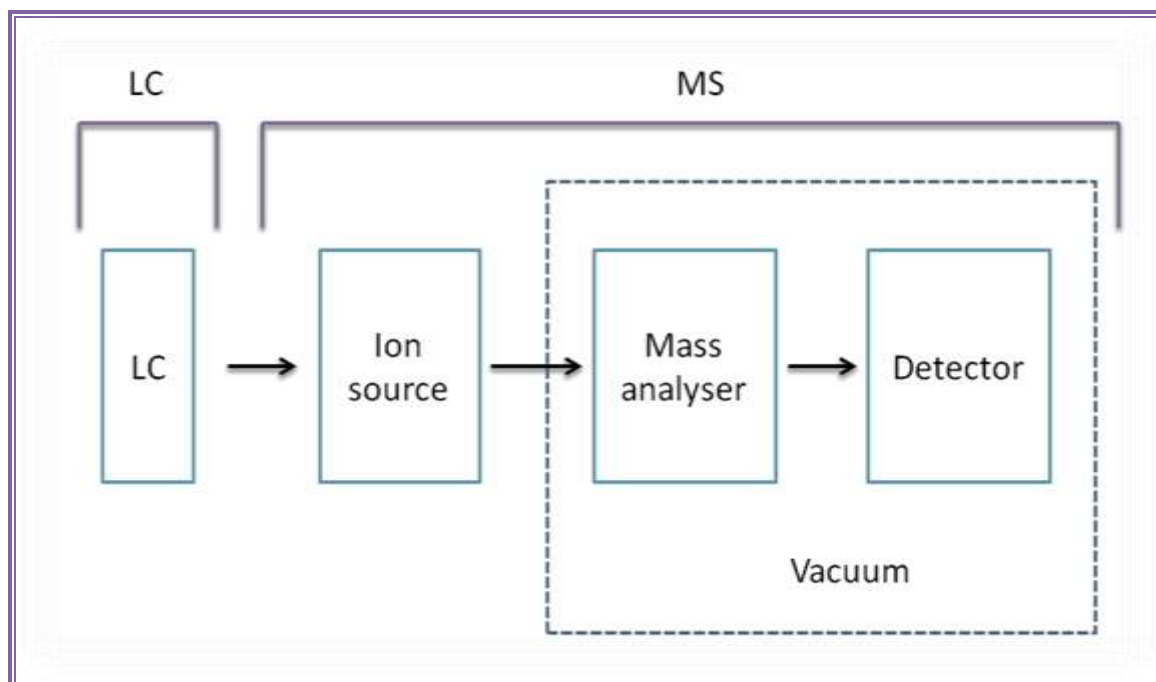
Figure 2.11: The Components of an LC-MS System

The mobile phase is an important component for both the separation and ionisation of compounds. It often consists of two parts, the aqueous phase and the organic phase. In SCs analysis, as with most LC-MS methods, methanol or acetonitrile are commonly used for the organic solvent of the mobile phase. Acetic acid, formic acid or their salts are mainly chosen as buffer additives. Buffers are commonly used in the mobile phase to enhance the ionisation of compounds. Reconstituting samples in the mobile phase before they are injected onto the system leads to increases in the response of the analyte [237]. The concentrations of buffer are very important because concentrations that are too low could lead to poor efficiency and peak shape, while concentrations that are too high could cause ion suppression of the compound.

The column is a very important component of the HPLC because the separation of analytes is achieved when those analytes pass through the column. Standard columns used in HPLC typically range from 2 to 4.6 mm internal diameter and

150, 250, 300 mm in length [238]. They are normally filled with modified silica gel because its pore structure, surface properties and particle shape help to get a good separation. They are usually protected by a guard column which is a disposable filter located at the top of the column. Columns can be classified according to their function into normal phase, reverse phase, size exclusion and ion exchange. In reverse phase, the stationary phases are non-polar such as C8- and C18-substituted silica, whereas the solvents are polar aqueous-organic mixtures such as acetonitrile-water or methanol-water. C18 columns are mainly used for the separation of SCs and their metabolites during LC. The interactions between the stationary phase and the sample analytes in the mobile phase influence the speed of the passing analytes and depend on their physical and chemical properties. The separation of the sample components in LC is greatly enhanced by gradient elution, which is changes the mobile phase composition during the chromatographic run. As a result, the detection limit is improved and the analysis time is reduced. Another elution method is isocratic elution, in which the composition of the mobile phase is kept constant [238].

LC-MS uses atmospheric pressure ionisation (API) to interface the LC with the MS. The interface helps to avoid excess contamination of the source through its position orthogonal to the source [237]. The two most common API techniques are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). The interface can be interchanged easily, in most instruments, allowing the use of either [237]. In contrast to GC-EI-MS, both ESI and APCI use soft ionisation to ionise the molecules and the pseudo molecular ion is formed [237]. If it is an acidic molecule a proton is removed $[M-H]^-$, or if a basic molecule a proton is added $[M+H]^+$. The fragmentation of the parent drug and its metabolites is similar for ESI+ collision and EI modes. However, the m/z of the fragments is different. It was found that most SCs are affected by the matrix compounds found in the sample [151, 191], particularly when ESI is used [239].

ESI has the ability to ionise larger compounds, such as SCs, therefore it has been used more frequently than APCI [237]. ESI can be applied to a wide spectrum of molecules of varying polarity and molecular weight. However, APCI has some advantages. For example, due to the different ionisation processes, APCI seems to be less susceptible to matrix effects than ESI [240]. However, it was found that the responses of SCs are lower using APCI than with ESI [192]. ESI and APCI

interfaces operate in positive and negative polarity. In SCs analysis, positive polarity is commonly performed [230]. The negative ionisation mode has been used for HU-210, CP 47,497 and its homologue due to the absence of nitrogen in the chemical structures of these compounds, in contrast to almost all other SCs [198].

ESI generates molecular ions in solution before the molecule enters the MS. Therefore, thermally labile compounds can be ionised without degradation [241]. Ions can be formed in ESI by exposing a metal capillary to a high voltage varying from 3-5 kV. The mobile phase is then evaporated and the ions enter the MS.

There are many mass analysers available for LC-MS including tandem quadrupole, time-of-flight and ion trap. The quadrupole is the most widely used analyser [242]. Quadrupole mass analysers contain four rods parallel to each other making a square. The ions are directed pass to the centre of the square. Electromagnetic fields are generated by exposing the rods to a voltage. These fields allow only ions of a certain mass/charge ratio to pass through the filter onto the detector. The quadrupole can be used singularly or in tandem (triple quadrupole). There are three quadrupoles in a tandem mass analyser (Figure 2.12): Q1 and Q3 are used as mass analysers whereas Q2 is used as a fragmentation zone [243]. Q1 selects a precursor ion. In Q2, fragmentation is achieved by applying collision energy and introducing a collision gas. The precursor ion is fragmented to form product ions. Q3 selects product ions of interest for detection.

MS detection can be performed in full scan, product ion scan, SIM and multiple reaction monitoring (MRM) modes. When using full scan, the quadrupole mass analysers scan over a range of mass/charge ratios. Whereas, when SIM is used, the quadrupole mass analysers identify specific ions chosen by the user.
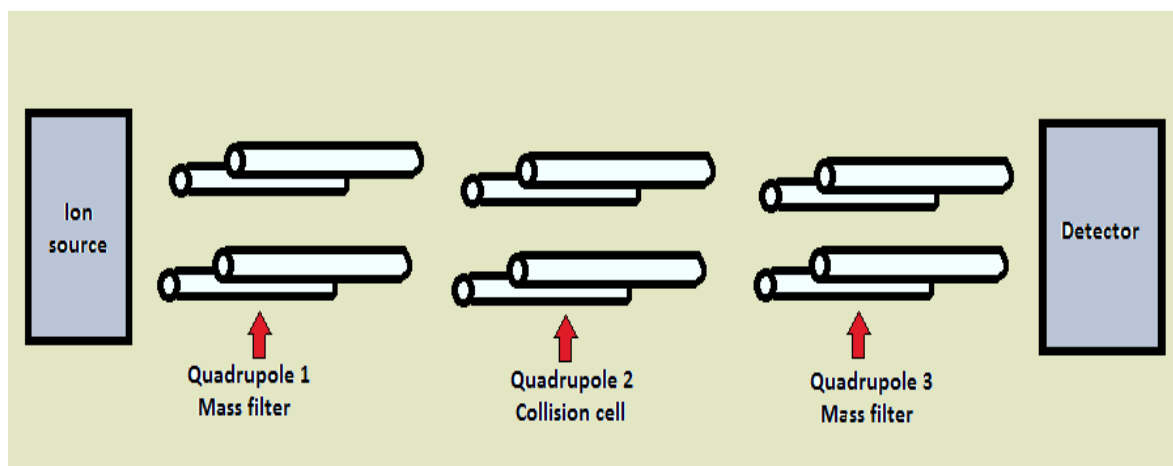
Figure 2.12: Schematic of Triple Quadrupole Mass Spectrometer

Full scan mode reduces sensitivity, however, and this may cause false negative results for analytes at low concentrations. The use of LC-MS/MS in MRM mode will increase the specificity and enhance the sensitivity. Fragment ions are observed using product ion mode method when the protonated molecular ion ($[M+H]^+$) is utilized as the precursor ion. Two product ions of acceptable abundance should be determined to ensure that the correct compound is detected and to achieve good sensitivity. SIM and MRM modes elevate sensitivity, however, it is important to have information about the compounds and fragmentation patterns. Tandem quadrupole mass analysers increase sensitivity and selectivity as well as giving more structural information.

Overall, LC-MS/MS plays an important role in routine forensic toxicology, particularly for quantification of the analytes identified. It has the advantage of being able to quantify high molecular mass, low-dose, polar and thermo-labile compounds [244]. In addition, it might be the best instrumentation to improve the detection of analytes in biological specimens [242]. Unlike GC-MS, LC-MS/MS is able to directly analyse almost all the compounds that are soluble in the mobile phase. Since most drugs are water soluble, LC-MS(MS) is allows the detection of a wide range of drugs. The majority of published methods for different types of human matrices containing SCs and their metabolites used LC techniques for the analyses. LC-MS/MS has also been used for the analyses of SCs in Spice products in several studies [65, 84].

2.3.10.4 LC-HR/MS

The newer technique LC–HR/MS has recently been introduced to forensic toxicology. Several non-targeted methods using the HR/MS technique for screening of drugs of abuse including SCs in biological matrices were published [206, 212, 222, 245].

Similar or overlapped mass spectra of SCs are sometimes obtained by GC-MS due to their structural similarities and isomeric forms. The misidentification of these drugs might increase when using only the information from the mass spectra without retention time information.

To overcome the misidentification of various SCs, which have similar chemical structures that can produce very similar fragmentation patterns or to identify new compounds in a biological material, HRMS or TOFMS can be used to determine the elemental composition of the structure and screen for parent drugs and their metabolites of SCs in specimens. The development of non-targeted methods using HRMS techniques facilitates the detection of known or unknown compounds at the time of analysis. It can distinguish between different substances with the same nominal molecular mass. Unlike conventional MS analysers, new HRMS technology enables the identification of analytes based on the exact molecular formula because it offers mass assignment with an accuracy of 0.001 atomic mass units (amu) [246].

This new technology allows for retrospective data inquiry after library updates like the World Anti-Doping Agency (WADA) accredited laboratories have done with athletes' urine samples. The main challenge of non-targeted LC-HRMS screening methods is the toned for a library with all the analytes in it. HRMS can give an elemental composition but you may not know what the analyte is. Another challenge is to achieve sufficient selectivity to identify low concentrations of SCs [247]. These methods are enabling forensic laboratories to obtain accurate MS and MS/MS data without pre-selecting substances.

2.3.10.5 Identification Criteria

Many identification parameters are available that can be used to increase the confidence of the toxicologist in the presence of toxicants in biological samples. Sample preparation and chromatographic separation are important issues to eliminate unwanted drugs and interference [248]. Contamination from the ion source can be addressed through the use of high organic modifier percentages to eliminate potential in-source dissociation of metabolites [248].

It has been recommended that a deuterated internal standard should be included whenever available to achieve an accurate identification and quantification procedure [249, 250]. Deuterated internal standards are the best way to minimize the matrix effect phenomenon and examine the efficiency of extraction and ionisation of analytes [250]. Different types of internal standard can be used in LC-MS applications including deuterium-labelled analogues, structurally similar compounds (e.g. homologue), and any other compounds (e.g. a drug) [251]. Analytes can be labelled with stable isotopes - hydrogen (deuterium is the most common isotope used in forensic laboratories - with masses different from the analytes by at least 3 mass units to avoid interference between analyte and its internal standard [250]. The use of any other compounds (e.g. a drug) is not recommended because they may be found in case samples due to drug ingestion, and can cause misinterpretation of results [244, 249].

Retention times play an important role in identification in all chromatography systems, and compounds should be satisfactorily separated from each other. The retention times of the analytes detected in real cases should match that of standards or positive controls included in the same run within a range of 2 percent or ±0.1 minutes (whichever is smaller) using GC or LC [252].

2.3.11   Synthetic Cannabinoids and Biological Matrices

Multiple analytical methodologies have been reported since 2010 for the detection of SCs in various body fluids, including blood, urine, oral fluid and hair. These methods have widely differing purposes, strengths and weakness. These publications study various aspects such as pharmacokinetics, pharmacodynamics, method development and clinical investigations. Some of them included toxic and lethal concentrations as a result of studying the adverse effects or drug-related deaths (DRD) involving these drugs.

The choice of the kind of sample matrix is a critical component of forensic toxicology investigation. It depends on many factors such as the availability of specimens and the purpose of the analysis. Urine is the most widely used matrix for SC detection [247], which is also likely to be the case for other drugs due to its ease of collection. Figure 2.13 illustrates the number of publications for each biological matrix since 2010.
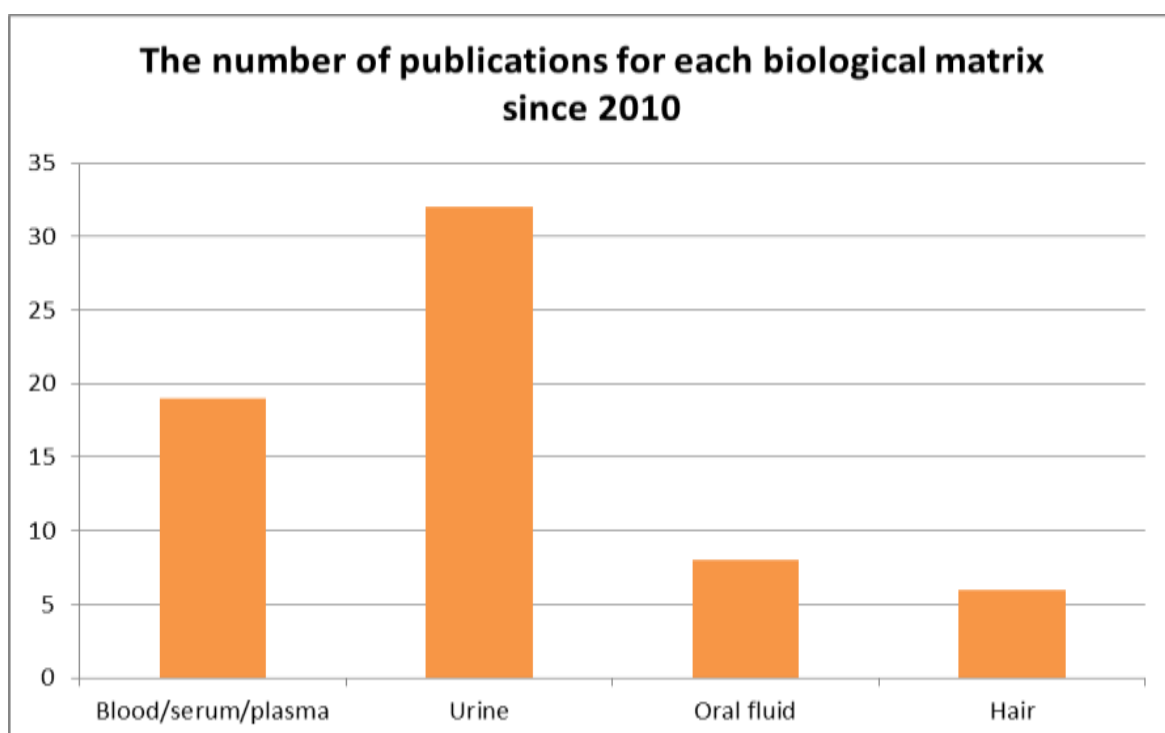
Figure 2.13: Methods for SC Detection in Different Biological Specimens Published Between 2010 and 2015

2.3.11.1 Blood, Plasma and Serum

Blood can offer unique advantages over other matrices. It can be used for the determination of recent drug use as in the case of identifying drivers under the

influence of illicit drugs [253, 254]. It also can be used for the determination of the ratio of parent drug to metabolites that could yield useful information relating to acute or chronic drug use [253]. However, blood sampling requires the presence of medical staff for the collection and there is a risk of infection.

The concentrations of SCs in blood are usually very low however, and as such, analytical methods for detection need to be very sensitive. Another difficulty in determining SCs in blood specimens is the window for detecting acute intake is short (hours–days) [149]. In the case of long-term use of SCs, the windows for detection can be longer due to their accumulation in fatty tissues [109].

19 methods for SC detection in blood, serum and/or plasma have been published between 2010 and 2014. A summary of the majority of these methods is found in Appendix 1. LLE was the most common technique used for the extraction of SCs from these matrices. These methods have some strengths and weaknesses at the same time. For example, a method, developed by Dresen *et al.* [192] for the quantitation of JWH-018, JWH-073, JWH 200, JWH-250, JWH-081, JWH-015, WIN 55,212-2 and methanandamide in serum, has less sample preparation but it has more matrix effects (40.2 to 134.7%). In general, no method is perfect: there is always a compromise.

2.3.11.2   Urine

Urine is the most widely used matrix for toxicological analysis in providing evidence of drug use and assessing drug exposure [247]. It is the preferred specimen for many laboratories because the collection of urine samples is easy and non-invasive and the concentrations of analytes are often higher when compared with samples of blood or oral fluid. Moreover, the detection time in urine is longer than blood (days–weeks) [255]. The prevalence of positive results in urine specimens for some compounds is higher than those observed in blood because the detection windows are longer for urine samples. However, there are some disadvantages with using urine as a sample. For example, it is not suitable for identifying drivers suspected of being under the influence of drugs as it only gives evidence of past use. In addition, urine concentrations cannot correlate with pharmacodynamic effects.

The parent compounds of SCs may not be detectable in urine due to their extensive metabolism; therefore it seems essential to focus on metabolites [114]. The metabolism of some of SCs are still under investigation which also makes analysis of urine samples more complicated.

32 articles published between 2010 and 2014 described method validation for the identification of SCs and the metabolites in urine [236, 256-260]. The first publication to identify the JWH-018 urinary markers after the smoking of illicit herbal mixtures was by Sobolevsky *et al*. in 2010 [79]. Urine samples were collected from three intoxicated people taken into police custody. The parent compound JWH-018 was not detected whereas the phase-1 metabolites were identified after de-conjugation. The authors suggested a low content of the metabolites phase-1 in urine. A summary of the majority of these published methods is found in Appendix 2.

SPE, LLE, PPT and dilution techniques have been used for the extraction of SCs from urine. Hydrolysis with acids [78, 81, 196] or enzymes [79, 120, 130, 131, 261] is required in the procedure of urine sample preparation for SCs because it was found that the percentage of conjugated form excreted of the hydroxylated metabolites of many SCs is very high [81, 131, 262]. It can be noticed that these methods vary in their scope of drugs analysed. For example, a method, developed by Grigoryev *et al*. [236] focused on the metabolites of UR-144 whereas a method developed by Holm *et al*. [58] studied 5F-AKB-48 and its metabolites. These variations in scope of drugs between the methods might explained by the time of the study, aims of the method or the availability of resources such as standards.

2.3.11.3 Alternative Samples

The use of alternative samples such as oral fluids and hair could be required in different cases when the desired samples are not available [263].

Drugs are readily detectible in oral fluid specimens. Oral fluid can be used as an alternative to blood for identifying drivers that are suspected of driving under the influence, and in place of urine samples when testing for drugs usage in the work place. It is easy and rapid to obtain and is less prone to adulteration [201].

In addition, oral fluid containing parent SCs makes the sample a good choice for testing in cases where metabolite reference standards may be lacking, or if the target metabolites are still unknown [218]. However, there are some disadvantages: the sample volume must be sufficient for the analysis, and this amount could be difficult to obtain from some SC abusers. Low sample volume can also occur without adequate devices for collecting the sample. Different collection devices have been used for oral fluid collection including DCD 5000 Dräger [201], Intercept [202] and Quantisal[TM] [214, 218]

Hair was first described as an alternative specimen for drugs testing in the field of toxicology in 1979, when used to document long-term drug exposure. Hair testing provides long-term histories via segmental analysis, because hair offers a larger detection window when compared with all the other types of specimens (up to months) [264]. Another advantage of hair as a sample is low potential for donor manipulation. However, hair testing has its disadvantages too. Environmental contamination, especially through smoking of drugs, can have an effect on hair analysis, leading to false-positive results [265]. According to the Society of Hair Testing (SoHT), preparation of a hair sample should include a washing step in the procedure prior to analysis to remove superficial or surface drug [266]. This allows for demonstrating that the drug being recovered by the extraction procedure is an internally consumed drug.

8 and 6 methods for SC detection in oral fluid [267] and hair [268] respectively were published between 2010 and 2014. A summary of these published methods is found in Appendixes 3 and 4.

To sum up, numerous methods have been developed to analyse these drugs for different purposes in blood, plasma, serum, urine, oral fluid and hair but only a few studies have been reported for analysing these drugs in post-mortem matrices. The main weaknesses of some of these methods may be the poor sensitivity, inappropriate scope of drugs due to the speed of the emergence of SCs or the lack of some validation parts. Blood and urine were selected as the matrices for the current study.

Chapter 3 - Method Development for Synthetic Cannabinoids Using GC-MS and LC-MS/MS

3.1 Introduction

Several studies indicate that SCs may cause serious toxicity and impairment to health (see 2.3.4) therefore, forensic toxicology laboratories have made concerted efforts to update the analytical methods that can detect the newer compounds. A number of simultaneous chromatographic assays for SCs and metabolites in biological samples have been developed using GC-MS and LC-MS/MS.

Identifying SCs in biological samples challenges toxicologists and drug testing programmes. The speed of the emergence of SCs has resulted in several analytical challenges for clinical and forensic laboratories. The initial simultaneous chromatographic assays, from 2008, studied the first generation of SCs such as JWH-018 and JWH-073. Certified drug reference material for use in positive identifications is not readily available. In blood and oral fluid, parent compounds are found at extremely low concentrations therefore methods for analysis of SCs in these matrices must be very sensitive. Libraries of reference mass spectra are not commercially available to assist the identifications, especially for the most recent drugs.

To overcome these emerging challenges, different strategies should be applied. Analytical methods for the identification of SCs should be continuously updated, improved and revalidated to cover the new compounds available. Because SCs contain a variety of functional groups, the extraction of SCs requires a general procedure. Very limited data on cut-offs, detection windows or expected concentrations of SCs have been published. Therefore, limits of detection (LODs) should be determined as low as analytically possible.

GC-MS and LC-MS/MS have different advantages and disadvantages. The majority of published methods use LC techniques for the analyses of different types of human matrices containing SCs and their metabolites. However, most of these methods need extensive extraction procedures or have lengthy chromatographic

run times that can affect the throughput capacity, particularly in the case of analysis of many samples.

3.2 Aim and Objectives

The aim was to develop a testing method using chromatographic techniques to quantitate a range of synthetic cannabinoids in urine samples.

The analytes including in the method were selected due to their apparent availability in Scotland and Saudi Arabia (See 2.3.2). These included HU-210, CP47,497, JWH-018 and one of its major metabolites JWH-018 4-OH pentyl, JWH-073 and one of its major metabolites JWH-073 3-OH butyl, JWH-250 and one of its major metabolites JWH-250 4-OH pentyl, JWH-200, AM-2201 4-OH pentyl, JWH-122 5-OH pentyl, JWH-210 5-OH pentyl, AM-1248, AB-FUBINACA, UR-144, A-796260, 5F-AKB-48 and one of its major metabolites 5F-AKB-48 (*N*-4 OH pentyl), and 5F-PB-22 and one of its major metabolites 5F-PB-22 3-carboxyindole (Figure 2.7).

The objectives included:

- Developing a method to detect synthetic cannabinoids in biological sample extracts using GC-MS.

- Developing a method to detect synthetic cannabinoids in biological sample extracts using LC-MS.

3.3 Chemicals and Reagents

JWH-018, JWH-073, JWH-250, HU-210, CP47,497, JWH-200, 5F-AKB-48, 5F-PB-22, AM-1248, AB-FUBINACA, A-796260, UR-144, JWH-018 4-OH pentyl, JWH-073 3-OH butyl, AM-2201 4-OH pentyl, JWH-250 4-OH pentyl, JWH-122 5-OH pentyl and JWH-210 5-OH pentyl were purchased from Sigma Aldrich (Basingstoke, UK). 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole were obtained from LGC Standards (Teddington, UK). JWH-073, JWH-250, JWH-200, 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole were purchased as 1 mg of powder, whereas the rest of the drugs and metabolites were purchased as solutions with the concentration of 100 µg/mL in methanol.

Deuterated standards JWH-018 4-OH pentyl-d5, JWH-250 4-OH pentyl-d5, AM-2201 4-OH pentyl-d5, JWH-073 3-OH butyl-d5 and JWH-200-d5 were obtained from Sigma Aldrich (Basingstoke, UK). All these drugs were purchased as solutions with the concentration of 100 µg/mL in methanol.

N,O-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + 1 % TMCS) were manufactured by Cerilliant (Texas, USA) and purchased from Sigma Aldrich (Basingstoke, UK).

Methanol (MeOH) (HPLC grade), ethyl acetate, acetonitrile (ACN), isopropanol, dichloromethane (DCM) n-hexane and methyl-tert-butyl-ether (MTBE) were obtained from VWR International Ltd, (Lutterworth, UK). Sodium acetate trihydrate, disodium hydrogen orthophosphate anhydrous, sodium dihydrogen orthophosphate monohydrate, ammonium acetate (HPLC grade), formic acid and beta-glucuronidase from Helix pomatia containing ≥85,000 units/ml of beta-glucuronidase and ≤7,500 units/ml of sulphatase per mL were supplied by Sigma Aldrich (Basingstoke, UK). Purified water was obtained from the in-house Merck Millipore system.

3.4   Solutions

3.4.1 Preparation of Drug Standard and Internal Standard Solutions

3.4.1.1   Stock Solutions

Stock solutions were prepared in methanol for each drug individually giving a concentration of 10 µg/mL. This was achieved by transferring 1 mL of each drug (100 µg/mL) into a single 10 mL volumetric flask, and making up to the mark with methanol. The entire amount was transferred to individual amber glass bottles and stored at –20°C for 6 months.

3.4.1.2   Working Solutions

Working solutions were prepared by dilution of stock solutions in methanol to obtain 1 µg/mL for each drug. This was achieved by adding 1 mL of the stock solution to a 10 mL volumetric flask and making up to volume with methanol,

then transferring to individual amber glass bottles and storing at −20°C for 6 months.

3.4.1.3   Preparation of Working Drug Standard Mixture Solution

A 0.5 µg/mL mixture solution containing 10 SCs was prepared in methanol by adding 500 µL of each drug (stock solution) to a 10 mL volumetric flask and making up to volume with methanol. All mixture solutions were transferred to amber glass bottles and stored at −20°C.

3.4.1.4   Preparation of Working Internal Standard Mixture Solution

The working internal standard mixture solution was prepared in methanol at 0.5 µg/mL by adding 500 µL of each drug (stock solution) to a 10 mL volumetric flask and making up to volume with methanol. All mixture solutions were transferred to amber glass bottles and stored at −20°C.

3.4.2 Preparation of Buffers

3.4.2.1   Preparation of 0.1 M, pH 6.0 Phosphate Buffer

1.7 g of disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 12.14 g of sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H2O$) were transferred to a 1 L beaker. They were dissolved in 800 mL of deionised water and the pH was adjusted to 6.0 with 0.1 M dibasic sodium phosphate or 0.1 M monobasic sodium phosphate. The entire amount was placed in a 1 L volumetric flask and made up to volume with deionised water. This solution was then mixed, and then stored at 4°C for up to 1 month.

3.4.2.2   Preparation of 1.0 M, pH 5.0 Sodium Acetate Buffer

13.6 g of sodium acetate trihydrate was dissolved in 6 mL of glacial acetic acid, made up to 1 L with deionised water. The pH was adjusted with acetic acid to result in pH 5.0. The solution was mixed and stored at 4°C for up to 1 month.

3.4.2.3   Preparation of 2 M Ammonium Acetate in Methanol

15.42 g of ammonium acetate was weighed out into a 100 mL volumetric flask and made up to the mark with methanol and stored at 4°C for up to 1 month.

3.4.2.4   Preparation of 2 mM Ammonium Acetate

1 mL of 2 M ammonium acetate was added to a 1 L volumetric flask and made up to the mark with deionised water and stored at 4°C for up to 1 month.

3.4.2.5   Preparation of Formic Acid 0.1% (pH=2.8)

1 mL of concentrated formic acid was transferred to a 1 L volumetric flask and made up to the mark with deionised water.

3.4.2.6   Infusion Solution

500 mL of methanol was added to a 1 L volumetric flask and made up to the mark with deionised water and then 1 mL of 2 mM ammonium acetate and 1 mL of formic acid were added.

3.4.3 Blank Matrix

3.4.3.1   Drug-free Urine

Blank urine from ten healthy volunteers was used. The urine samples were stored at 4°C. Existing blank urine samples from a previous research project, obtained with informed consent and appropriately stored in Forensic Medicine and Science, were used. The chair of the University Medical Research Ethics Committee, Dr Jesse Dawson, Institute of Cardiovascular and Medical Sciences, University of Glasgow confirmed that approval would not be needed for these samples.

3.4.3.2   Blank Blood

Expired packed red blood cell pouches were provided from the Scottish National Blood Transfusion Service (SNBTS) (submission reference number 14-07) based at Gartnaval General Hospital (Glasgow, UK). They were stored at −20°C on receipt within Forensic Medicine and Science. In order to prepare blank blood, packed red blood cells were defrosted and suspended in a ratio of 1:1 with 1% saline solution which was prepared by dissolving 9.5 g of sodium chloride in 1 L of deionised water.

3.5 Method Development Using GC-MS

3.5.1 Instrumentation

An Agilent Technologies GC-MS; (Autosampler 7683B, Gas Chromatograph 7890A and Mass Spectrometer 5975C), using a HB-5MS + DG column Crawford Scientific (30 m x 0.25 mm id x 0.25 μm) was used. Data analysis was performed using GC/MSD ChemStation Software (E.02.02.1431).

3.5.2 Derivatisation

At this stage of method development, only JWH-018, JWH-073, JWH-250, JWH-200, HU-210 and CP47,497 standards were available. 100 μL unextracted drug standards for JWH-018, JWH-073, JWH-250, JWH-200, HU-210 and CP47,497 at concentrations of 1000 ng/mL in MeOH were individually evaporated under a stream of nitrogen to dryness at room temperature and were redissolved in 50 μL MeOH. HU-210 and CP47,497 were derivatised with 50 μL BSTFA + 1% TMCS for I hour at 70°C. None of the JWH compounds form TMS derivatives as they do not have suitable functional groups. All analytes were then transferred to autosampler vials.

3.5.3 GC-MS Procedure

1μL of each standard sample was used for injection on to GC-MS to obtain their mass spectra. Table 3.1 summarises the GC-MS procedure and Table 3.2 shows the selected ions and retention times for JWH-018, JWH-073, JWH-250, HU-210 and CP47,497.

Table 3.1: GC-MS Procedure

GC-MS Parameters	Setting
Inlet temperature	230 °C
Injection volume	1 μL
Injection port type	Splitless mode
Flow rate of carrier gas	1.5 mL/min
Column	HP-1 30 m x 0.25 mm id x 0.25 μm
Oven programme	50 °C initial & ramped to 320 °C @ 20 °C/min. Hold 4 minutes
Interface temperature	230°C
Ionisation mode	EI mode, ionisation energy 70 eV
Detection mode	Full Scan over the range m/z 40-600
Run time	17.5 minutes

3.5.4 Sensitivity Assessments of Unextracted Standards

Sensitivity assessment was carried out to evaluate the analytical method. The sensitivity of each SC was assessed using serial dilution of unextracted drugs standards for each drug individually at the following concentrations: 1000, 500, 250, 125, 62.5 and 31.25 ng/mL.

Table 3.2: Selected Ions and Retention Times for CP47,497, HU-210, JWH-250, JWH-073 and JWH-018.

Drug	Quantitative Ion (*m/z*)	Qualitative Ion 1 (*m/z*)	Qualitative Ion 2 (*m/z*)	Retention Time (min)
CP47,497	377	462	287	11.9
HU-210	446	530	359	13.3
JWH-250	214	144	335	13.9
JWH-073	327	200	310	14.6
JWH-018	341	284	214	15.0

3.5.5 Results of GC-MS Method Development

JWH-018, JWH-073, JWH-250, HU-210 and CP47,497 were detected by GC-MS except JWH-200 which was not eluted from the column and this may because it has a high molecular weight. Figure 3.1 shows GC-MS chromatograms and full-scan mass spectra of 0.5 ng on-column of un-extracted standards for these drugs. The developed method gave a poor response for all analytes. JWH-018, JWH-073 and JWH-250 were undetectable at 0.25 ng on-column and, CP47,497 and HU-210 were undetectable at 0.031 ng on-column.

Due to the low concentrations (1 ng/mL) detected in reported biological case samples in the literature (see 2.3.6 and 2.3.7), the developed method was altered several times to improve the sensitivity. Several GC oven programmes in combination with changes to the flow rate were tested. The method was also altered from SCAN to SIM, thus allowing for higher sensitivity. The injection port temperature was reduced from 265°C to 230°C to prevent injection port degradation but less efficient vapourisation. The sensitivity remained unsatisfactory for the project aims. Indeed, SCs are not ideal compounds for GC-MS analysis because they have high molecular weights. It was concluded that the only option was to try a different technique more suitable to these compounds (LC-MS/MS).
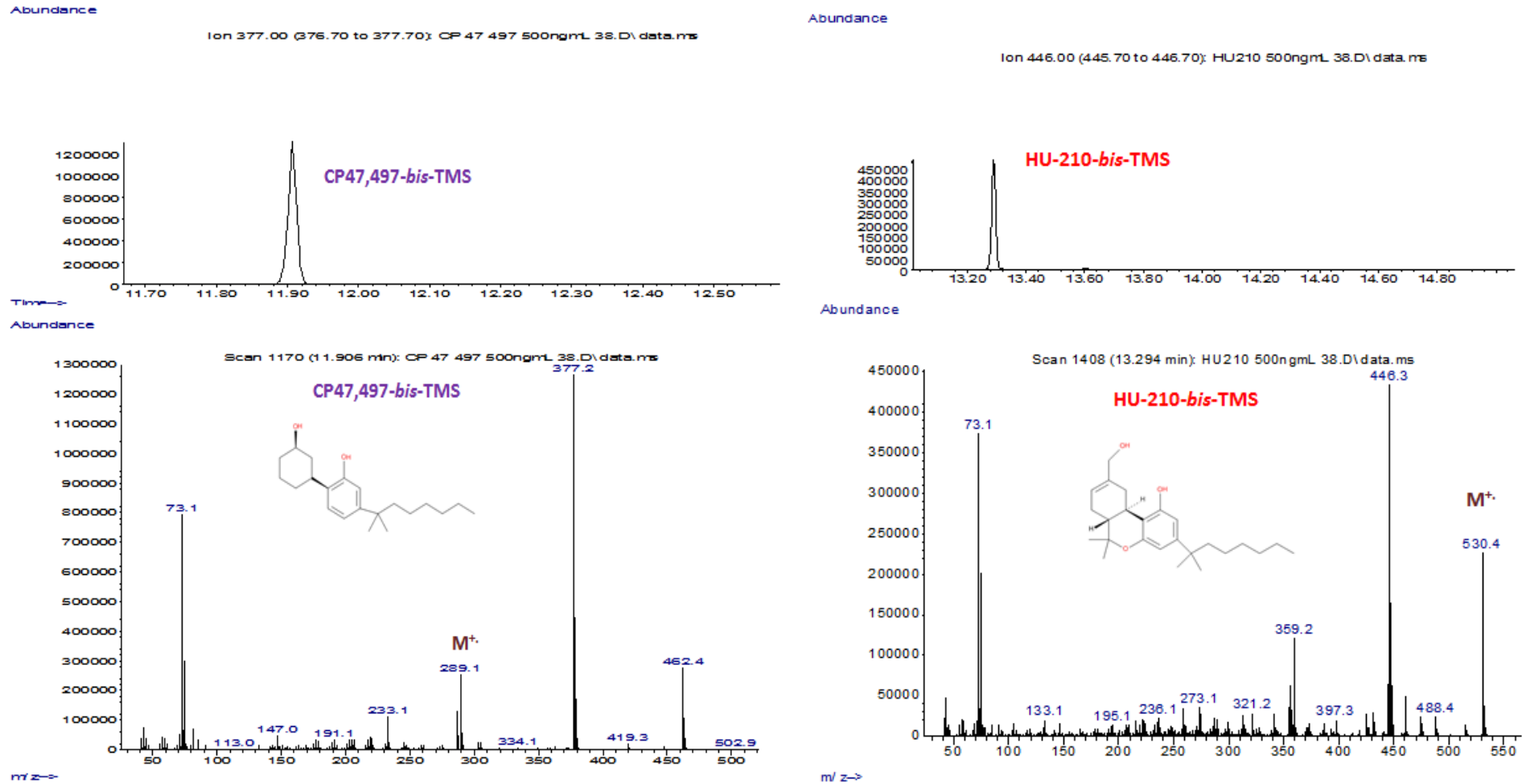
Figure 3.1: GC-MS Chromatograms and Full-Scan Mass Spectra of 0.5 ng on-column of CP47,497-*bis*-TMS, HU-210-*bis*-TMS, JWH-250, JWH-073 and JWH-018 (Unextracted)
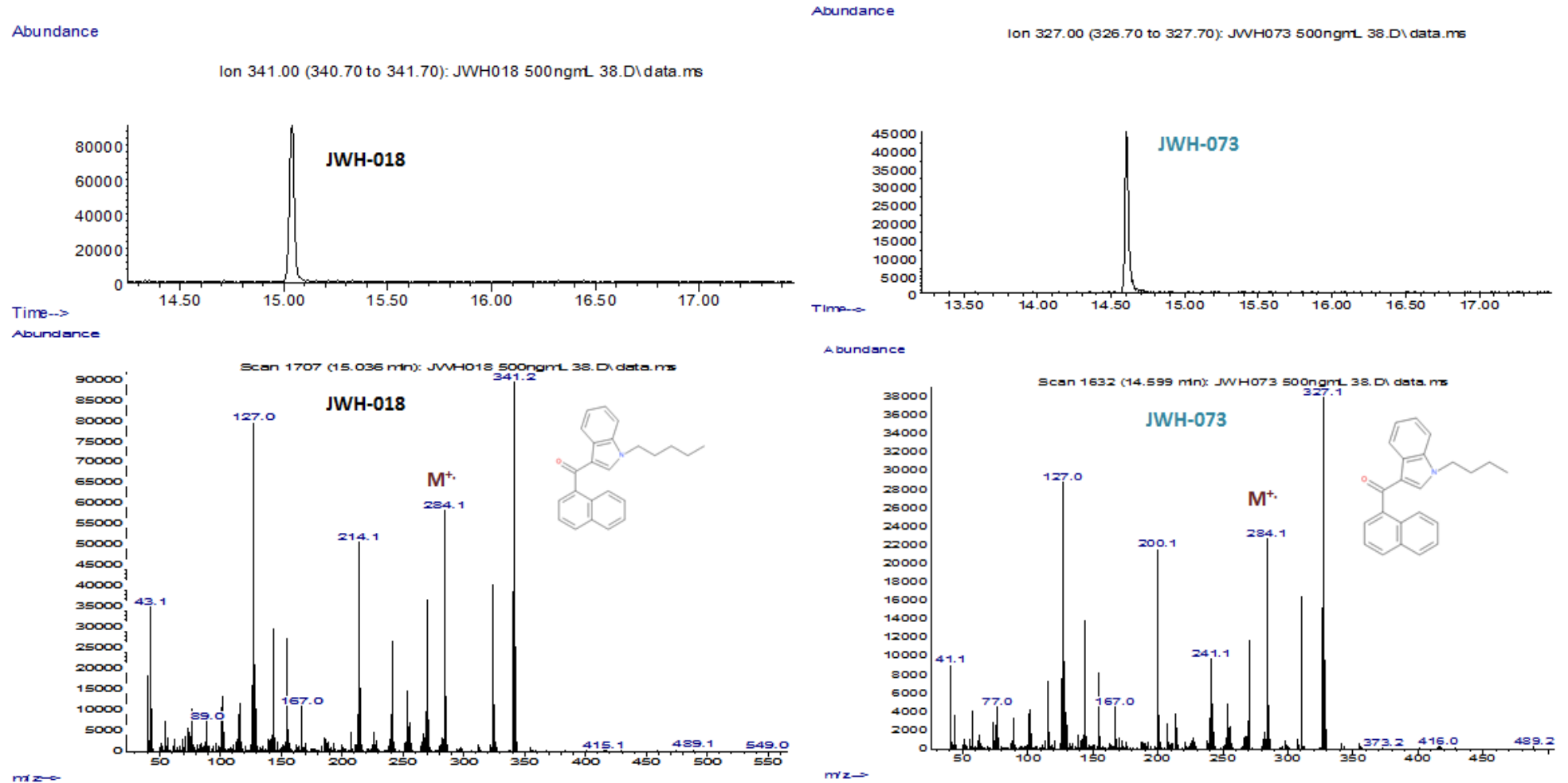
Figure 3.1 (Continued): GC-MS Chromatograms and Full-Scan Mass Spectra of 0.5 ng on-column of CP47,497-*bis*-TMS, HU-210-*bis*-TMS, JWH-250, JWH-073 and JWH-018 (Unextracted)
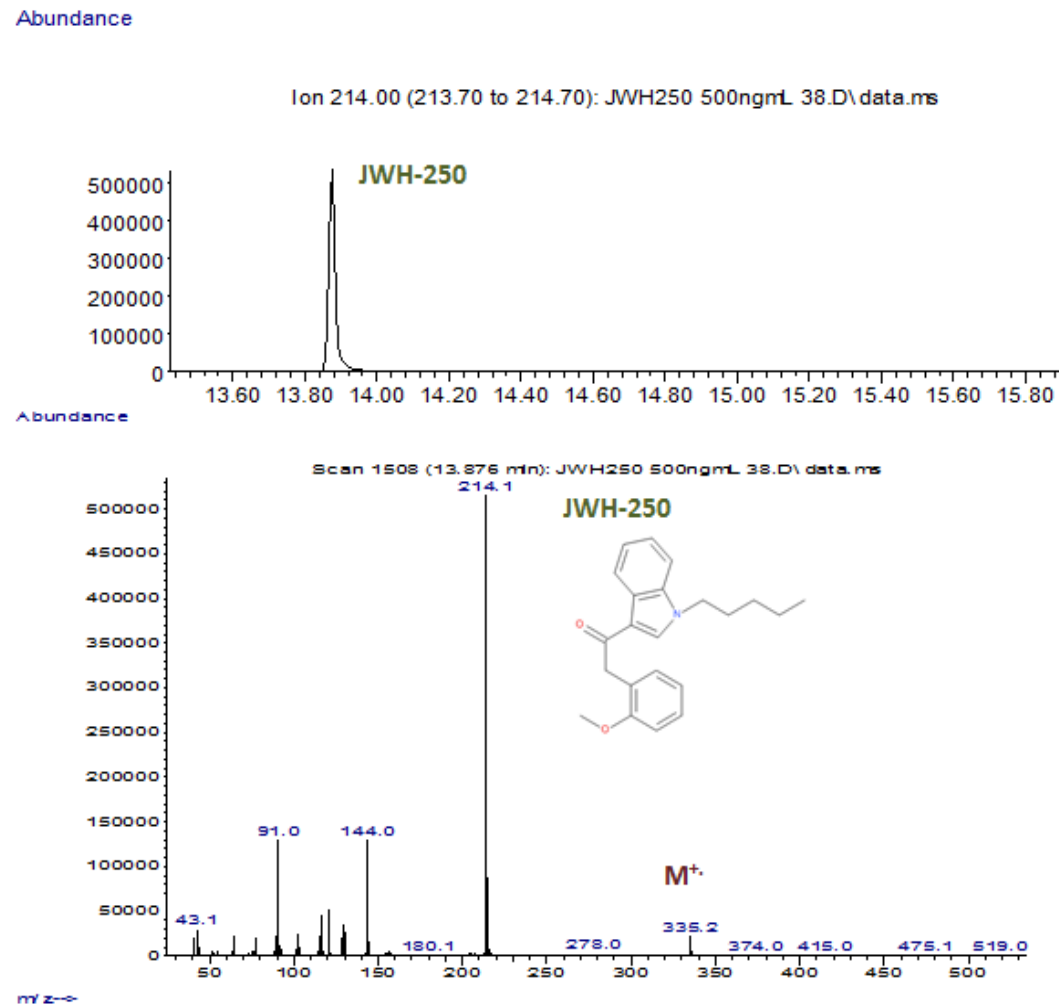
Figure 3.1 (Continued): GC-MS Chromatograms and Full-Scan Mass Spectra of 0.5 ng on-column of CP47,497-*bis*-TMS, HU-210-*bis*-TMS, JWH-250, JWH-073 and JWH-018 (Unextracted)

3.6 Method Development Using LC-MS

3.6.1 Method Designing

The development of an LC-MS/MS method is usually time-consuming, especially if the method requirements are challenging. Therefore, strategies for LC-MS/MS method development should be used to save time and money as well as achieving adequate sensitivity and selectivity. These strategies involve research and planning, and development of three main separate methodologies; MS/MS, LC and sample preparation (Figure 3.2). Iterative approaches may be required for optimising an LC-MS/MS method since changes in one method can affect another.
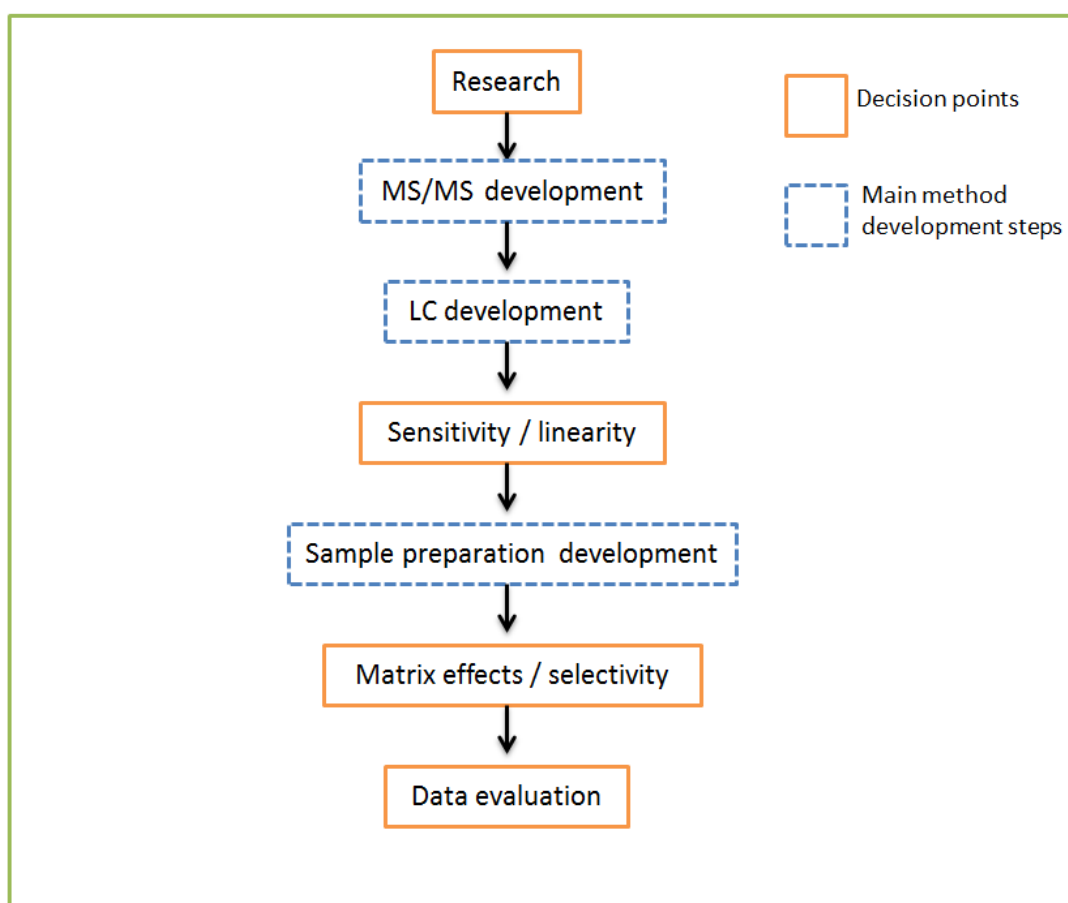
Figure 3.2: Stepwise Optimisation of LC-MS/MS Method

An MS/MS method development can give the developer a clear picture of whether the compounds of interest are detectable in this type of technique. It can also provide the optimum MS conditions for the compound detection. During this step of the method development process, the mode of ionisation (e.g. ESI, APCI) and the polarity should be evaluated to achieve the optimum set of

conditions. LC method development plays an important role to ensure the compounds of interest separate from each other and from endogenous or exogenous interferences. The last step is the sample preparation method development. Selection of the appropriate sample preparation is a crucial step. It depends on many factors such as the type of matrix, the availability of materials, duration of use of the assay and the purpose of analysis. The method of sample preparation should eliminate endogenous molecules such as phospholipids that may cause ion enhancement or ion suppression in LC-MS. In addition, it should concentrate analytes of interest to enhance assay sensitivity.

3.6.2 Optimisation of MS/MS Method

All SC compounds and their internal standards were ionised using ESI mode and generated prominent protonated molecular ions $[M+H]^+$ in positive-ion mode because they are basic molecules.

10 SCs and 4 internal standards were prepared individually in the infusion solution (see 3.4.2.6), at a concentration of 1 μg/mL and then a constant stream of each drug solution was directly introduced into the ESI interface using a syringe infusion pump. This can determine whether the compounds will ionise in the mass spectrometer and allows for parameters to be 'tuned' to the compounds, leading to optimal conditions for ionisation and therefore optimal sensitivity. All SCs and internal standards were individually tuned in order to detect the precursor ions and then optimise their product ion responses, with individual fragmentor voltages and collision energies. In the tuning process, four steps were performed for each analyte as follows:

1) MS1 scan method in order to determine the precursor ions.

2) Fragmentor voltage optimisation for each precursor ion.

3) Product ion mode method in order to determine the product ions using the data from the previous steps.

4) Optimising the collision energy of each transition through a range 0 – 60 V by increasing the value of collision energy by 5 V each time.

MRM mode was used to monitor 2 ion transitions (quantifier and qualifier) for each analyte because of sensitivity requirements and 1 transition was used for the internal standard. The fragmentor voltage was optimised for the analytes to maximize precursor ion intensity. The fragmentor voltage for all the analytes investigated in this study was 140 V. The collision energy was optimised to achieve the highest abundance of product ions. The optimal collision energy of the majority of these analytes was less than 40 eV. The major product ions were selected for quantification in all analytes.

Although there are LC-MS/MS methods in the literature for the determination of CP47,497 and HU-210, it was not possible to detect them in either positive or negative mode. It was unclear whether the lack of detection of CP47,497 and HU-210 resulted from contamination in ion source or because they are weakly acidic compounds with phenolic hydroxyl groups. These 2 drugs belong to the first generation of SCs therefore it was decided to exclude them from the method.

Optimised results for precursor ions, product ions, fragmentor voltages, collision energies, collision cell exit potential and retention times for 10 SCs and 4 internal standards are shown in Table 3.3. The optimal conditions were achieved using nitrogen gas heated to 300°C and delivered at 10 L/min, a capillary voltage of 4,000 V and a nebulizer pressure at 15 PSI in order to obtain the highest sensitivity.

Table 3.3: Optimisation of MRM Transitions, Fragmentor Voltage, Collision Energy, Collision Cell Exit Potential and Retention Time of 10 SCs and 4 Internal Standards

Analyte	Q1 mass (*m/z*)	Q3 mass (*m/z*)		Frag[a] (V)	CE[b] (eV)	CXP[c] (V)	RT[d] (min)
		Quantifier	Qualifier				
JWH-018 4-OH pentyl	358.2	155.1	127.0	140	5	4	4.5
JWH-073 3-OH butyl	344.2	155.1	216.0	140	20	4	4.3
AM-2201 4-OH pentyl	376.2	155.1	248.0	140	30	4	3.9
JWH-250 4-OH pentyl	352.3	121.1	186.0	140	20	4	3.5
JWH-122 5-OH pentyl	372.0	169.0	230.0	140	20	4	5.4
JWH-210 5-OH pentyl	386.1	183.0	155.0	165	20	4	6.2
JWH-018	342.2	155.1	214.0	140	30	4	7.4
JWH-073	328.2	155.1	200.1	140	20	4	7.0
JWH-250	336.2	121.1	200.1	140	20	4	6.9
JWH-200	385.2	155.1	114.0	140	20	4	3.4
JWH-018 4-OH pentyl-D5	363.1	155.1		140	20	4	4.4
JWH-250 4-OH pentyl-D5	357.2	121.0		140	20	4	3.4
AM-2201 4-OH pentyl-D5	381.1	154.9		140	20	4	3.8
JWH-073 3-OH butyl-D5	349.2	155.1		140	20	4	4.2

[a]Fragmentor voltage. [b]Collision energy. [c]CXP Collision cell exit potential. [d]RT Retention time.

3.6.3 Development of LC Method

A reversed-phase column such as C18 is usually suitable for non-polar and lipophilic compounds. Therefore, a C18 column was used in these experiments. Suitable LC conditions were identified in the planning stage from the literature [79, 127, 259] and used initially. A suitable eluent for ESI should contain an organic modifier (acetonitrile or methanol) and a volatile buffer.

An Agilent LC/MS-MS triple quadrupole 6420 mass spectrometer equipped with an Agilent 1260 Infinity autosampler, binary pump with degasser was used. A Phenomenex Gemini C18 (150 x 2.0 mm, 5 µm) column coupled with a C18 guard column (4.0 × 2.0 mm) was used with a gradient elution system consisting of two mobile phases, A) 2 mM ammonium acetate and 0.1% formic acid in de-ionised water and B) 2 mM ammonium acetate and 0.1% formic acid in methanol, at a flow rate of 0.3 mL/min. The temperature of the column oven was 40°C.

It was decided to make a balance between a useable analysis time and a good chromatographic separation. Several gradient systems were applied to get the best separation and improve the resolution for all the analytes with satisfactory run time in one analysis. This was achieved by modifying the aqueous/organic phase percentage of the mobile phase.

The Gemini C18 column gave good separation of all SCs with a run time of 14 min (Figure 3.3). The method run time was reduced from 14 to 11.5 minutes by modifying the initial mobile phase composition from 50:50 B/A to 70:30 B/A. This is because the reversed-phase column contains a non-polar stationary phase and, when using mobile phase with higher organic content, analytes would elute out more quickly. The gradient mobile phase system started at 70:30 B/A, increasing to 95:05 B/A within 2.5 minutes. This percentage was maintained for 3 minutes before being decreased to 70:30 B/A for 6.5 minutes in order to condition the column before the next injection. Data analysis was performed using Agilent MassHunter™ Workstation software (version: B.05.00).

3.6.4 Initial Sensitivity and Linearity Assessments

Following development of the instrumental method, sensitivity and linearity assessments were carried out to evaluate the suitability of the analytical method before starting the development of sample preparation.

The sensitivity is based on the Limit of Detection (LOD). LOD is considered the lowest concentration of analyte that gives a reproducible instrument response with signal-to-noise (S/N) ratio greater than 3 [269]. Acceptable LODs should achieve the purpose of the method. In the current study, the main aim was to develop an analytical method for the identification of SCs in urine samples. Thus, LODs were assessed based on the concentrations that were found in case urine sample in the literature. The sensitivity of the method for each SC was assessed using serial dilution of un-extracted drug standard solutions within the expected range of the LODs at concentrations of 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100 ng/mL.

All the analytes gave good responses at a concentration of 0.39 ng/mL with signal-to-niose ratios in excess of 3 for the quantification transition, which allowed continuation of the development of the current method (Figure 3.3).

In order to evaluate the linearity, the peak area ratios of each SC to the respective internal standard were calculated and calibration curves were generated by plotting the concentration against the area ratio. The correlation coefficient (R^2) was calculated for each graph. The linearity of un-extracted SCs investigated in this study was evaluated over the concentration range 0.39–100 ng/mL for a 10 µL injection. SCs were found to have a linear response over the concentration range 0.39–100 ng/mL in un-extracted samples with R^2 values greater than 0.994 (Figure 3.4).
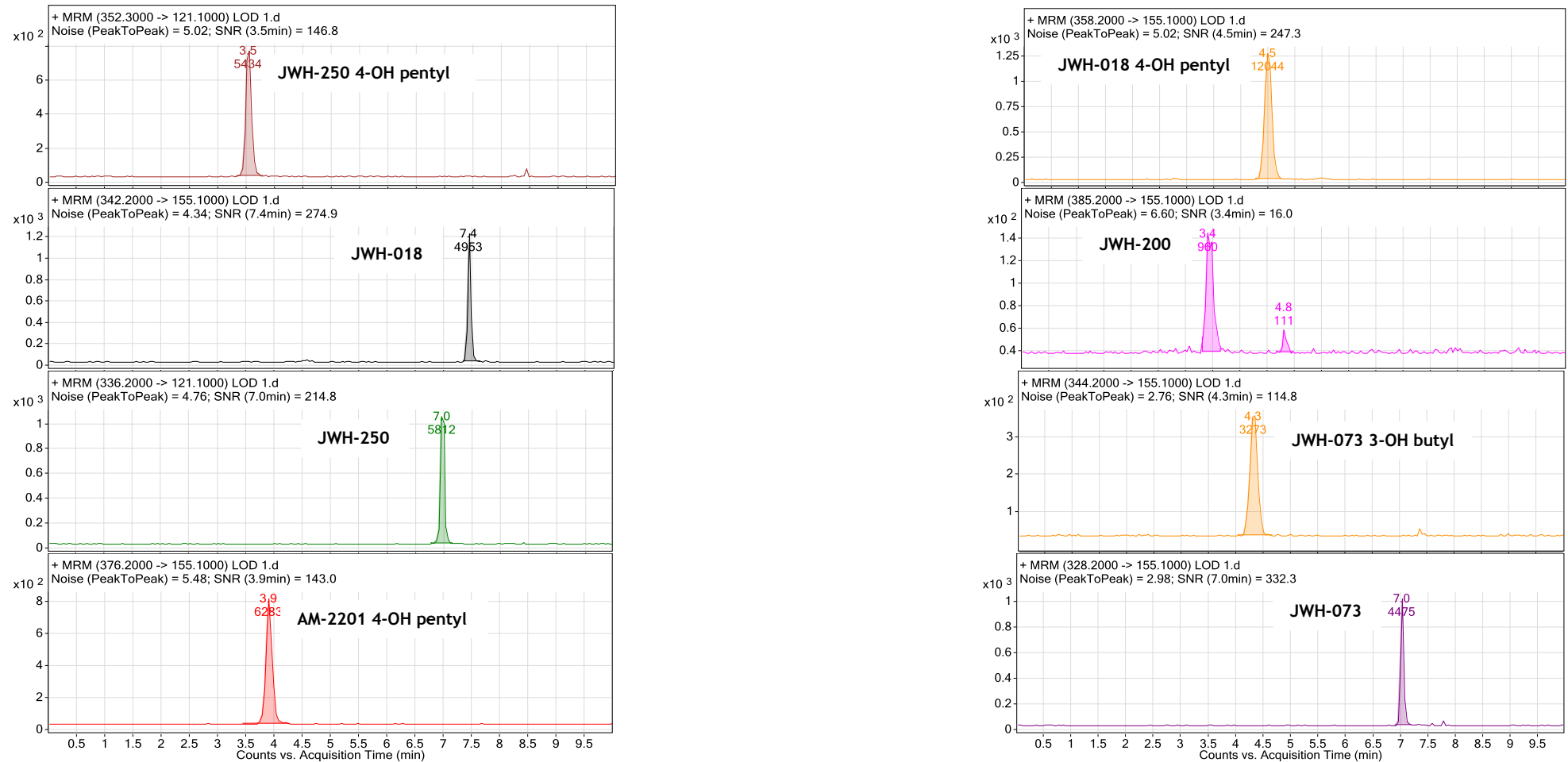
Figure 3.3: LC-MS/MS Quantifier MRM Transition Chromatograms of 0.039 ng on-column of 8 SCs Using a Standard Mixture
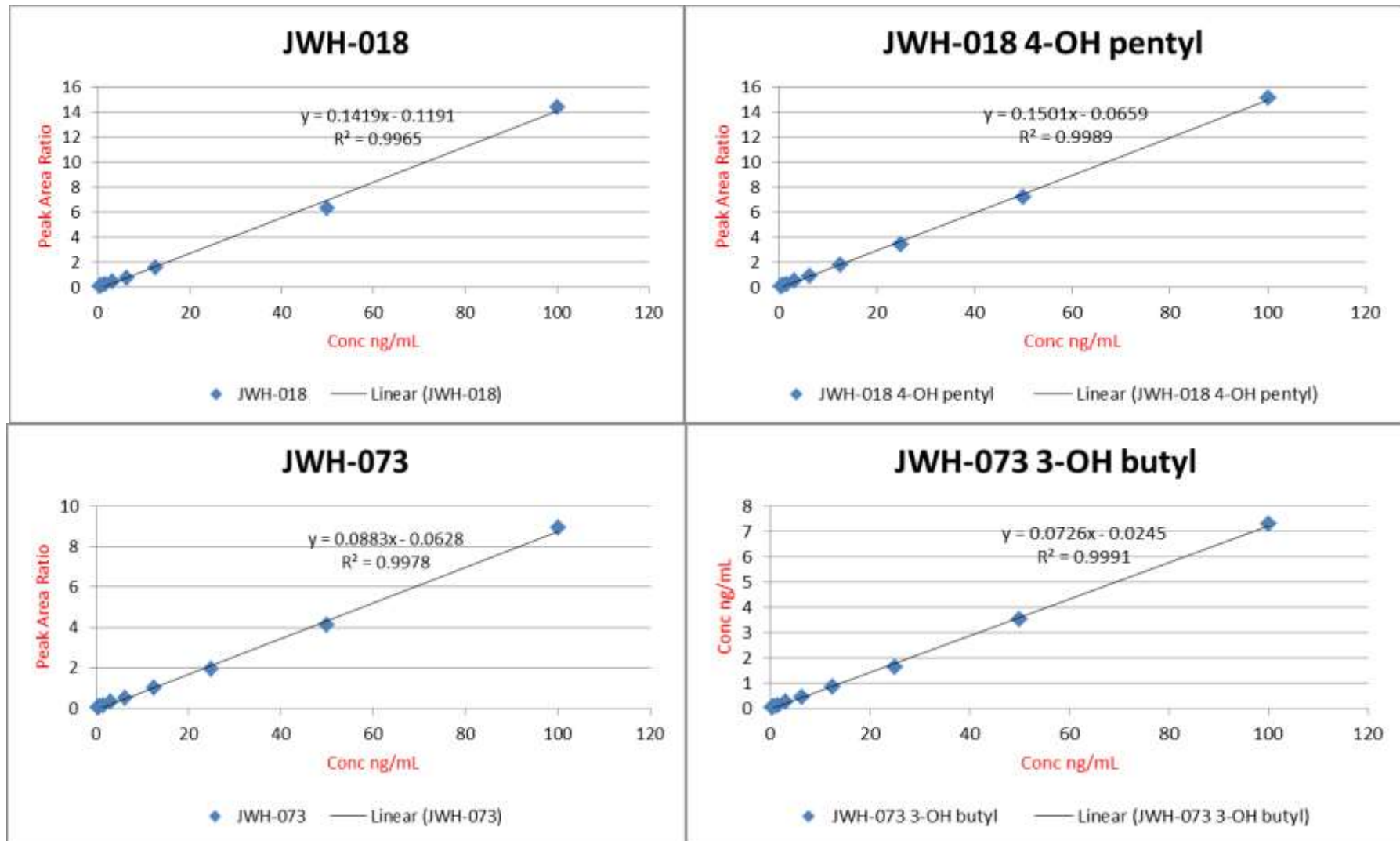
Figure 3.4: Calibration curves and Linear Correlation Coefficients (R^2) for Unextracted SC Standards over the Concentration Range 0.39-100 ng/mL in Un-extracted Samples (10 µL injection volume) Analysed by LC-MS/MS
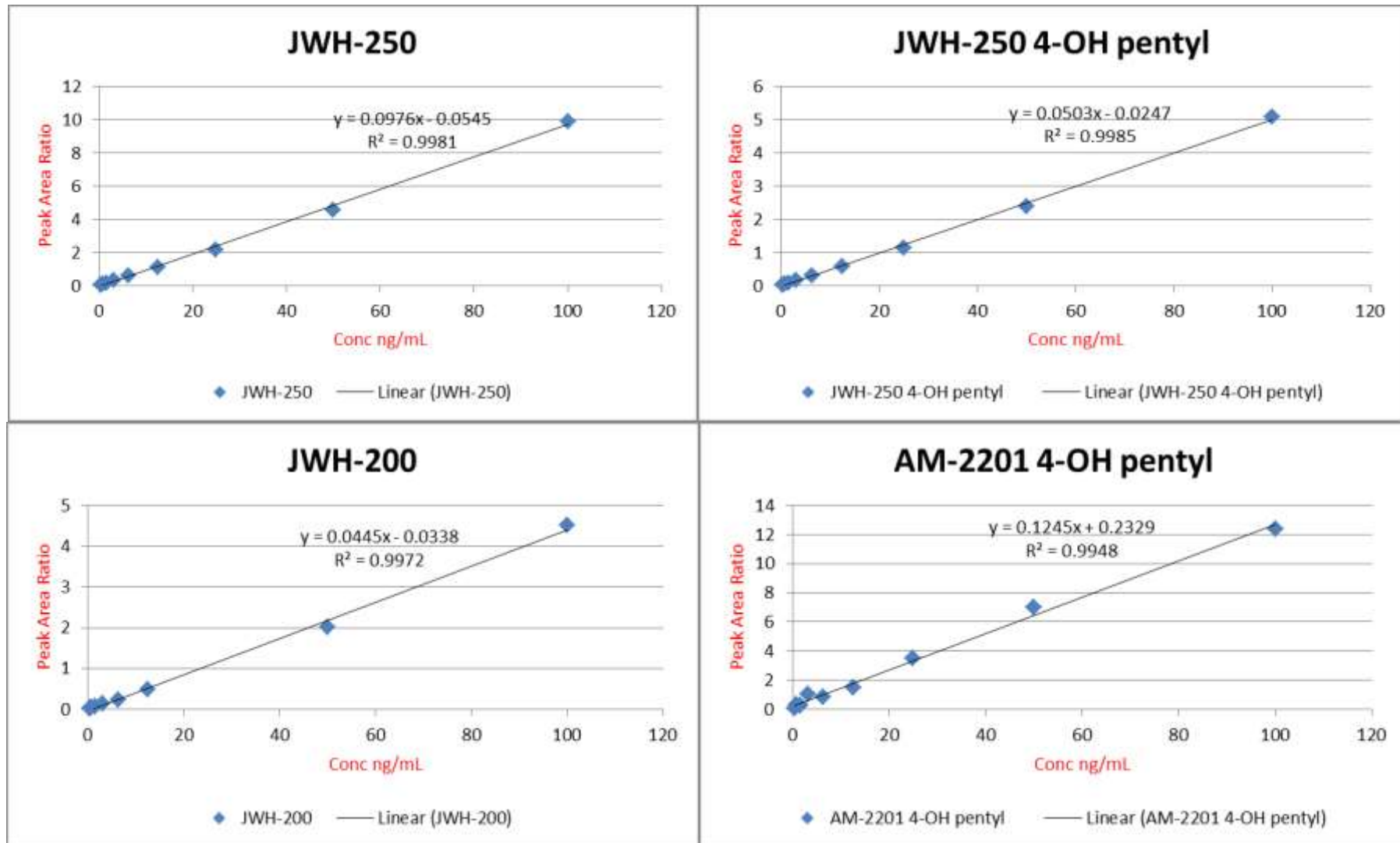
Figure 3.4: Calibration curves and Linear Correlation Coefficients (R^2) for Unextracted SC Standards over the Concentration Range 0.39-100 ng/mL in Un-extracted Samples (10 μL injection volume) Analysed by LC-MS/MS (Continued...)

3.6.5 Development of Extraction Method

In order to analyse SCs in biological samples, the compounds first need to be extracted from the matrix and concentrated. This is likely to be the most technically demanding step in the method development process. The procedure needs to be able to remove as many interferences as possible without diminishing the recovery of the compounds. Iterative extraction experiments were carried out to optimise the best conditions for the extraction of 10 SCs as a mixture from urine. Recoveries, matrix effects, the cleanliness of the extracts, time of extraction and peak shapes were evaluated when optimising extraction methods. It was decided to evaluate the recovery of three extraction techniques; dilute and shoot, PPT (with/without evaporation step) and an LLE method using methyl-*tert*-butyl-ether, modified from the literature. Sample recovery can be described as the percent of analyte that was recovered during the extraction process.

3.6.5.1   Dilute and Shoot

A volume of 100 µL blank urine was transferred to 3 mL vial followed by 40 µL of mixed standard solution at 0.5 µg/mL. And then 30 µL of 1 M sodium acetate buffer (pH 5.0) and 20 µL of ß-glucuronidase were added. A 50 µL internal standard mix solution at a concentration of 0.5 µg/mL was added to the sample. This was vortexed briefly to mix and heated (in heating block) at 60°C for 1h for enzymatic hydrolysis. The samples were left to cool down for approximately 3 minutes. A volume of 100 µL was transferred to a labelled Eppendorf vials and diluted with 900 µL of mobile phase. These were centrifuged at 13,000 rpm for 15 minutes. The samples were then transferred to labelled LC autosampler vials. A 10 µL volume was injected into the LC-MS/MS.

3.6.5.2   Protein Precipitation

40 µL of 0.5 µg/mL mixed standard solution was added to blank urine (100 µL) in a 3 mL vial. 30 µL of 1 M sodium acetate buffer (pH 5.0) and 20 µL of ß-glucuronidase were added. This was vortexed briefly to mix. The samples were incubated at 60 °C for 1h for enzymatic hydrolysis. The samples were left to cool down for approximately 3 minutes. A volume of 600 µL acetonitrile was added and samples were then transferred to labelled Eppendorf vials. These were

vortexed briefly to mix and then centrifuged at 13,000 rpm for 15 minutes. The supernatants were transferred to 3 mL vials. 50 µL internal standard mix solution at a concentration of 0.5 µg/mL was added to the supernatants which were then evaporated to dryness at 40°C. Finally, the samples were reconstituted in 100 µL of 50:50 A/B mobile phase mixture and transferred to labelled LC autosampler vials. A 10 µL volume was injected into the LC-MS/MS.

The same procedure was also applied without evaporation of the supernatant. Supernatant (200 µL) was transferred to a labelled LC vial and a 10 µL volume injected.

3.6.5.3 Liquid-Liquid Extraction

A volume of 100 µL of blank urine was transferred to a 3 mL vial followed by 40 µL of the mixed standard solution at 0.5 µg/mL. Then 30 µL of 1 M sodium acetate buffer (pH 5.0) and 20 µL of ß-glucuronidase were added. This was vortexed briefly to mix and heated at 60°C for 1h for enzymatic hydrolysis. The samples were left to cool down for 3 minutes. A volume of 300 µL methyl-*tert*-butyl-ether was added and the mixture was then transferred to a labelled Eppendorf vial. The samples mixed by shaking for 10 minutes and then centrifuged at 13,000 rpm for 15 minutes. The upper organic layer was transferred to a 3 mL vial. 10 µL internal standard mix solution at a concentration of 0.5 µg/mL was added to the upper organic layer which was then evaporated to dryness at 40°C. Finally, the samples were reconstituted in 100 µL of 50:50 A/B mobile phase mixture and transferred to a labelled LC vial. A 10 µL volume was injected into the LC-MS/MS.

3.6.5.4 Results of Recovery of Extraction Methods (Dilute and Shoot, PPT and LLE)

3 extraction techniques were used to extract 8 SCs out of urine: dilute and shoot, PPT (with/without evaporation step) and LLE. It was decided to exclude SPE methods of extraction optimisation for many reasons. It is often time consuming in method development due to the multiple steps involved with sample preparation, particularly for the washing and conditioning of the cartridge. It is also very expensive, especially in the case of high throughput of samples. The selectivity achieved when using LLE is generally as good as that

obtained with SPE [270]. Moreover, urine is quite a clean sample compared with blood or oral fluid therefore LLE or PPT might be satisfactory for sample preparation.

Choosing which extraction approach is best can be determined in practice. The comparison of the recovery of the three methods is necessary to decide which method is the best for further use, without changing any factor. Recoveries of analytes were calculated at a concentration of 200 ng/mL of urine and extracted in duplicate. At the same concentration duplicate un-extracted standards were also prepared without internal standard and kept in the fridge during the extraction. Internal standard mix solution was added to each vial for extracted and unextracted standards after the extraction. After analysing the samples, the peak area ratios between the compound and its respective deuterated standard were calculated. The recovery of each compound was calculated using the following equation [237]:

$$Recovery(\%) = \frac{Peak\ Area\ Ratio\ of\ Extracted\ Standards}{Peak\ Area\ Ratio\ of\ Unextracted\ Standards} \times 100 \qquad \textbf{Equation 3}$$

The chromatograms for the compounds were also analysed in order to determine which extraction gave the cleanest extract.

In the dilute and shoot method, the time of sample preparation compared with PPT and LLE was generally shorter. However, it is clear that, it is not the best method for the analysis of SCs. Urine samples following dilute and shoot method were not clean (brownish). JWH-200, JWH-018 and JWH-073 were not well recovered (21.8, 39.1 and 43.6% respectively). One potential reason for this might be because this method does not remove any interferences, however the use of a deuteron-labelled internal standard might compensate for matrix effects.

In PPT extraction, it seemed that recoveries of analytes with evaporation were slightly lower than without evaporation. JWH-200 showed poor recovery using the PPT method with evaporation step (17.7%) and JWH-018 and JWH-073 were not well recovered (36.2% and 37.6% respectively). For PPT extraction without the evaporation step, the recoveries for all parent analytes investigated in this

study were lower than 52%. The urine samples in PPT extraction without an evaporation step were slightly dirty which might cause as many chromatographic problems as the dilute and shoot method. The lower apparent recovery of some analytes using the PPT extraction method is probably because it does not remove many of the matrix interferences.

The LLE method compared with simple dilution and PPT methods gave cleaner extracts. This is potentially the reason why the analytes had nice chromatography. LLE attained recoveries higher than 69% for all the compounds. In addition, SCs are highly hydrophobic and LLE is a suitable method for the extraction of these types of compounds from biological matrices. An LLE method has relatively low costs and is easy to transfer from one laboratory to another. For all these reasons, it was decided to use LLE as the extraction method for all the compounds and to try to improve the sample preparation and the recovery of the analytes.

The results for recoveries for all extraction techniques used in this study are shown in Figure 3.5.
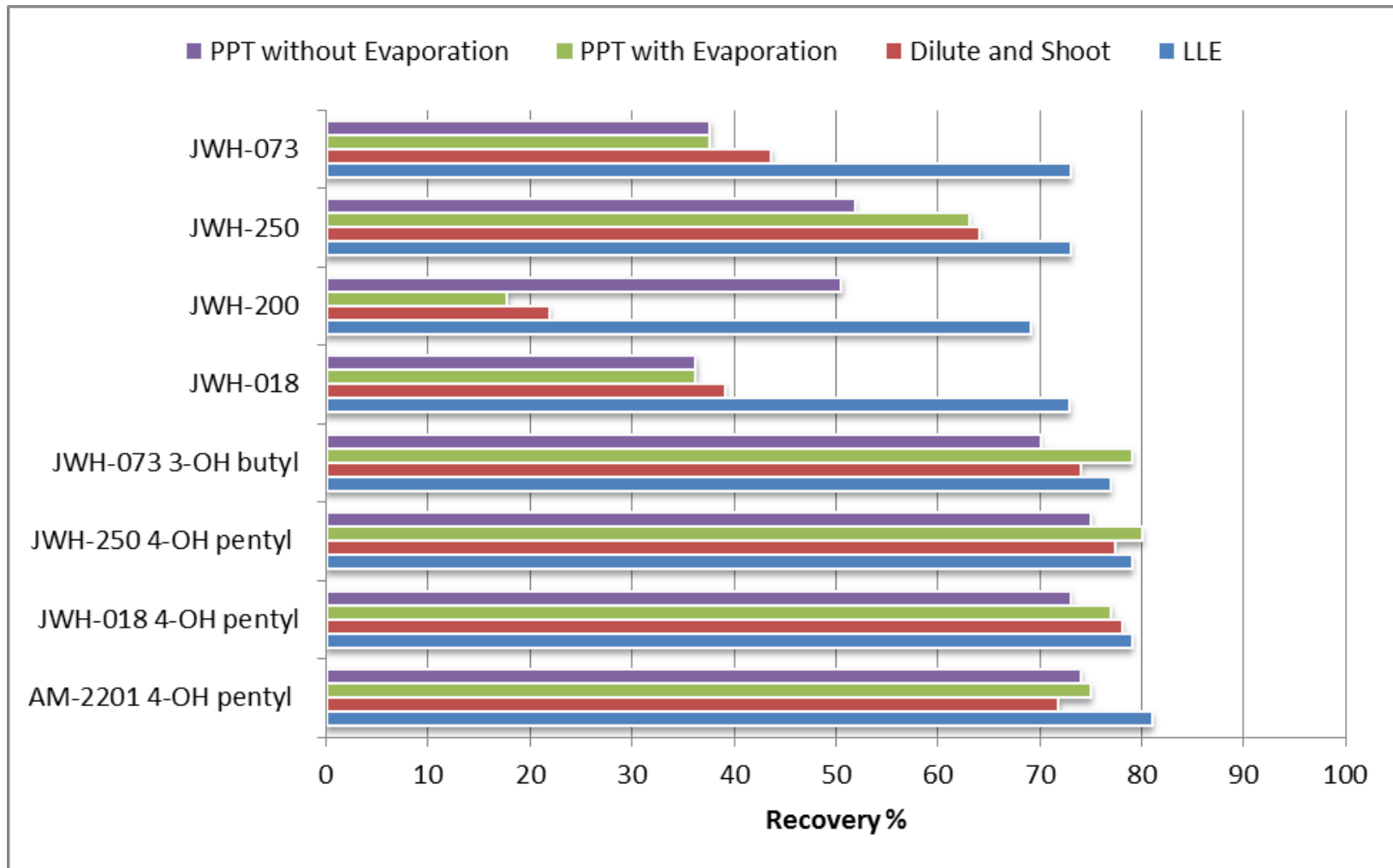
Figure 3.5: Recoveries of 8 SCs from Urine at a Concentration of 200 ng/mL by Dilute and Shoot, PPT with Evaporation, PPT without Evaporation and LLE Extraction Methods

3.6.5.5 Extraction Optimisation – Comparison of 6 LLE Methods (3 Solvents vs 2 Buffers)

It was decided to continue using the LLE method and to try to improve the sample preparation and the recoveries of the analytes. The aim of this study was to assess recoveries using three LLE solvents, MTBE, DCM and *n*-hexane, and two buffers with different pH, 0.1 M phosphate buffer (pH 6.0) and 1 M sodium acetate buffer (pH 5.0).

It was found that the time of extraction was slightly long due to the transfer step of the samples between 3 mL and Eppendorf vials. Therefore, the volume of urine was increased to 1 mL and the procedure was slightly changed as follows:

A volume of 1 mL blank urine was transferred to a test tube followed by 400 µL of mixed standard solution at 0.5 µg/mL. Then 300 µL of **(buffer)** and 20 µL of β-glucuronidase were added. This was vortexed briefly to mix and heated at 60°C for 1h for enzymatic hydrolysis. The samples were left to cool down for 3 minutes. A volume of 3 mL **(solvent)** was added and then mixed by shaking for 10 minutes. The samples were centrifuged at 3500 rpm for 10 minutes. The upper or lower organic layer (depending on the solvent) was transferred to a labelled 3 mL vial. 100 µL of internal standard mix solution at a concentration of 0.5 µg/mL was added and then the mixture was evaporated under a nitrogen stream at 40°C. The samples were reconstituted in 100 µL of 50:50 A/B mobile phase mixture and transferred to a labelled LC vial. A 10 µL volume was injected into the LC-MS/MS.

Recoveries of analytes were calculated at a concentration of 200 ng/mL of urine and were bead on duplicate extractions; refer to Equation 3 above for the calculation.

3.6.5.6 Results of Recovery of 6 LLE Methods (3 Solvents vs 2 Buffers)

Practical work on different buffers and pH values might help to choose suitable conditions of ionisation especially in the case of the analysis of many dissimilar compounds. SCs included in this method are non-ionised and have indole rings which don't protonate easily.

An organic solvent should be selected depending on the analyte's solubility in the particular solvent to obtain maximum recovery. The method was evaluated by using three LLE solvents, MTBE, DCM and *n*-hexane, and two buffers, 0.1 M phosphate buffer (pH 6.0) and 1 M sodium acetate buffer (pH 5.0).

LLE with MTBE as a solvent gave better recoveries for most of the analytes than DCM and *n*-hexane. Recovery depends primarily on solubility of the analyte in the solvent - the partition coefficient. Recovery was greater than 75.3% for all the analytes using LLE/MTBE/1 M sodium acetate buffer (pH 5.0). JWH-018 showed poor recovery using LLE/DCM/0.1 M phosphate buffer (pH 6.0) method (16.3%). The results for recoveries for all these methods are shown in Figures 3.6, 3.7 and 3.8.

Nonpolar compounds (e.g. SCs) by their nature prefer to stay in the organic phase more than in the aqueous phase. MTBE and n-hexane have a lower density than water and, unlike DCM, the organic phase forms the upper layer during phase separation that makes the collection simpler and minimises dripping losses [270]. Time of extraction was shorter when using MTBE as a solvent, allowing more samples to be analysed in each batch.

It was decided to use the LLE/MTBE/1 M sodium acetate buffer (pH 5.0) method because it gave higher recoveries for most of the analytes compared with the other methods and for its ease in practice.
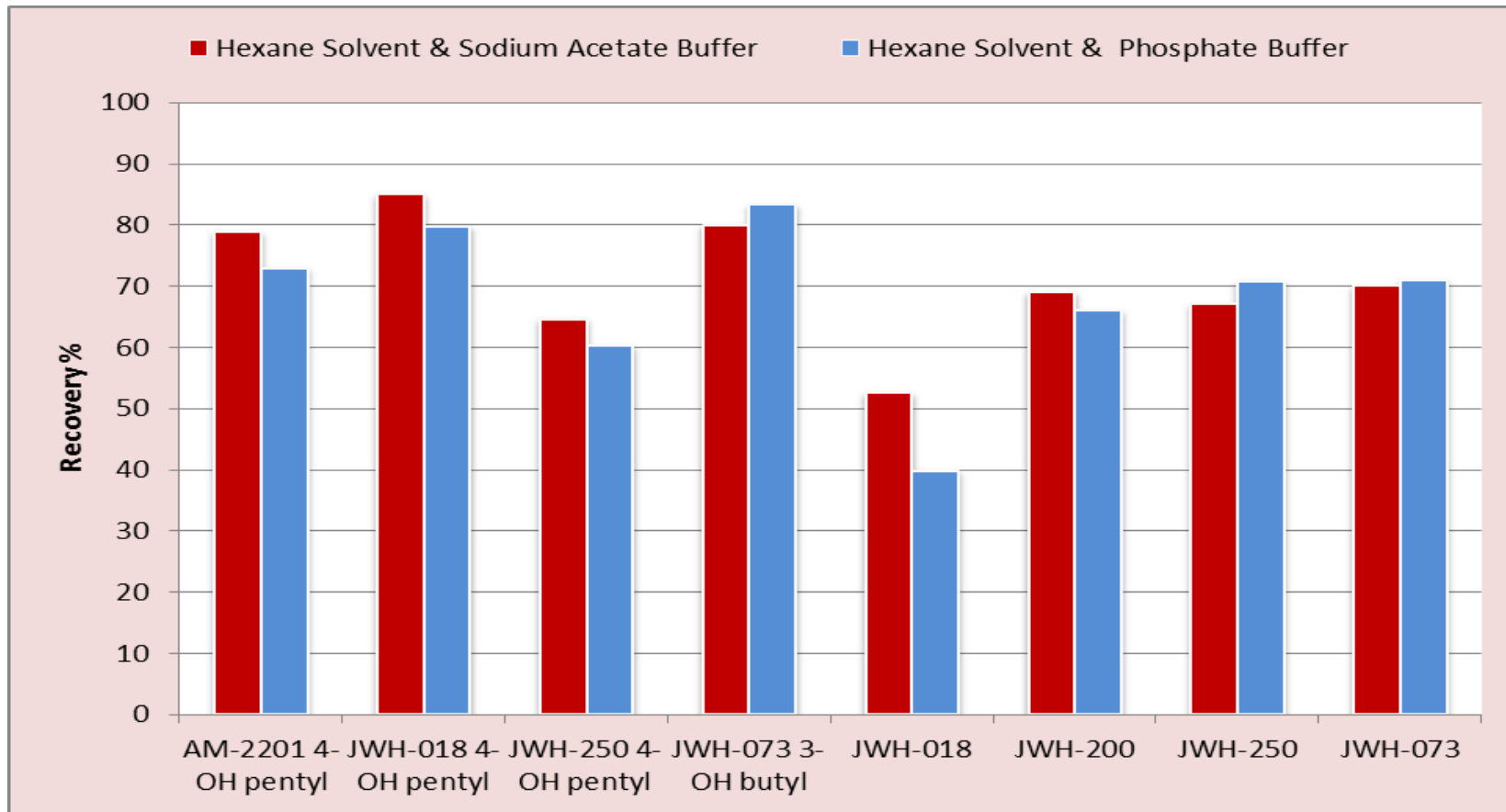
Figure 3.6: Recoveries of 8 SCs Using LLE Methods with (Hexane Solvent & Sodium Acetate Buffer (red)) and (Hexane Solvent & Phosphate Buffer (blue))
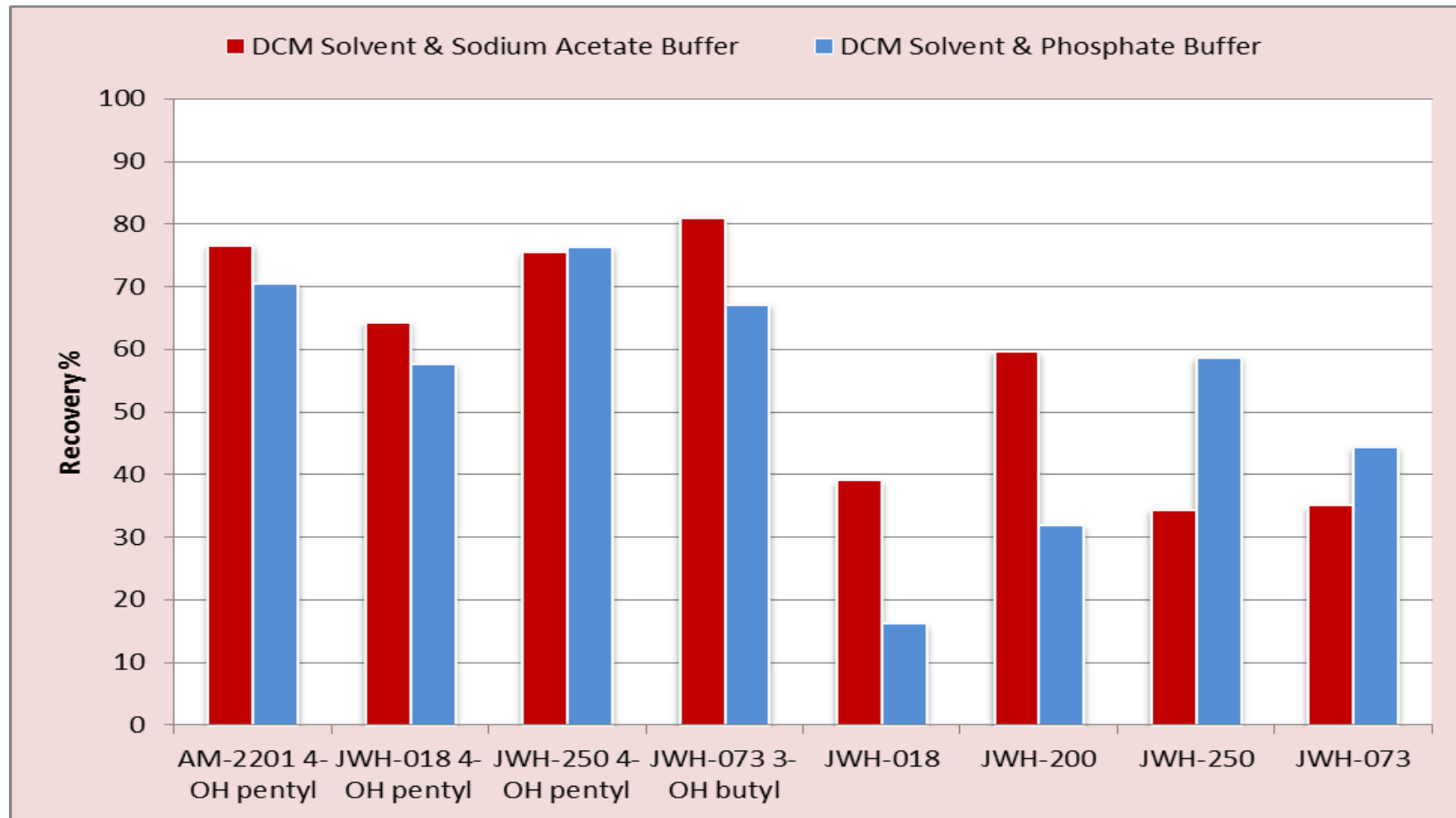
Figure 3.7: Recoveries of 8 SCs Using LLE Methods with (DCM Solvent & Sodium Acetate Buffer (red)) and (DCM Solvent & Phosphate Buffer (blue))
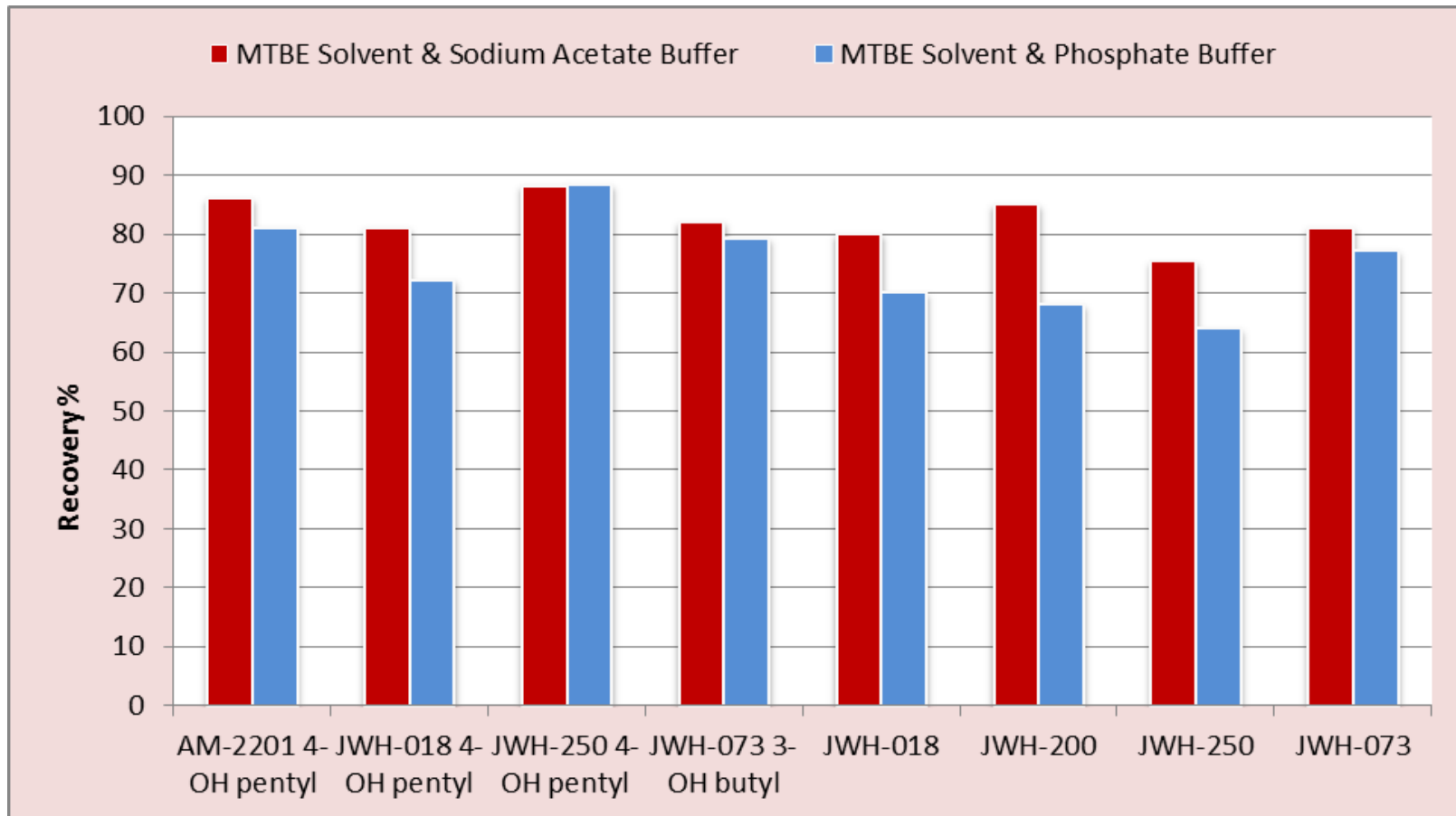
Figure 3.8: Recoveries of 8 SCs Using LLE Methods with (MTBE Solvent & Sodium Acetate Buffer (red)) and (MTBE Solvent & Phosphate Buffer (blue))

3.6.5.7 Extraction Optimisation – Matrix Effects and Sensitivity Assessments

At this stage of method development JWH-122 5-OH pentyl and JWH-210 5-OH pentyl were obtained from the supplier and added to the standard mixture. It was decided to use the LLE method with MTBE as a solvent and 1 M sodium acetate (pH 5.0) as a buffer. Matrix effects and sensitivity were assessed for the developed method using two sample volumes, 0.1 and 1 mL, in duplicate. The matrix effects of the developed method were further assessed with a sample volume of 0.5 mL using two sample reconstitution volumes, 100 and 200 µL of mobile phase (50:50 V/V A/B). The main parameters of the procedures are given in Table 3.4.

Table 3.4: Comparison of 3 Sample Volumes

Sample Volumes Urine (mL)	Mixed STD Solution (µL)	Buffer (µL)	Solvent (mL)	Centrifuge rpm/min	IS Mix Solution (µL)
0.1	40	30	0.3	13000/15	10
0.5	200	150	1.5	3500/10	50
1.0	400	300	3	3500/10	100

The matrix effect was studied to assess ionisation enhancement/suppression for the developed method using the post-extraction addition approach (Matuszewski strategy) [271]. Mixed standard solution was added to each vial for extracted standards after the extraction. To calculate the matrix effects, a pure standard at the same concentration in mobile phase was prepared in duplicate at the same time. After analysing the samples, the matrix effect was calculated for each compound using the following equation:

$$ME(\%) = \frac{Peak\ Area\ Ratio\ of\ Post\text{-}extraction\ Spike}{Peak\ Area\ Ratio\ of\ Pure\ Analytes\ in\ Reconstitution\ Solvent} \times 100 \quad \textbf{Equation 4}$$

The sensitivity of the method for each SC was assessed instrumentally using blank urine samples spiked with the serially-diluted standard solutions. The LOD is considered to be the lowest concentration of analyte that gives a reproducible instrument response with S/N ratio greater than 3.

3.6.5.8   Results of Matrix Effects and Sensitivity Assessments

It was found that the most effective way to minimise matrix effects is to use stable isotope-labelled internal standards [272]. However, this is not always possible for various reasons. With respect to SCs, it is very difficult to obtain stable isotope-labelled internal standards for all analytes of interest due to the rapid proliferation of SCs on the world market.

Matrix effects and sensitivity were assessed for the developed method in urine using two different sample volumes; 0.1 and 1 mL. The LLE method using MTBE as solvent, 1 M sodium acetate (pH 5.0) as a buffer and 0.1 mL as a sample volume gave better results for matrix effects for most of the analytes, with response ratios in the range 71.1-115% compared to those for a 1 mL sample volume, which had more ion suppression (Figure 3.9). A potential reason for this might be because the method using 0.1 mL as a sample volume gave a cleaner extract which led to reduced matrix effects and potentially more accurate quantitations. It is clear that this would be better for the instrument and for conserving sample.

However, the developed method gave a very good sensitivity when using 1 mL as a sample volume with LODs ranging from 0.05 to 0.2 ng/mL with signal-to-niose ratios in excess of 3 for the quantification transition, compared to those obtained with a sample volume of 0.1 mL. LODs with 0.1 mL samples were not 10 times higher for most of the analytes. Potential reasons for this might be because the accuracy of measuring the LODs or due to the matrix effects. LOD results of all SCs investigated in this study for both methods are presented in Figure 3.10.

Based on these results, it was decided to continue to use the developed method with a sample volume of 0.5 mL as a compromise without decreasing the sensitivity too much.
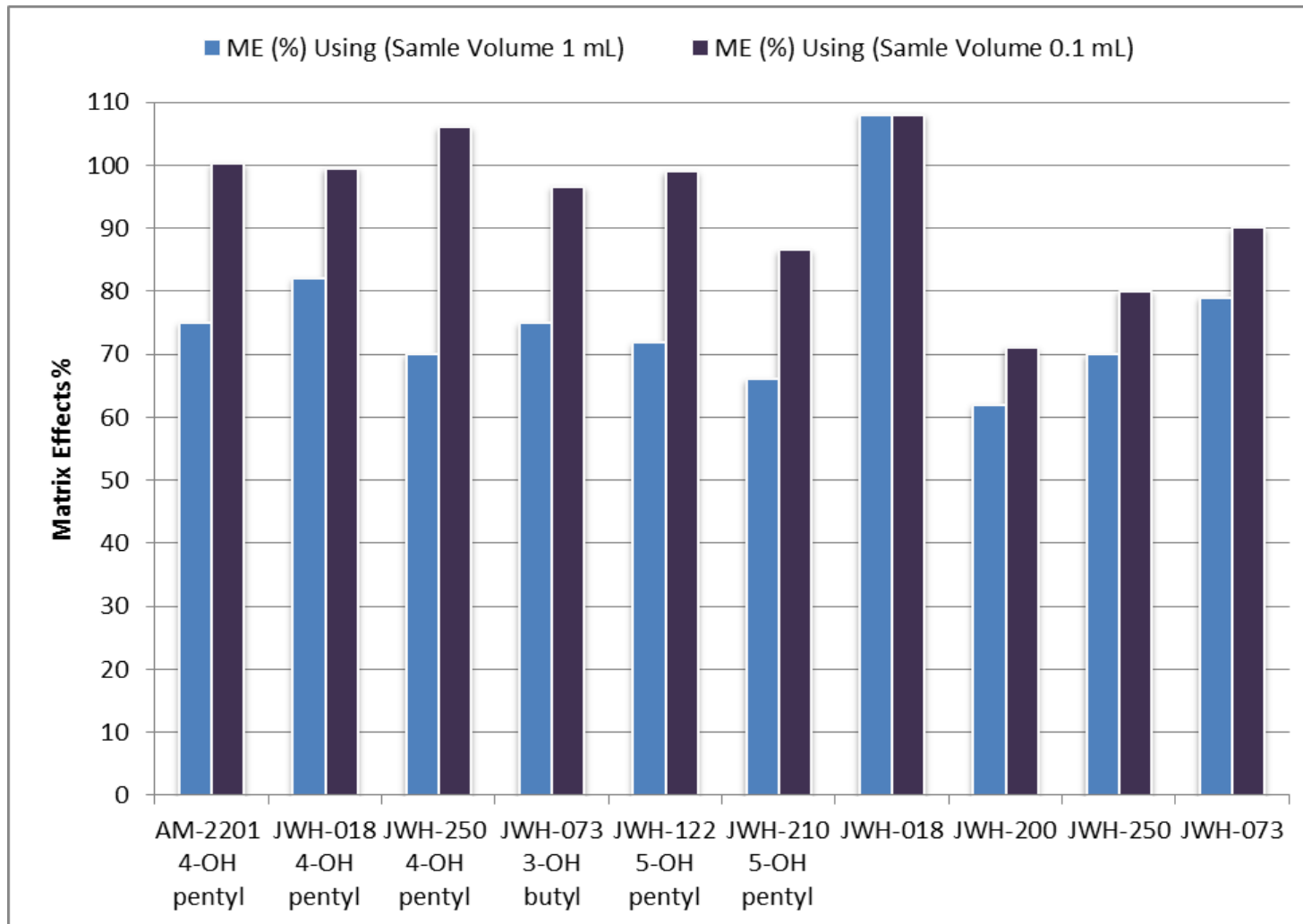
Figure 3.9: Comparison of Matrix Effects During LC-MS/MS Analysis of 10 SCs in Urine at a Concentration of 200 ng/mL Using Two Sample volumes
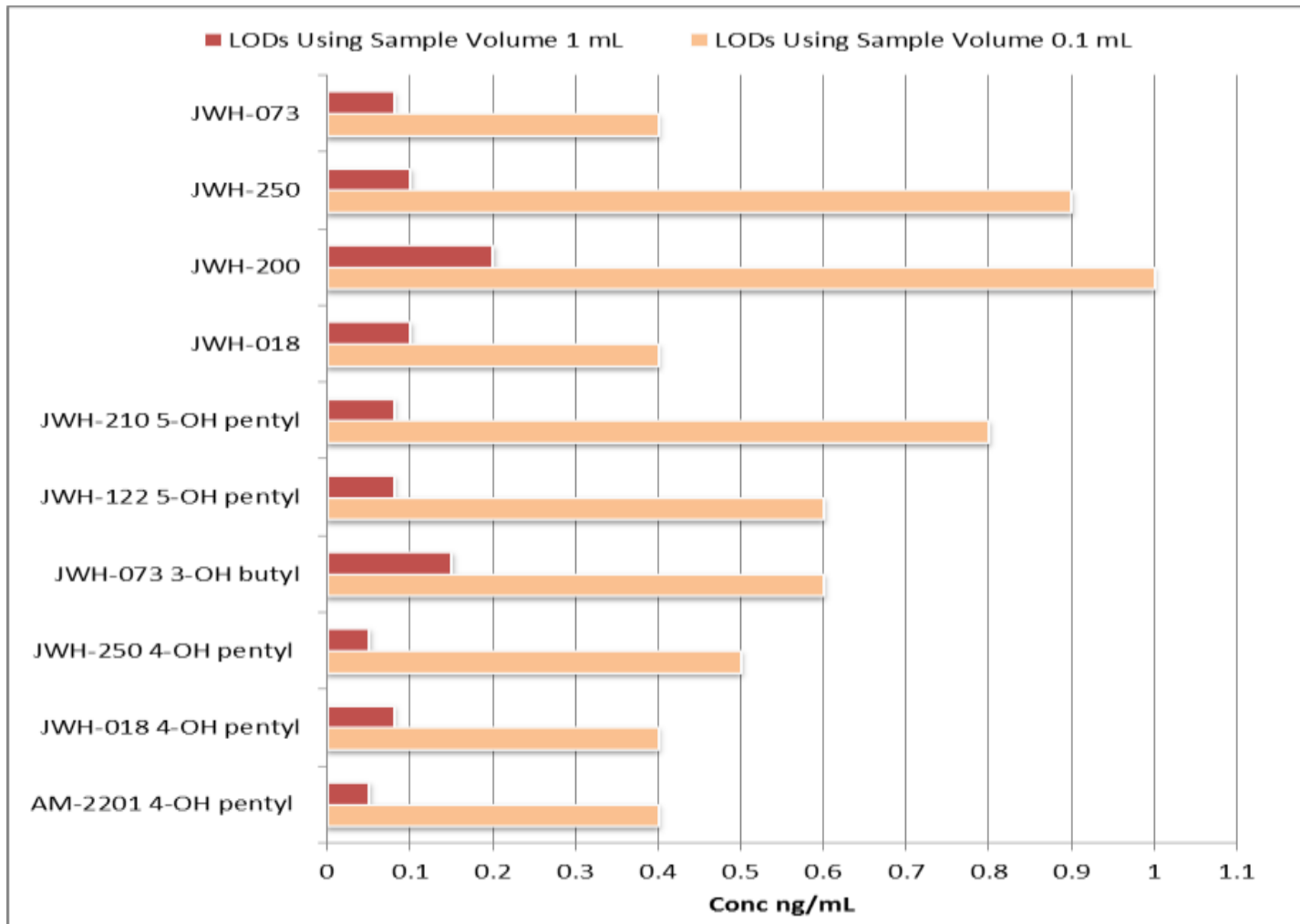
Figure 3.10: LODs for the Analysis of 10 SCs in Urine by LC-MS/MS Based on 2 LLE Methods Using Sample Volumes of 1.0 and 0.1 mL.

3.6.5.9   Extraction Optimisation – Sample Reconstitution

Two reconstitution volumes; 100 and 200 µL were used for the same extracted sample to achieve the best results with respect to sensitivity and matrix effects. The developed method using 0.5 mL sample and 200 µL reconstitution volume gave lower matrix effects while at the same time LODs under these conditions were still about 0.1 ng/mL. Matrix effects were acceptable, ranging from 76 to 110.5% for all analytes at concentrations of 200 ng/ml in urine (Figure 3.11).

The dilution of samples by increasing the reconstitution volume reduced the matrix effects and helped to improve the linearity. A potential reason for this might be because the use of low concentrations prevents the MS source from saturating or may due to the detector.
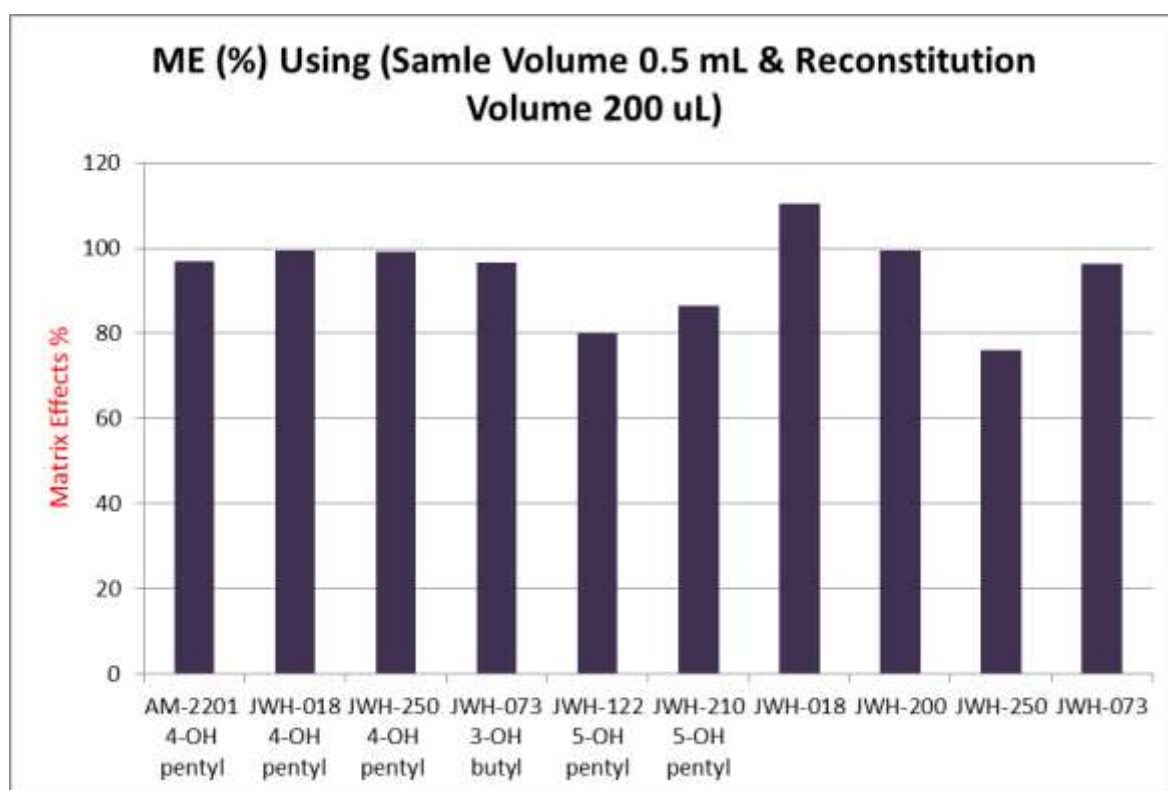
Figure 3.11: Matrix Effects for the LC-MS/MS Analysis of 10 SCs in Urine at a Concentration of 200 ng/mL Using the Final Developed Method

3.7 Conclusions

The sensitivity of GC-MS was unsatisfactory, which would make the determination of SCs in biological samples, particularly blood, more challenging. In contrast, the newer technique of LC-MS/MS seems to be more convenient for the detection of SCs and their metabolites in biological samples because it provides better sensitivity; indeed, most of the published literature uses LC-

MS/MS. It has become more common for the analysis of illicit drugs in biological specimens, specifically for simultaneous multicomponent analysis. It is a much softer option to GC-MS, so can be used for non-volatile, high molecular weight and thermally labile compounds. Sample preparation time is shorter compared to GC-MS because the derivatisation of polar functional groups is not required. Moreover, the variation of derivatisation agents yields different spectra for the compound and this can lead to an unwanted diversity between laboratories. LC-MS/(MS) has been shown to be a perfect supplement to GC-MS/(MS).

A simple and cost-effective LC-MS/MS method using LLE for the simultaneous quantification of 4 SCs and 6 of their metabolites in urine has been developed. It is sometimes difficult to judge if a method is 'good enough' because there is always a possibility of improving it at every stage. However, it is all about balancing things and the current developed method seems to be fit for the purpose of the project. The sensitivity of the method was satisfactory and LODs were low enough to detect SCs that have been seen in other cases. It is clear that LC-MS/MS is one of the best chromatographic techniques for the analysis of SCs. Prior to using the developed method to analyse samples, the overall performance had to be evaluated through a formal validation procedure.

Chapter 4 - Method Validation of 18 Analytes in Blood and Urine Using LC-MS/MS

4.1 Introduction

Reliable results are very important in forensic toxicology investigations which in turn need reliable analytical methods that give accurate and reproducible data. Methods in forensic toxicology should be able to distinguish between a diversity of drugs which might be sharing the same chemical behaviour and could be present in the samples at the same time. Therefore, prior to using a new analytical method, it should be validated according to international recommendations to ensure that the method is fit for use for a particular analysis. The validation of a method proves the scientific soundness of the characterisation or measurement. It demonstrates that a developed method measures the correct compound in the correct amount for the intended samples. The validated method also allows the analyst to establish the performance limits of the method as well as to understand the behaviour of the method. According to the International Organization for Standardization (ISO), validation can be defined as "verification, where the particular requirements are adequate for an intended use" [273].

Before starting any validation experiments, a validation plan should be in place. A validation plan should contain the parameters which need to be examined depending on the requirements for a method [274, 275]. Thus, the method can be validated for use as a qualitative or quantitative method. For a qualitative method, few parameters require to be examined. Specificity/selectivity, precision (intra and inter-day precision), the limit of detection and stability of analytes have to be investigated. In the case of a quantitative method, more parameters also need to be optimised; accuracy, linearity within the quantitation range, carryover and recovery. It has been recommended that matrix effects should be evaluated in the method validation process for quantitative methods using LC-MS/MS [276, 277].

Useful protocols for method validation have been published by different organisations to ensure high quality that contributes to achieving the most reliable results, such as the United Kingdom and Ireland association of forensic

toxicologists forensic toxicology laboratory guidelines (2010) [278] the SOFT/AAFS Forensic Toxicology Laboratory Guidelines (2006) [279] and the standard practices for method validation in forensic toxicology which was published in 2013 by the Scientific Working Group for Forensic Toxicology (SWGTOX) [269]. Several articles [274, 280] have been published discussing important considerations in analytical method validation which may be used as guidance.

Due to the rapid appearance of SCs in the market, it was necessary to add a further 6 drugs (5F-AKB-48, 5F-PB-22, AM-1248, AB-FUBINACA, A-796260, UR-144) and 2 metabolites (5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole) (See Figure 2.7). Initial experiments were carried out on these drugs and it was found that they were compatible with the already developed method.

Both urine and blood samples might play an essential role in the forensic analysis of SCs. While urine is the best matrix to indicate past exposure of ingested drugs, blood is the preferred sample in post-mortem toxicology. The analysis of blood increases the chance of identifying the ingested parent compound. Analysing blood is also important for assessing whether the deceased was under the influence of a drug at the time of death.

As mentioned previously, SCs undergo extensive metabolic conversion and the parent compounds are rarely observed in urine [236]; therefore it seems essential to focus on metabolites. It was found that structurally similar SCs follow common metabolic pathways leading probably to produce metabolites with similar metabolic transformations [281]. Dresen *et al*. [192] reported that it is very important in developing analytical methods to detect parent compounds of SCs in the blood and also to correlate SCs in blood to their major urinary metabolites.

For all these reasons, it was decided to validate the optimised method in Chapter 3 for the quantitation of a selection of SC parent compounds along with their corresponding metabolites in blood and urine using LC-MS/MS. The method was validated according to the SWGTOX guidelines [269] for urine and whole blood.

4.2   Materials and Methods

4.2.1 Chemicals and Reagents

The chemicals and reagents were the same as those described in Section 3.3

4.2.2 Calibrators, Internal Standards and Controls

Stock and working solutions were the same as those described in Section 3.4.1

Two mixture solutions (0.5 µg/mL) were prepared as follow:

(A) '0.5 µg/mL mixture solution 1' containing 10 SCs was prepared in methanol and used to make up calibrators at 0.5, 1.0, 2.0, 5.0, 10.0, 25, 50, and 100 ng/mL in blood.

(B) '0.5 µg/mL mixture solution 2' containing 8 metabolites of SCs was prepared in methanol used to make up calibrators at 1.0, 2.0, 5.0, 10.0, 25, 50, and 100 ng/mL in urine.

The internal standard solution contained JWH-018 4-OH pentyl-D5, JWH-250 4-OH pentyl-D5, AM-2201 4-OH pentyl-D5, JWH-073 3-OH butyl-D5 and JWH-200-D5 and was prepared in methanol at 0.5 µg/mL. All stock and working solutions were transferred to amber glass bottles and stored at –20°C for up to 6 months. Three quality control (QC) working solutions at concentrations of 5, 40 and 75 ng/mL were prepared in methanol for all the analytes.

4.2.3 Instrumentation

An Agilent LC/MS-MS triple quadrupole 6420 mass spectrometer equipped with an Agilent 1260 Infinity autosampler, binary pump with degasser was used. Chromatographic separation (run-time 11.5 mins) was achieved using a Phenomenex Gemini C18 (150 x 2.0 mm, 5µm) column and a gradient elution system consisting of two mobile phases; 2mM ammonium acetate and 0.1% formic acid in de-ionised water and in methanol. The temperature of the column oven was 40°C. The gradient mobile phase system started at 70:30 B/A, increasing to 95:05 B/A within 2.5 minutes. This percentage was maintained for

3 minutes before being decreased to 70:30 B/A for 6.5 minutes in order to condition the column before the next injection. Agilent LC-MS/MS equipped with an electrospray ionisation interface, operated in positive polarity, was used for analysis. Multiple Reaction Monitoring (MRM) mode was used to monitor 2 ion transitions (quantifier and qualifier) for each analyte and 1 transition was used for the internal standard. Data analysis was performed using Agilent MassHunter™ Workstation software (version: B.05.00). The MS/MS parameters for all SCs used in this project are detailed in Table 4.1.

4.2.4 Extraction Methodology

A volume of 0.5 mL blank urine was transferred to a test tube followed by 200 µL of mixed standard solution at 0.5 µg/mL. Then 150 µL of 1 M sodium acetate buffer (pH 5.0) and 20 µL of ß-glucuronidase were added. This was vortexed briefly to mix and heated at 60°C for 1h for enzymatic hydrolysis. The samples were left to cool down for 3 minutes. A volume of 1.5 mL methyl-*tert*-butyl-ether was added and then mixed by shaking for 10 minutes. The samples were centrifuged at 3500 rpm for 10 minutes. The upper organic layer was transferred to a labelled 3 mL vial. 50 µL of internal standard mix solution at a concentration of 0.5 µg/mL was added and then the mixture was evaporated under a nitrogen stream at 40°C. The samples were reconstituted in 200 µL of 50:50 A/B mobile phase mixture and transferred to a labelled LC vial. A 10 µL volume was injected into the LC-MS/MS.

4.2.5 Limit of Detection and Lower Limit of Quantification

LOD of an analyte can be defined as that concentration giving a signal significantly different from the background signal. Several approaches for determining LOD are possible, depending on whether the procedure is instrumental or non-instrumental [274, 282]. It can be determined by visual inspection, from the S/N ratio or using a statistical method based on the standard deviation (SD) of the response and the slope.

Table 4.1: Multiple Reaction Monitoring Transitions, MS/MS Parameters and Retention Times for SCs, their Metabolites and Internal Standards

Analyte	Q1 mass (*m/z*)	Q3 mass (*m/z*)		Frag[a] (V)	CE[b] (eV)	CXP[c] (V)	RT[d] (min)
		Quantifier	Qualifier				
JWH-018 4-OH pentyl	358.2	155.1	127.0	140	5	4	4.5
JWH-073 3-OH butyl	344.2	155.1	216.0	140	20	4	4.3
AM-2201 4-OH pentyl	376.2	155.1	248.0	140	30	4	3.9
JWH-250 4-OH pentyl	352.3	121.1	186.0	140	20	4	3.5
JWH-122 5-OH pentyl	372.0	169.0	230.0	140	20	4	5.4
JWH-210 5-OH pentyl	386.1	183.0	155.0	165	20	4	6.2
5F-AKB-48 (*N*-4 OH pentyl)	400.1	135.0	107.0	150	25	4	6.5
5F-PB-22 3-carboxyindole	250.1	118.0	206.0	145	20	4	2.8
JWH-018	342.2	155.1	214.0	140	30	4	7.4
JWH-073	328.2	155.1	200.1	140	20	4	7.0
JWH-250	336.2	121.1	200.1	140	20	4	6.9
JWH-200	385.2	155.1	114.0	140	20	4	3.5
5F-AKB-48	384.2	135.0	-	210	22	4	5.5
5F-PB-22	377.1	232.1	144.0	235	27	4	7.7
AM-1248	391.1	135.0	112.0	190	30	4	2.0
AB-FUBINACA	369.1	252.9	324.1	100	25	4	3.5
A-796260	355.2	125.0	114.0	155	20	4	4.3
UR-144	312.1	125.0	214.0	160	25	4	7.8
JWH-018 4-OH pentyl-D5	363.1	155.1		140	20	4	4.4
JWH-073 3-OH butyl-D5	349.2	155.1		140	20	4	3.4
AM-2201 4-OH pentyl-D5	381.1	154.9		140	20	4	3.8
JWH-250 4-OH pentyl-D5	357.2	121.0		140	20	4	3.4
JWH-200-D5	390.1	155.0		185	20	4	3.4

[a]Fragmentor voltage. [b]Collision energy. [c]CXP Collision cell exit potential. [d]RT Retention time.

In this project, the S/N ratio approach was used. LOD is considered the lowest concentration of analyte that gives a reproducible instrument response with S/N ratio greater than 3 [269]. Specifically, LOD can be identified when the lowest

concentration of analyte met these identification criteria; quantifier ion peaks greater than 3 times the background noise and presence of qualifier ions at the known retention time. LOD is not a rugged or robust parameter. Minor changes in the analytical system (e.g. instrumental conditions, matrix effects, purity of reagents) can affect the LOD. The lower limit of quantification (LLOQ) is considered the lowest concentration that gives a reproducible instrument response with acceptable precision (<20% coefficient of variation) and S/N ≥ 10 [274]. MassHunter™ software was used to calculate the S/N ratio.

LOD and LLOQ were determined instrumently for each analyte of interest by lowering the concentrations of calibration standards in blood and urine at eight different concentrations: 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5 and 0.75 ng/mL. The samples were prepared in duplicate and analysed using the optimised method.

4.2.6 Linearity

Linearity was determined by preparing and analysing eight calibrators over the concentration range of 0–100 ng/mL (0.5, 1, 2, 5, 10, 25, 50, 100 ng/mL) in blood and seven calibrators (1, 2, 5, 10, 25, 50, 100 ng/mL) in urine. Five separate calibrations were prepared freshly in duplicate by spiking blank blood and urine with working mixture solutions 1 and 2 respectively and extracted in accordance with the method reported in Chapter 3, over 5 different days. Drug-free matrix and internal standard mixture added at 50 ng/mL were included with each run.

Calibration curves were obtained by plotting peak area ratios of analytes to internal standards against concentration. The linear correlation coefficient, R^2, often used as criterion of linearity, was calculated. Acceptable values should be greater than 0.99.

4.2.7 Selectivity and Specificity

Selectivity is tested to confirm the ability of a method to distinguish the target analyte(s) in a complex matrix without any potential interference from other matrix components of similar behaviour (e.g. degradation products, impurities

and metabolites) whereas specificity can be described as the ultimate selectivity where the possibility of interference is 0% [283, 284].

The selectivity of the method was assessed by analysing 10 different sources of blank blood and urine (each), and comparing their chromatograms with those of corresponding standards spiked with 10 SC and 8 metabolites in blood and urine respectively at a concentration of 50 ng/mL. Specificity was evaluated by spiking drug-free matrix with each analyte and internal standard individually, at a concentration of 50 ng/mL. For the exogenous interference from other drugs, it was evaluated by analysing drug-free matrix containing tetrahydrocannabinol, amphetamine, cocaine, benzoylecgonine, anhydroecgonine methyl ester, ecgonine methyl ester, methadone, codeine, morphine, diazepam, gabapentin and mephedrone at a concentration of 500 ng/mL. All of them were separately spiked into blood and urine, and extracted.

PB-22 and its fluorinated analogue 5F-PB-22 share some metabolites [88]; therefore the identification of unique metabolites for proof of compound consumption in forensic toxicology is a crucial issue. 5F-PB-22 3-carboxyindole is a potential marker for 5F-PB-22 with 2 MRM transition (250.1> 118.0) and (250.1> 206.0) [88]. A similar phenomenon may also occur in the case of other fluorinated analogues of SCs. 5F-AKB-48 (*N*-4 OH pentyl) is a good target metabolite for 5F-AKB-48 [58]. All the metabolites of SCs in the urine method used in this project were the major metabolites and good targets for their parents. Using authentic matched blood and urine samples can often overcome that issue.

4.2.8 Carryover

As part of method validation requirements, carryover has to be investigated under analysis conditions. Carryover is the inadvertent transfer of analyte into a subsequent sample during instrumental analysis. Carryover of analyte from one sample to the subsequent sample might cause an inaccurate result when using instrumental methods [269]. Carryover was tested by triplicate injecting of blank samples after two injections of an extract of blood/urine containing all analytes at a concentration of 100 ng/mL. To establish if there is carryover, the chromatograms were analysed visually.

4.2.9 Bias and Precision

Bias (accuracy) is defined as the degree of closeness of the mean concentration obtained by the analytical method to the true concentration of the analyte under the prescribed conditions [285]. Precision can be described as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous samples [274].

Method bias/precision is determined using two main condition sets called intra-day and inter-day bias/precision and which are also known as repeatability and reproducibility, respectively. Intra-day bias/precision is measured by analysing replicate samples containing drugs in the same day using the same analytical method and extraction. The inter-day bias/precision is determined in a similar manner to the intra-day bias/precision but on different days.

Bias and precision were determined by replicate analysis of drug-free matrix spiked at three different concentrations (low, medium and high) as QCs. Each batch of QCs involved a freshly prepared calibration curve to calculate the concentrations using the optimised method. Intra-day bias and precision were calculated from five replicates per QC in one batch. For the determination of inter-day bias and precision, five replicates per QC were analysed on five different days (n=25). Bias and precision were calculated using the following formula [269]:

$$\overline{x} = \frac{\sum x_i}{n} \qquad \text{Equation 5}$$

$$SD = \sqrt{\frac{\sum (x - \overline{x})^2}{n-1}} \qquad \text{Equation 6}$$

$$Bias(\%) = \frac{\overline{x}}{x} \times 100 \qquad \text{Equation 7}$$

$$Intra\text{-}day\,run\,(\%CV) = \frac{SD\;for\;single\;run\;of\;samples}{\overline{x}\;for\;single\;run\;of\;samples} \times 100 \qquad \text{Equation 8}$$

$$\text{Inter-day run (\%CV)} = \frac{\text{SD for each concentration over 5 runs}}{\bar{x} \text{ for each concentration}} \times 100$$

Equation 9

Where $\bar{x}$ is mean measured concentration, SD is standard deviation, x is nominal concentration and CV is the co-efficient of variation.

Bias was expressed as a percentage of the nominal concentration. The maximum acceptable bias is ±15% and ±20% at high and low concentrations respectively [274]. Precision was expressed as the co-efficient of variation (%CV). The %CV should not exceed 15% at each concentration [274].

4.2.10   Recovery and Matrix Effects

Recovery can be defined as the percentage of the analyte of interest originally present in the sample that reaches the end of the procedure [275]. The assessment of the matrix effects on ionisation of analytes is an important aspect of validation of LC-MS method due to the susceptibility of the technique to ion enhancement or suppression that could lead to erroneous quantitative results. Several papers describing the evaluation and strategies for reducing matrix effects have been published [271]. Recoveries and matrix effects were measured using the approach of Matuszewski [269].

3 sets of drug-free matrix spiked with analytes at three different concentrations (5, 50 and 100 ng/mL) were prepared in triplicate as follows: neat standard (set 1), drug-free matrix from 10 different sources spiked with mixture solutions 1 and 2 as appropriate after LLE (set 2), and drug-free matrix from 10 different sources spiked with mixture solutions 1 and 2 as appropriate before LLE (set 3).

For the recoveries, internal standards were added after extraction to allow direct comparison with un-extracted standards and recoveries of each compound were investigated using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Peak Area Ratio of Pre-extraction Spike}}{\text{Peak Area Ratio of Post-extraction Spike}} \times 100 \quad \textbf{Equation 10}$$

The recovery of an analyte need not be 100%: a low recovery can be accepted if bias, precision and LLOQ of the method are within the acceptable limits. It has been recommended that the recovery should be reproducible and at least 50% for all concentrations tested. Recovery may be reported higher than 100% that probably indicates the presence of matrix effects, particularly when using LC-MS/MS [271].

Matrix effects of endogenous components were calculated using the Equation 4. Matrix effects are considered acceptable if they do not exceed ±25% in the absence of matrix. Matrix effect values less than 100% indicate ion suppression and values greater than 100% indicate ion enhancement.

4.2.11   Stability

4.2.11.1 Stability as Part of Validation

Analytical challenges might arise from the degradation of drugs and their metabolites which should be considered when planning an analytical strategy. Therefore, the validation of the analytical method should demonstrate the extent to which the analytes in a given matrix under specific conditions are stable for given time intervals in order to ensure accurate quantitative results [285], particularly if no information is available from previous work [274].

Stability studies measure the differences in drug concentration that could be encountered between the time of sampling and the time of analysis [286]. Two concentrations have to be tested at low and high points of the calibration range [286]. The stability of parent SCs in blood and their metabolites in urine were evaluated using three types of stability study;

   A) Benchtop Stability

This refers to the stability of the analyte under the conditions of sample preparation. It should be evaluated to discover if preservatives have to be added to the sample during the preparation in order to prevent degradation of the analytes. Drug-free blood and urine samples were spiked with 10 SCs and 8 metabolites individually at 2 concentrations QC1 and QC2 (5 and 75 ng/mL). To determine time zero concentrations, freshly spiked samples were immediately

extracted and analysed in triplicate. Short-term stability was evaluated for fortified drug-free matrix stored in the dark in glass tubes for 24 hours at room temperature (18 ±1°C). Stability samples were prepared and stored under previous conditions until analysis. On the day of each experiment, samples were extracted and a freshly prepared mixture of internal standard solutions was added to each specimen and processed in triplicate along with freshly spiked calibration standards and then analysed.

B) Freeze-thaw Cycle Stability:

Freeze-thaw cycle stability is also very important because biological samples are often frozen and thawed (e.g. for reanalysis). Drug-free blood and urine samples were spiked with 10 SCs and 8 metabolites individually at 2 concentrations QC1 and QC2 (5 and 75 ng/mL). Short-term stability was evaluated for fortified drug-free matrix after three freeze-thaw cycles at (-20 ±1°C). Samples were left at room temperature to defrost after 24, 48 and 72 hours and analysed in triplicate after the three cycles.

C) In-process Stability:

In-process (autosampler) stability is a critical issue in the case of unexpected delays in analysis (e.g. breakdown of instrument). The stability of analytes was also evaluated in-process stability using reconstituted extracted sample at 24 hours after extraction at approximately 20°C.

Stability of benchtop and in-process were calculated by dividing the calculated concentrations after 24 hours by the initial concentrations of analytes and multiplying by 100. Stability of freeze-thaw cycle was calculated by dividing the calculated concentrations after the three cycles by the initial concentrations of analytes and multiplying by 100. If the bias was ±10% of the time zero concentration, the analyte was considered stable. The experiments were conducted together with the bias and precision experiments.

4.2.11.2 Long Term Stability Study of 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole in Methanol/Acetonitrile Solutions for Up to 3 Months

A long-term stability study was conducted using methanol and acetonitrile spiked with 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole.

12 mL of methanol or acetonitrile was spiked with 5F-AKB-48 (*N*-4 OH pentyl) and separately with 5F-PB-22 3-carboxyindole at a concentration of 80 ng/mL. Each solution was transferred equally to 3 vials and stored under three different conditions; at room temperature (18 ±1°C), in the fridge at (4 ±1°C), and in the freezer at -20 ±1°C until analysis.

After freshly spiking the solutions were analysed in duplicate to establish time zero concentrations. On the day of the experiment, freshly prepared internal standard solution was added to each sample in duplicate along with freshly spiked calibration standards and then analysed. Samples were analysed on the first, third, fifth and seventh day during the first week, then once a week for 12 weeks. Stability was calculated by dividing the calculated concentration at the selected days by the initial concentrations of analytes and multiplying by 100.

4.3 Results and Discussion

4.3.1 Chromatography

Good chromatography of all SCs was achieved using a gradient elution system containing 2 mM ammonium acetate and 0.1% formic acid. The analysis of SCs in both the blood and urine specimens was operated in MRM acquisition mode.

The chromatograms of the 10 parent SCs in whole blood and 8 corresponding metabolites in urine are shown in Figures 4.1 and 4.2, respectively and the chromatograms of the 5 internal standards can be seen in Figure 4.3.
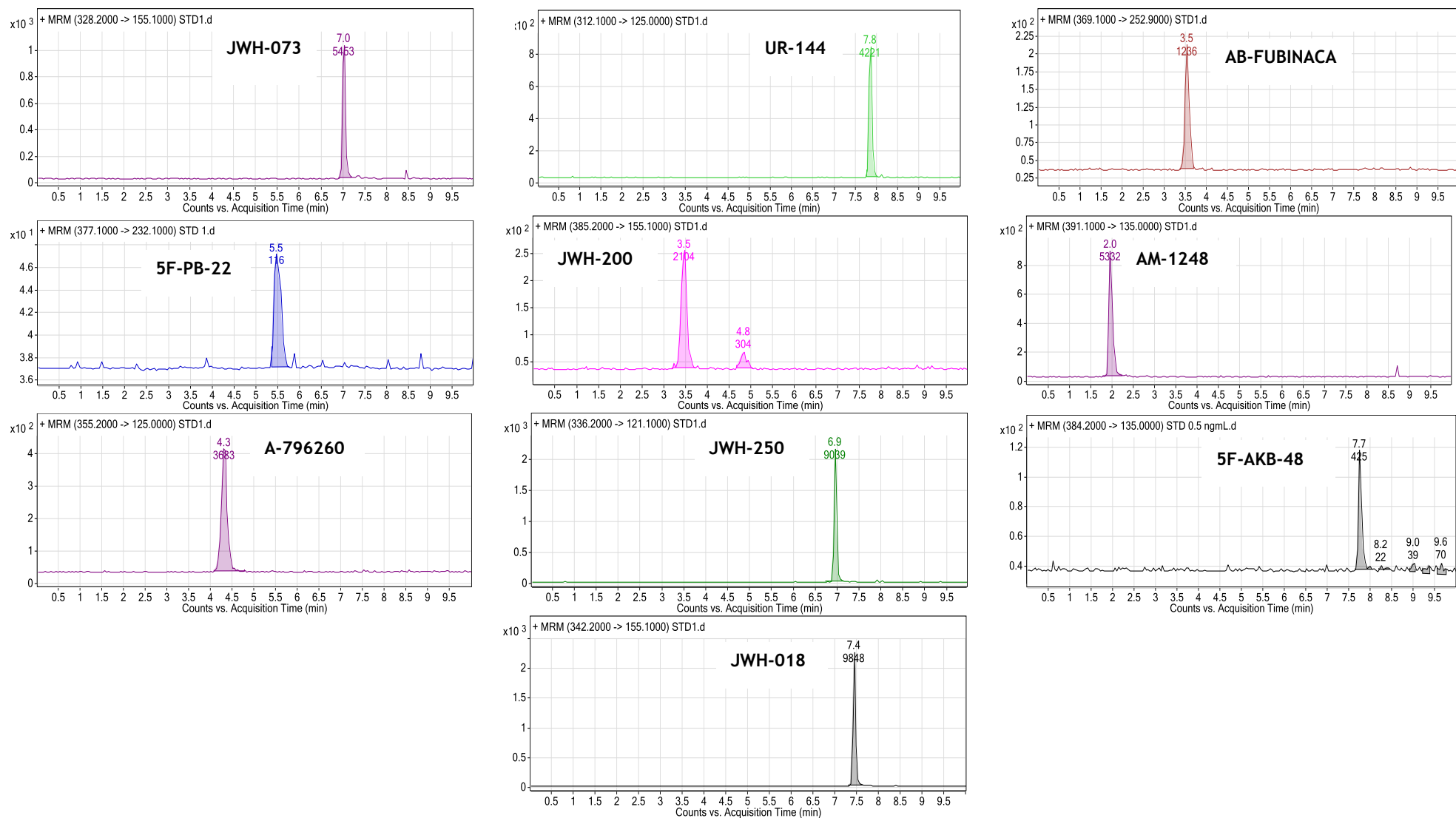
Figure 4.1: Examples of LC-MS/MS Quantifier MRM Chromatograms of Parent SCs in Blood at concentrations of 0.5 ng/mL
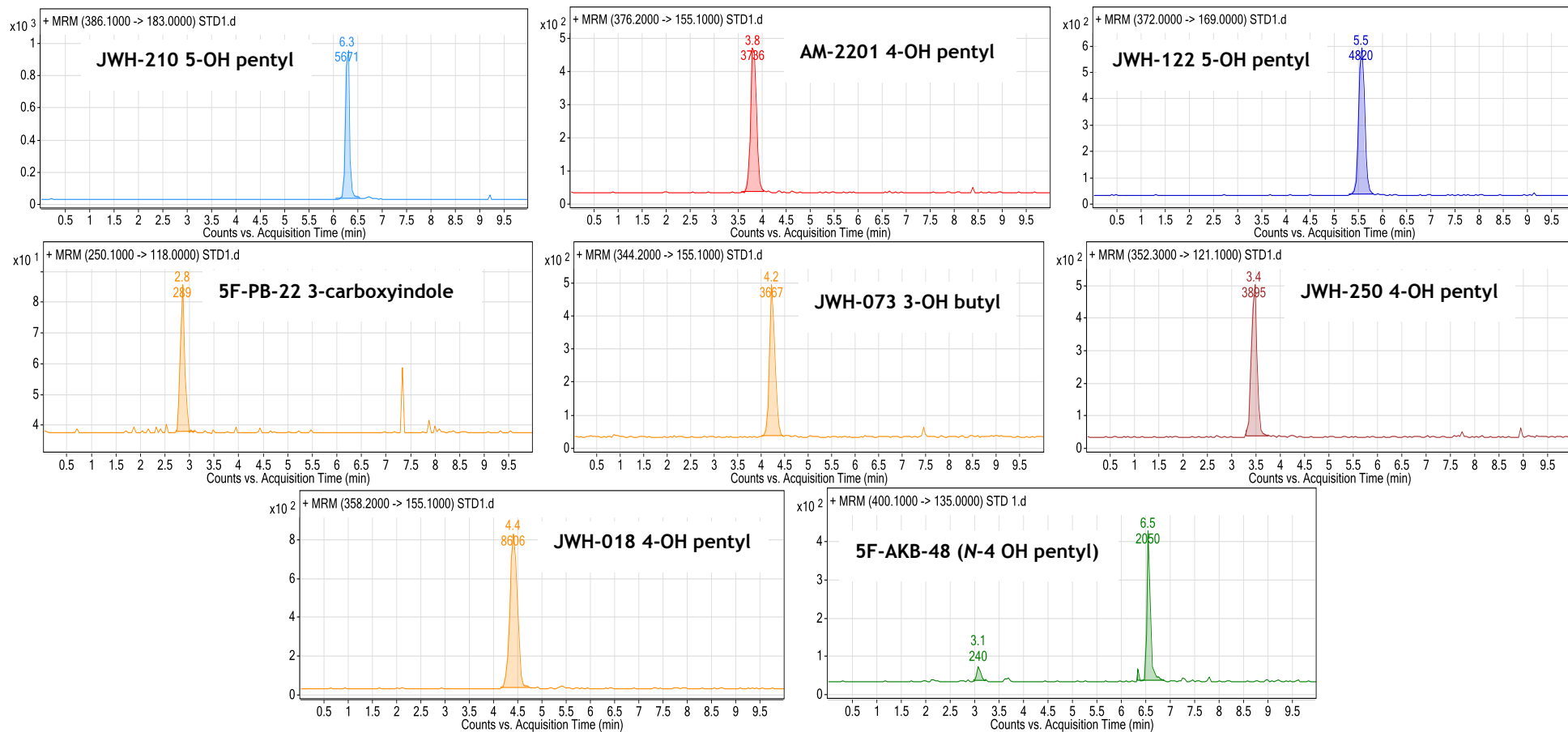
Figure 4.2: Examples of LC-MS/MS Quantifier MRM Chromatograms of SC Metabolites in Urine at concentrations of 1.0 ng/mL
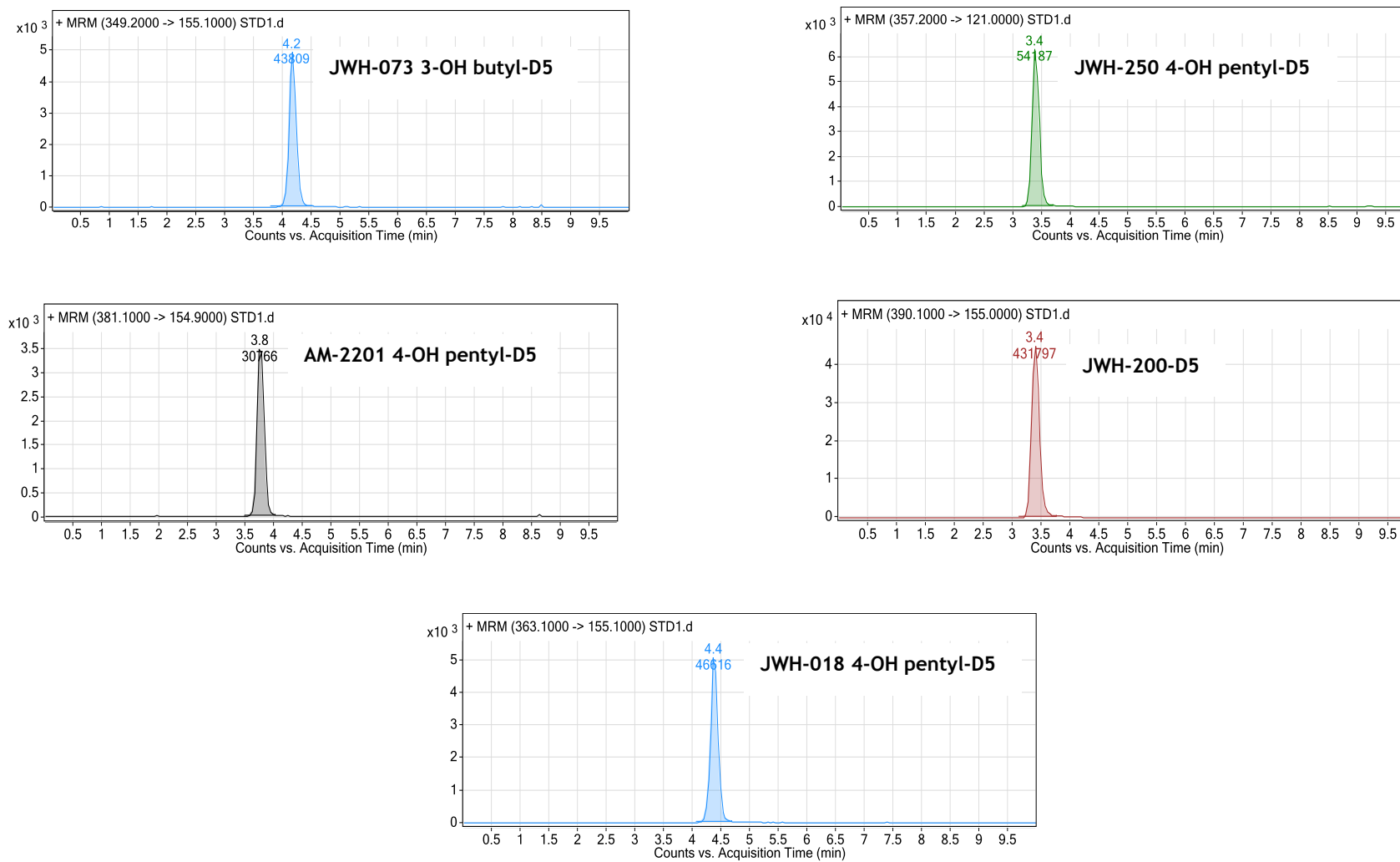
Figure 4.3: Examples of LC-MS/MS Quantifier MRM Chromatograms of Internal Standards of SCs in Blood at concentrations of 50 ng/mL

4.3.2 Limit of Detection and Lower Limit of Quantification

Sensitivity of the method was assessed by determining the LOD and LLOQ instrumentally for each analyte. LODs were determined by lowering the concentrations of calibration standards in blood and urine samples to the range of expected LODs: 0.010, 0.025, 0.050, 0.075, 0.100, 0.250, 0.500 and 0.750 ng/mL.

LOD was determined to be the lowest concentration which meets all identification criteria including quantifier ion peaks greater than 3 times the background noise and presence of qualifier ions at the known retention time. The concentrations ranged from 0.010 to 0.750 ng/mL. LODs were all very low and fitted within concentrations found in blood and urine in case samples from Spice users [3, 85, 146, 154, 256, 259, 260]. The LLOQs were administratively set at 0.5 and 1.0 ng/mL for blood and urine methods respectively. These were the lowest concentrations that met all quantitation acceptance criteria for each analyte of interest. LODs values are shown in Table 4.2 along with LLOQs.

4.3.3 Linearity

The peak area ratios of each SC to its assigned internal standard were calculated, and calibration curves were generated by plotting the peak area ratio against the concentration. The correlation coefficient, R^2, was calculated for each linear regression curve.

The calibration curves were linear for all analytes within the range 0.5 or 1.0–100 ng/mL with R^2 values of 0.996 or higher (Table 4.2). Figure 4.4 illustrates examples of the calibration curves for the parent SCs in blood and their metabolites in urine. In addition, Figure 4.5 shows the residual data for these calibration curves confirming their linearity.

Table 4.2: Experimentally Determined LODs, LLOQs and Correlation Coefficients for SCs in Blood and SC Metabolites in Urine Specimens

Analyte	Internal Standard	LOD (ng/mL)	LLOQ (ng/mL)	Calibration Range (ng/mL)	(R2) (range) (n=5)
Blood					
JWH-018	JWH-018 4-OH pentyl-D5	0.05	0.5	0.5-100	0.997-0.999
JWH-073	JWH-073 3-OH butyl-D5	0.075	0.5	0.5-100	0.998-0.999
JWH-250	JWH-250 4-OH pentyl-D5	0.05	0.5	0.5-100	0.998-0.999
JWH-200	JWH-200-D5	0.1	0.5	0.5-100	0.997-0.999
5F-AKB-48	JWH-250 4-OH pentyl-D5	0.25	0.5	0.5-100	0.997-0.999
5F-PB-22	JWH-250 4-OH pentyl-D5	0.25	0.5	0.5-100	0.997-0.999
AM-1248	AM-2201 4-OH pentyl-D5	0.1	0.5	0.5-100	0.996-0.999
AB-FUBINACA	JWH-200-d5	0.1	0.5	0.5-100	0.997-0.999
A-796260	JWH-250 4-OH pentyl-D5	0.1	0.5	0.5-100	0.997-0.999
UR-144	JWH-250 4-OH pentyl-D5	0.075	0.5	0.5-100	0.996-0.999
Urine					
JWH-018 4-OH pentyl	JWH-018 4-OH pentyl-D5	0.01	1.0	1.0-100	0.998-0.999
JWH-073 3-OH butyl	JWH-073 3-OH butyl-D5	0.05	1.0	1.0-100	0.998-0.999
AM-2201 4-OH pentyl	AM-2201 4-OH pentyl-D5	0.05	1.0	1.0-100	0.998-0.999
JWH-250 4-OH pentyl	JWH-250 4-OH pentyl-D5	0.05	1.0	1.0-100	0.998-0.999
JWH-122 5-OH pentyl	JWH-018 4-OH pentyl-D5	0.075	1.0	1.0-100	0.996-0.999
JWH-210 5-OH pentyl	JWH-250 4-OH pentyl-D5	0.075	1.0	1.0-100	0.996-0.999
5F-AKB-48 (*N*-4 OH pentyl)	JWH-250 4-OH pentyl-D5	0.1	1.0	1.0-100	0.997-0.999
5F-PB-22 3-carboxyindole	JWH-250 4-OH pentyl-D5	0.25	1.0	1.0-100	0.997-0.999
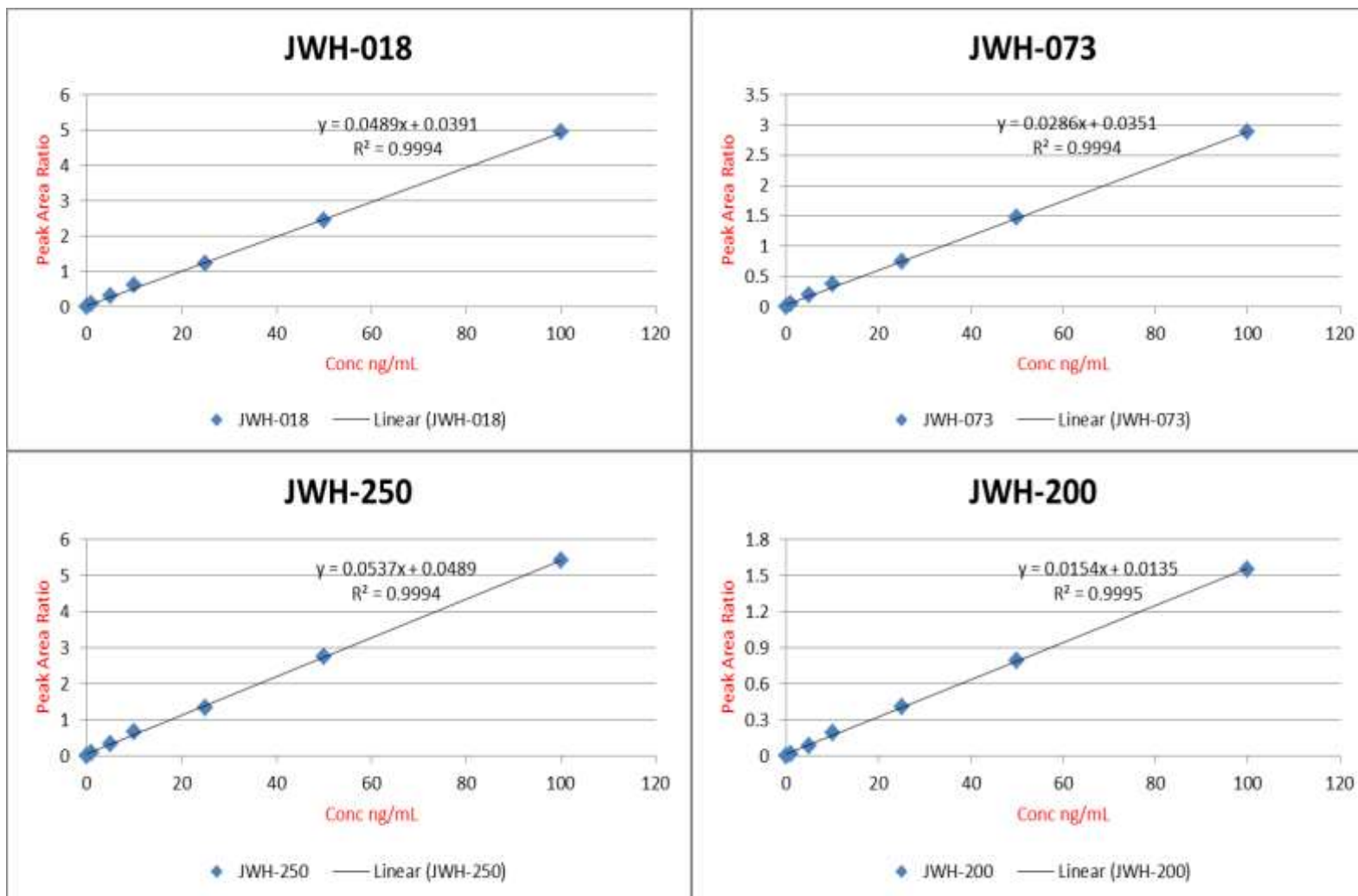
Figure 4.4: Calibration Curves for 10 SCs in Blood and 8 of their Metabolites in Urine
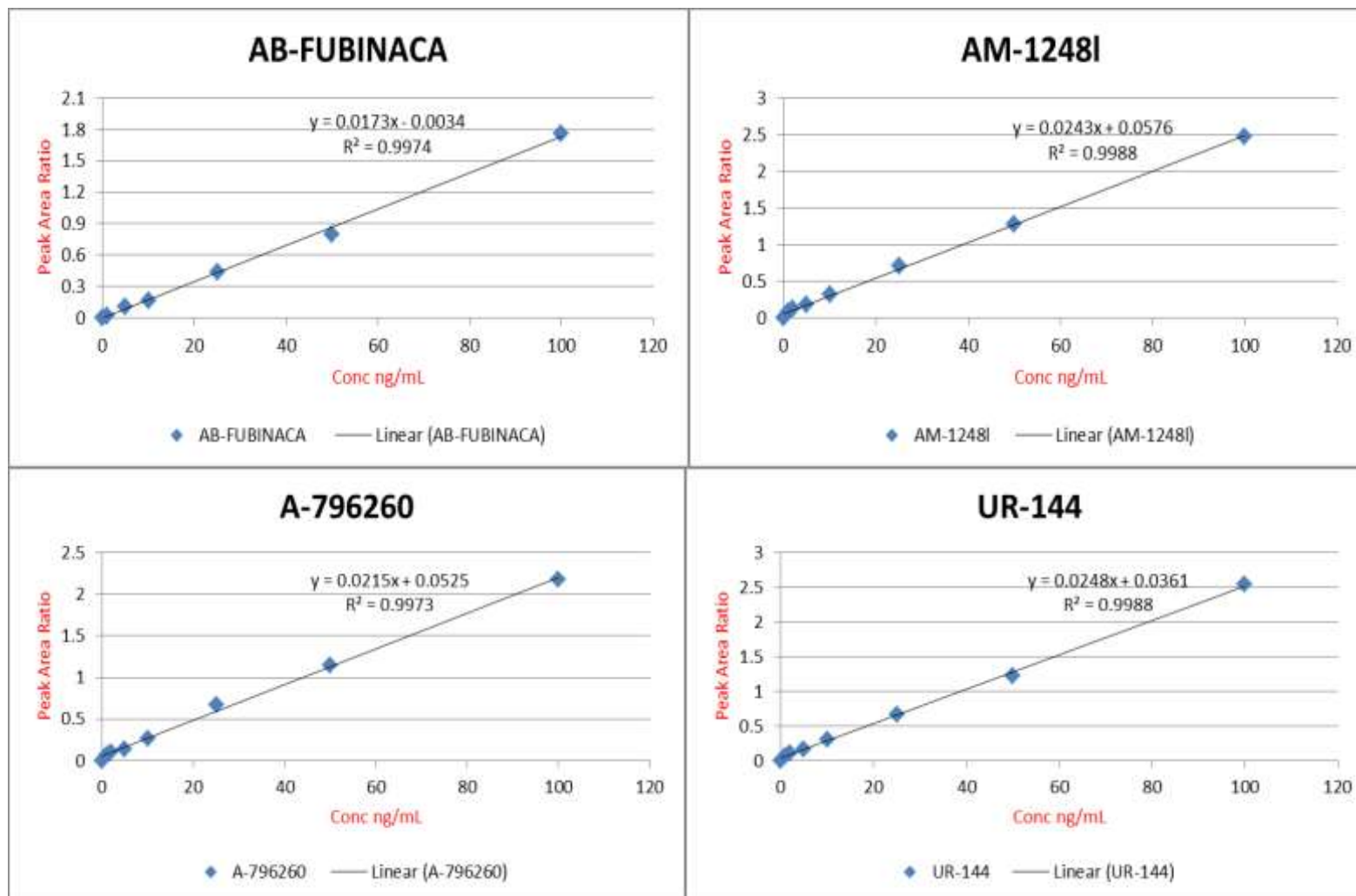
Figure 4.4 (continued): Calibration Curves for 10 SCs in Blood and 8 of their Metabolites in Urine
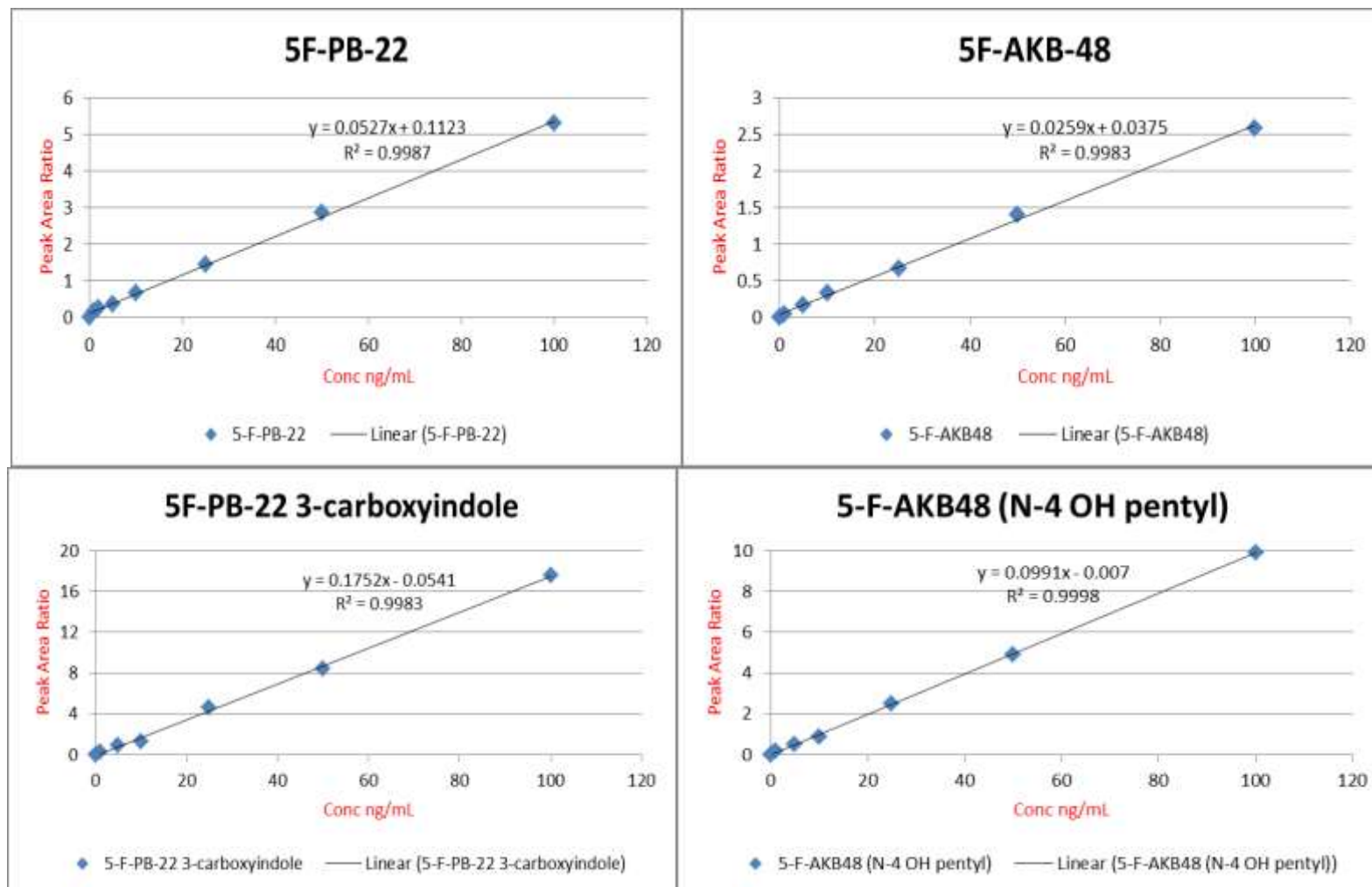
Figure 4.4 (continued): Calibration Curves for 10 SCs in Blood and 8 of their Metabolites in Urine
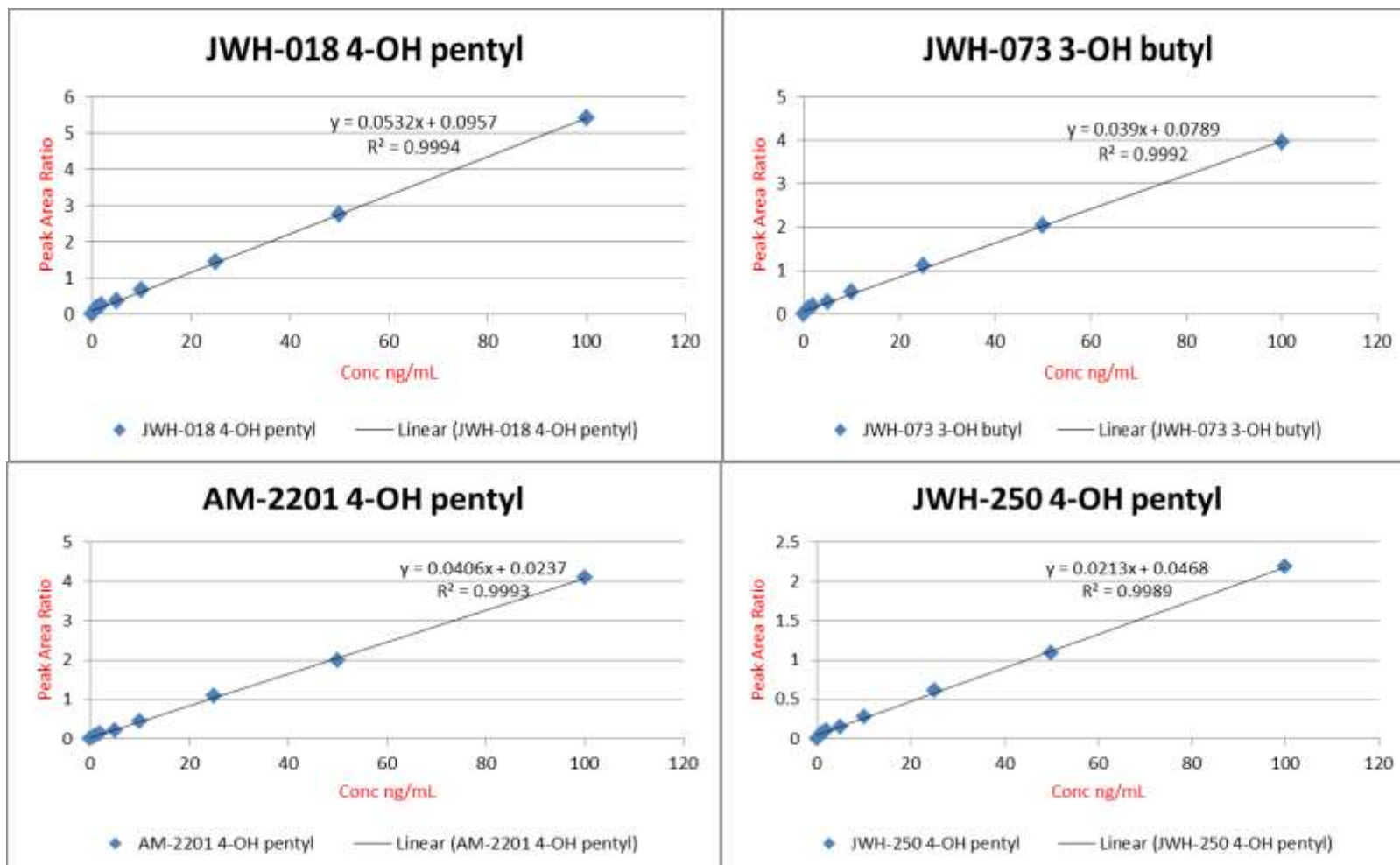
Figure 4.4 (continued): Calibration Curves for 10 SCs in Blood and 8 of their Metabolites in Urine
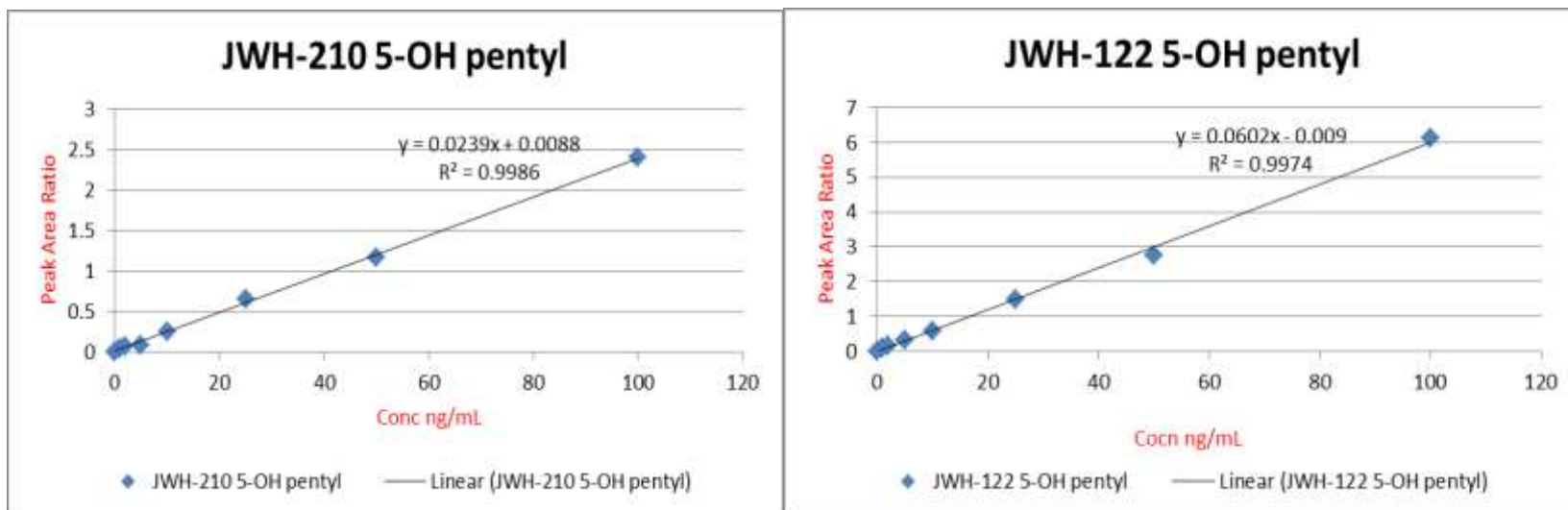
Figure 4.4 (continued): Calibration Curves for 10 SCs in Blood and 8 of their Metabolites in Urine
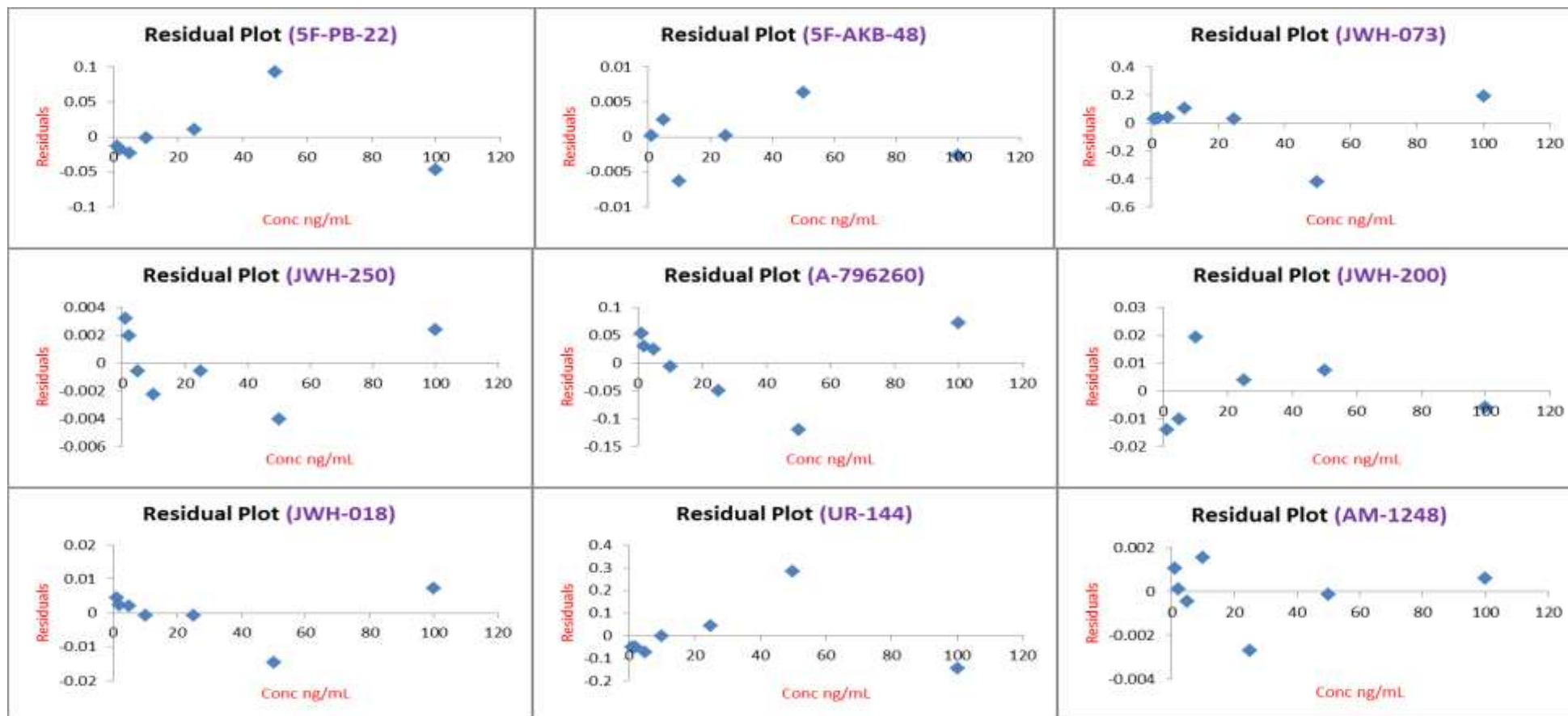
Figure 4.5: Residual Plots for 10 SCs in Blood and 8 of their Metabolites in Urine
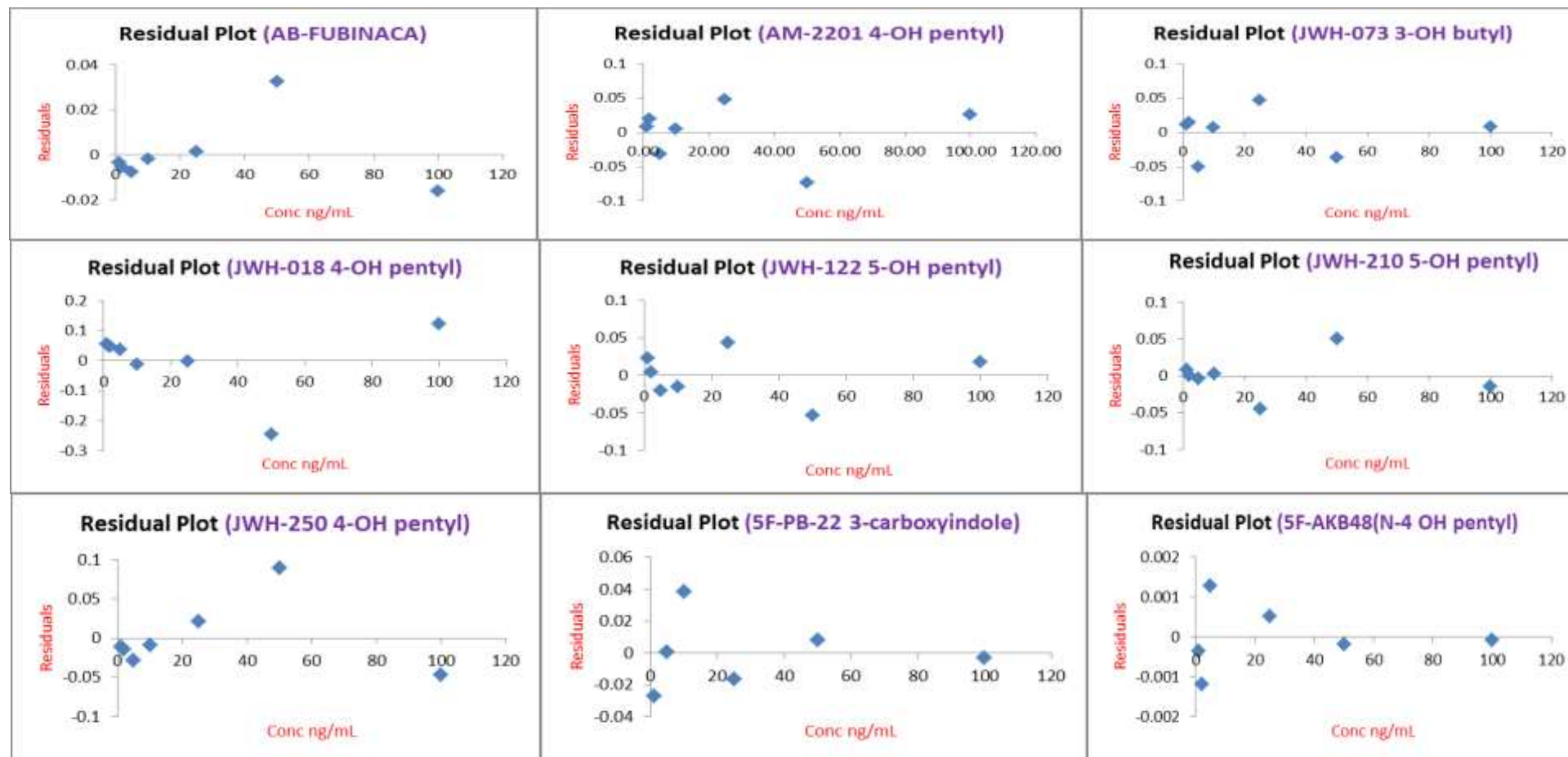
Figure 4.5: (continued) Residual Plots for 10 SCs in Blood and 8 of their Metabolites in Urine

4.3.4 Selectivity and Specificity

Typical endogenous components (e.g. proteins, lipids, minerals, salts and small molecules) are the most abundant source of possible interferants [210]. Selectivity studies were carried out using 10 drug-free sources of blood and urine (each) to be sure no endogenous interference occurred. Specificity studies were also carried out by spiking drug-free matrix with each analyte and internal standard individually. Exogenous interference from other drugs was evaluated by analysing drug-free matrix containing 12 of the most commonly detected drugs in forensic cases (tetrahydrocannabinol, amphetamine, cocaine, benzoylecgonine, anhydroecgonine methyl ester, ecgonine methyl ester, methadone, codeine, morphine, diazepam, gabapentin and mephedrone) at a concentration of 500 ng/mL. All these drugs were separately spiked into drug-free blood and urine and extracted.

Clean base lines with negligible matrix components were found. No interfering peaks were detected from the other common drugs at the retention times of any of the analytes used in both methods.

4.3.5 Carryover

Carryover was evaluated by triplicate injections of drug-free blood and urine extracts after two injections of extracts of blank blood containing parent SCs and drug-free urine containing SC metabolites at the highest concentration of the calibration range (100 ng/mL). No carryover was observed in any of the retention time windows corresponding with the transitions of SC parent compounds or their metabolites.

4.3.6 Bias and Precision

Intra- and inter-day bias and precision were assessed for both methods at three different concentrations (5, 40, and 75 ng/mL). Intra-and inter-day accuracy results for 10 parent SCs in blood and 8 of their metabolites in urine are presented in Table 4.3. Good accuracy was obtained for all analytes within the acceptable range of ±15% of the nominal concentrations. The intra-day accuracy ranged from 90.2-110.6% and 91.9-109.8% for blood and urine respectively. The

inter-day accuracy ranged from 87.1-110.7% and 87.4-108.8% for blood and urine respectively.

Table 4.3: Intra- and Inter-Day Assay Accuracy Results for SCs in Blood (A) and SC Metabolites in Urine (B) at Low (5 ng/mL), Medium (40 ng/mL) and High (100 ng/mL) concentrations

Analyte	Intra-day Accuracy (%)			Inter-day Accuracy (%)		
	Low (*n*=5) Mean	Medium (*n*=5) Mean	High (*n*=5) Mean	Low (*n*=25) Mean	Medium (*n*=25) Mean	High (*n*=25) Mean
(A) Blood						
JWH-018	105.5	94.1	96.8	94.5	93.4	93.6
JWH-073	95.5	96.5	106.1	108.8	106.2	95.7
JWH-250	96.2	99.1	104.2	101.2	103.4	95.4
JWH-200	107.8	105.0	99.5	110.7	105.1	96.3
5F-AKB-48	102.6	96.3	95.1	89.4	92.9	91.7
5F-PB-22	95.8	98.0	101.7	92.7	89.7	88.4
AM-1248	104.2	94.9	108.9	90.5	90.9	95.1
AB-FUBINACA	96.3	97.2	91.2	88.8	91.2	92.6
A-796260	110.6	105.2	92.4	92.4	93.1	94.4
UR-144	90.2	95.7	94.3	87.1	94.5	96.2
(B) Urine						
JWH-018 4-OH pentyl	105.1	101.6	103.0	96.5	99.4	97.8
JWH-073 3-OH butyl	106.2	97.2	99.3	92.8	98.0	97.8
AM-2201 4-OH pentyl	104.0	99.1	95.1	89.9	98.0	94.9
JWH-250 4-OH pentyl	100.1	99.1	91.9	88.2	99.6	97.2
JWH-122 5-OH pentyl	99.5	92.0	96.1	104.6	88.4	95.4
JWH-210 5-OH pentyl	96.6	99.4	102.0	101.4	105.6	87.4
5F-AKB-48 (*N*-4 OH pentyl)	109.8	103.0	96.2	98.1	101.4	98.8
5F-PB-22 3-carboxyindole	94.1	105.0	104.0	108.8	96.3	94.8

Precision values were acceptable and less than 15% for blood and urine methods (Table 4.4). The intra-day precision values were less than 9.5% and 9.0% for blood and urine respectively. The inter-day precision values for both methods were less than or equal to 13.5%.

Table 4.4: Intra- and Inter-Day Assay Precision Results for SCs in Blood (A) and SC Metabolites in Urine (B) at Low (5 ng/mL), Medium (40 ng/mL) and High (100 ng/mL) concentrations

Analyte	Intra-day Precision (%)			Inter-day Precision (%)		
	Low (*n*=5) Mean	Medium (*n*=5) Mean	High (*n*=5) Mean	Low (*n*=25) Mean	Medium (*n*=25) Mean	High (*n*=25) Mean
(A) Blood						
JWH-018	2.5	2.1	1.9	4.6	5.2	3.4
JWH-073	4.5	2.2	4.1	6.2	7.1	6.9
JWH-250	6.2	3.5	3.2	9.8	4.6	5.5
JWH-200	3.7	3.1	2.7	4.9	7.8	6.7
5F-AKB-48	4.7	5.3	4.2	7.7	7.1	6.3
5F-PB-22	9.3	5.8	7.5	12.9	5.1	7.3
AM-1248	3.3	4.6	2.1	9.6	6.6	6.8
AB-FUBINACA	7.6	7.8	5.9	6.4	7.4	8.4
A-796260	5.8	7.1	6.2	13.2	5.1	4.1
UR-144	8.2	5.1	7.2	8.7	8.4	4.7
(B) Urine						
JWH-018 4-OH pentyl	8.9	4.6	4.5	10.2	3.4	4.3
JWH-073 3-OH butyl	7.0	3.6	6.3	9.7	2.4	3.1
AM-2201 4-OH pentyl	3.4	3.3	3.7	10.0	3.9	2.1
JWH-250 4-OH pentyl	3.3	1.0	7.0	7.3	2.4	4.7
JWH-122 5-OH pentyl	6.5	4.1	5.7	13.4	9.4	11.0
JWH-210 5-OH pentyl	6.8	1.9	2.8	4.4	10.8	5.9
5F-AKB-48 (*N*-4 OH pentyl)	3.9	7.8	1.4	12.4	11.5	6.5
5F-PB-22 3-carboxyindole	3.0	1.6	4.7	8.7	5.2	11.5

4.3.7 Recovery and Matrix Effects

The evaluation of recoveries and matrix effects was carried out at three (low, medium and high) concentrations within the linear range, using 10 different sources of urine and 10 different sources of whole blood. Recoveries and matrix effects were measured using the approach of Matuszewski *et al*.[269].

Urine method recoveries meet the validation criteria. They were from 77.4 to 108.1%; while matrix effects were from 81.1 to 108.0% for all analytes of

interest at concentrations of 5, 50 and 100 ng/mL. It is clear that the average value of the ion enhancement or ion suppression of all urinary metabolites investigated in this study were within the accepted limit of the validation procedure (±25%).

For the blood method, the recoveries of parent SCs ranged from 71.1 to 126.3% and matrix effects were from 75.0 to 90.4% at concentrations of 5, 50 and 100 ng/mL (except for UR-144 (48.9%-51.5%) and JWH-200 (136.3%-138.1%)). It was clear that UR-144 and JWH-200 both had unacceptable matrix effects when extracted from blood, despite the compensating effects of deuteron-labelled internal stanards. One potential reason for this unacceptable ion suppression of UR-144 and ion enhancement of JWH-200 might be because UR-144 is a late-eluting compound. It was found that late-eluting interferents can appear in subsequent runs, leading to ion suppression [287]. This could arise from cholesterols, phospholipids or column components that elute during the later portion at the same time as these analytes [287]. It was very difficult to determine the actual reason for the unacceptable ion enhancement of JWH-200. However, the basicity of the morpholine ring of its structure may contribute in this issue [89].

Results for recoveries and matrix effects are presented in Table 4.5.

4.3.8 Stability

Drugs can break down in a biological specimen even after collection to form other compounds [286]. Breakdown will lead to a reduction in the concentration of the original drug and an increase in the concentrations of the breakdown/instability products. Breakdown involves enzymatic or chemical processes (e.g. oxidation, hydrolysis and de-esterification), typically in blood. In some extreme circumstances, the original drug can completely disappear. The stability of parent SCs in blood and their metabolites in urine was evaluated under various conditions.

Table 4.5: Recoveries and Matrix Effects for SCs in Blood (A) and SC Metabolites in Urine (B) Using 3 Different Concentrations (5, 50 and 100 ng/mL) and 10 Different Drug-free Matrices (*n*=5 per Concentrations per Matrix).

Analyte	Recovery (%)			Matrix effect (%)		
	Low (*n*=5) Mean	Medium (*n*=5) Mean	High (*n*=5) Mean	Low (*n*=5) Mean	Medium (*n*=5) Mean	High (*n*=5) Mean
(A) Blood						
JWH-018	75.5±4	76.9±4	74.5±3	77.5±2	75.0±3	76.8±3
JWH-073	84.1±3	83.2±3	84.4±3	79.9±3	80.0±2	82.7±2
JWH-250	100.5±4	97.5±3	97.9±2	86.1±4	84.9±4	87.2±3
JWH-200	72.7±5	71.1±4	74.2±4	137.4±7	136.3±4	138.1±7
5F-AKB-48	118.9±3	120.1±2	122.1±2	75.5±3	77.2±2	77.7±2
5F-PB-22	105.2±2	100.0±3	101.1±2	84.9±3	85.1±3	84.4±4
AM-1248	80.1±3	79.5±3	84.2±3	86.2±4	85.2±3	84.2±3
AB-FUBINACA	78.1±3	81.5±4	81.3±4	84.7±5	83.3±3	90.4±4
A-796260	84.4±3	79.5±3	77.4±4	84.4±3	89.5±4	88.0±3
UR-144	124.2±5	126.3±5	120.4±3	50.9±7	51.5±5	48.9±6
(B) Urine						
JWH-018 4-OH pentyl	86.7±3	84.4±2	100.1±2	82.6±2	91.9±3	93.0±2
JWH-073 3-OH butyl	93.7±2	95.2±3	108.1±3	84.8±3	94.2±2	88.2±2
AM-2201 4-OH pentyl	89.5±2	80.6±2	100.7±2	81.9±3	83.4±2	90.1±3
JWH-250 4-OH pentyl	87.1±2	91.8±3	107.9±3	81.8±3	91.4±3	94.2±3
JWH-122 5-OH pentyl	81.2±4	96.7±5	100.4±3	81.2±3	86.8±3	84.0±4
JWH-210 5-OH pentyl	79.3±4	79.2±3	86.7±4	93.2±4	81.1±3	90.3±3
5F-AKB-48 (*N*-4 OH pentyl)	89.1±3	80.8±2	93.1±4	89.7±2	93.1±2	84.5±2
5F-PB-22 3-carboxyindole	77.4±4	83.1±4	82.3±3	87.7±3	108.0±4	89.7±3

4.3.8.1   Benchtop Stability

For the urine, the stability results were within the acceptance criteria of ±10%, which means that all metabolites of SCs investigated in this study are considered stable in urine at room temperature (18°C) for up to 24 hours. For blood, all parent SCs were stable at room temperature for 24 hours except UR-144 and 5F-PB-22, which showed degradation of 26.9 and 33.6% of their nominal concentration, respectively (Table 4.6).

UR-144 has a tetramethylcyclopropyl group containing ring strain, which is a kind of instability that occurs when bonds in a molecule form angles that are abnormal, and hence it is prone to ring-opening (i.e. degradation) [200]. 5F-PB-22 was reported to degrade significantly due to the instability of the ester bond in its structure [88]. Ester groups are reactive and easily hydrolysed. Many parameters can contribute in this issue such as enzymatic processes in blood or reactions during sample preparation [88]. Peters *et al.* [288] reported that most drugs are probably stable in biological fluids when they are kept in appropriate storage conditions. However, some drugs which contain ester bonds are usually unstable in body fluids.

4.3.8.2   Freeze-thaw Cycle Stability

All parent SCs in blood and metabolites in urine were stable after being subjected to three freeze-thaw cycles (from -20°C to room temperature) (Table 4.6).

4.3.8.3   In-Process Stability

Analyte autosampler stability was assessed by re-injecting extracted low and high QC specimens in triplicate after 24 hours, and comparing with the time zero concentrations. Extracts of blood and urine samples were stored on the autosampler until the next analysis. In urine extracts, no significant degradation of the analytes was observed in the autosampler (at a room temperature of approximately 20°C) for up to 24 hours. Although UR-144 and 5F-PB-22 were not stable in whole blood their stability was acceptable after extraction and reconstitution in 200 µL of 50:50 mobile phase mixture.

Generally, the decrease in concentration is dependent on many factors including solvents, compounds, concentrations, the sample matrices and storage temperature.

Table 4.6 summarises the stability results.

Table 4.6: Stability of Blood (A) and Urine (B) Analytes

Analyte	24 h Room Temperature (%)		Freeze- Thaw Cycles (%)		24 h Autosampler Stability (%)	
	Low (*n*=3) Mean	High (*n*=3) Mean	Low (*n*=3) Mean	High (*n*=3) Mean	Low (*n*=3) Mean	High (*n*=3) Mean
(A) Blood						
JWH-018	92.3±4	91.5±3	95.5±4	93.7±2	91.2±4	93.1±2
JWH-073	90.1±4	90.5±4	96.1±3	95.1±4	93.5±3	92.8±4
JWH-250	90.9±3	91.1±4	97.8±3	94.6±3	95.2±4	96.0±3
JWH-200	94.0±5	94.9±3	101.2±4	105.1±3	96.1±4	92.1±3
5F-AKB-48	93.2±2	91.4±2	94.8±3	95.4±3	92.2±4	91.5±3
5F-PB-22	**70.5**±4	**73.1**±6	92.4±5	90.4±4	93.2±3	91.4±5
AM-1248	92.4±3	92.0±4	93.2±4	97.8±5	91.2±5	94.7±5
AB-FUBINACA	92.4±4	94.4±3	98.1±3	100.5±3	92.4±6	95.1±4
A-796260	95.5±6	93.5±5	97.9±5	96.4±3	92.2±7	91.9±4
UR-144	**68.4**±5	**66.4**±3	92.4±3	91.7±3	91.6±4	90.4±5
(B) Urine						
JWH-018 4-OH pentyl	99.4±3	99.2±2	105.8±3	100.4±2	98.2±2	103.9±2
JWH-073 3-OH butyl	96.2±4	89.8±2	96.3±3	93.6±3	97.6±4	96.6±4
AM-2201 4-OH pentyl	88.2±2	91.9±2	100.7±3	89.5±2	94.9±3	100.8±3
JWH-250 4-OH pentyl	100.8±3	94.5±3	93.6±4	90.8±3	101.0±2	99.1±3
JWH-122 5-OH pentyl	92.6±3	88.6±2	89.6±3	91.9±3	96.2±2	94.2±2
JWH-210 5-OH pentyl	94.6±4	88.3±5	94.5±4	90.3±3	95.8±5	95.0±3
5F-AKB-48 (*N*-4 OH pentyl)	91.2±3	88.8±3	92.2±4	92.5±3	98.5±4	97.1±4
5F-PB-22 3-carboxyindole	90.5±4	88.4±3	94.5±3	91.4±3	92.9±3	94.1±3

4.3.8.4   Long Term Stability Study of 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole in Methanol/Acetonitrile Solutions for Up to 3 Months

This study showed good stability for 5F-AKB-48 (*N*-4 OH pentyl) in both acetonitrile and methanol stock solutions under three different conditions; at room temperature (18±1ºC), in the fridge at (4±1ºC), and in the freezer at -20±1ºC for 3 months.

5F-PB-22 3-carboxyindole exhibited good stability in acetonitrile stock solutions under all storage conditions during the investigated time frame (Figure 4.6), whereas in methanol stock solutions lost 9% of its original concentration by the end of the 10th week at room temperature (Figure 4.7). 5F-PB-22 3-carboxyindole may be more susceptible to instability than 5F-AKB-48 (*N*-4 OH pentyl) which can be improved by using acetonitrile as a solvent.
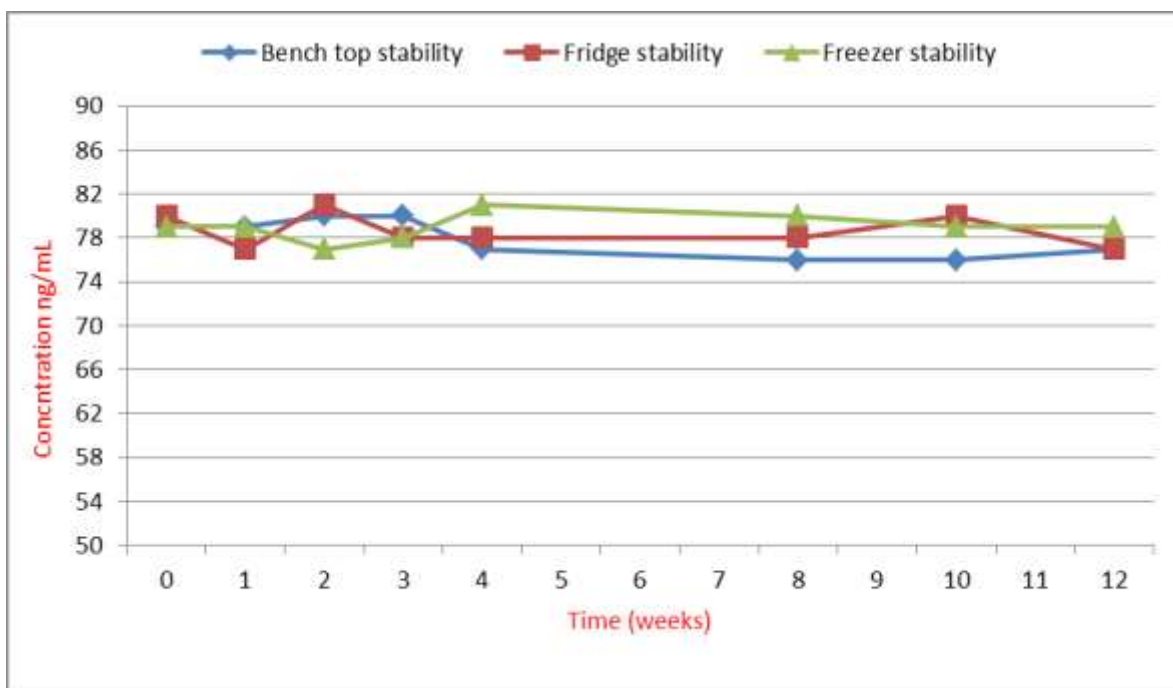
Figure 4.6: Stability of 5F-PB-22 3-carboxyindole in ACN Stock Solution
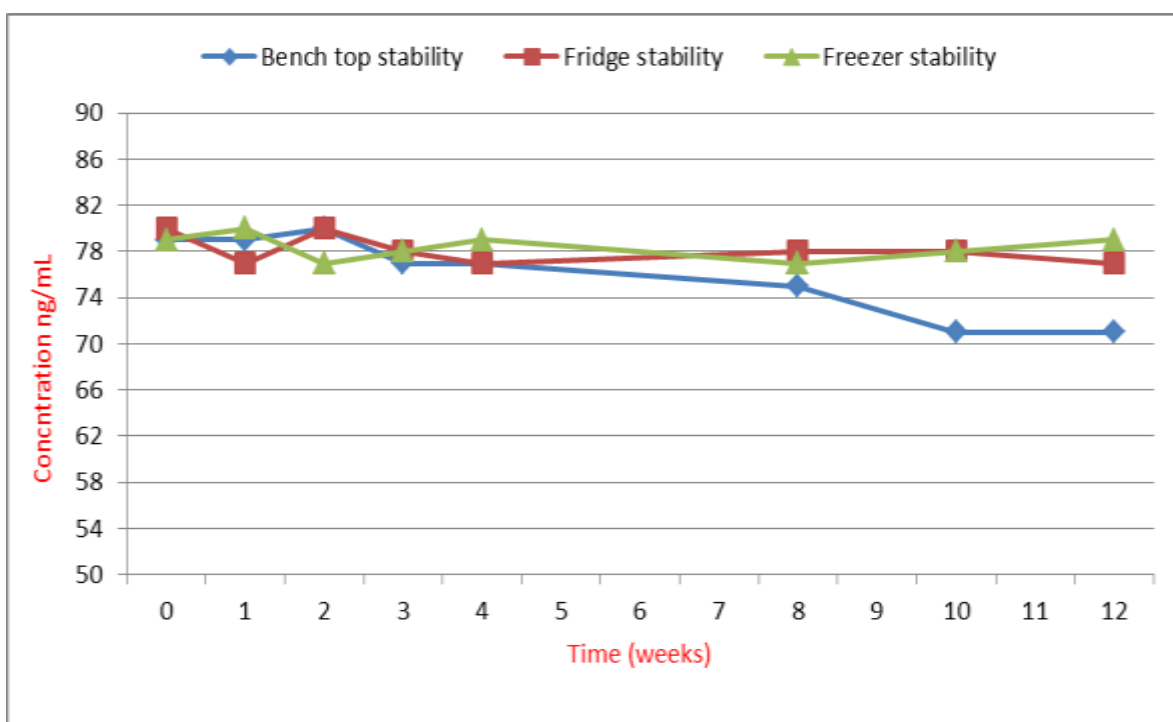
Figure 4.7: Stability of 5F-PB-22 3-carboxyindole in MeOH Stock Solution

4.4 Conclusions

The LC-MS/MS method developed has been validated according to SWGTOX Standard Practices for Method Validation in Forensic Toxicology [269] for the simultaneous determination of 10 SCs in whole blood and 8 corresponding metabolites in urine. All compounds were successfully extracted using LLE and achieved recoveries greater than 71.1% and 77.4% for all drugs in blood and urine respectively. The method was developed for a small sample volume (0.5 mL) and achieved acceptable matrix effects (within ±25%) for all drugs (except UR-144 and JWH-200). Therefore, the method is invalid for the quantitation for these two drugs. The present study suggests that the difference in chemical structure of SCs produced the variation in recoveries with the LLE.

Although a large number of analytes were included in the blood and urine methods, acceptance criteria for linearity, accuracy, precision, and recovery were achieved for all analytes. There were no endogenous or exogenous interferences which produced false-positive results for any of the analytes of interest. Since all analytes showed satisfactory stability at −20°C, it is highly recommended to store samples in freezer as well as to transport samples frozen to avoid any significant decrease in concentration of analytes of interest during transportation. The influence of storage methods on the stability of 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole in methanol and acetonitrile stock solutions was evaluated and found 5F-PB-22 3-carboxyindole lost about 9% in methanol solution after 10 weeks at room temperature.

Implementation of both methods can provide significant information for interpreting the forensic toxicological results for the toxicologist. The analytical results from urine can be used to indicate past exposure to SCs and may provide information concerning SCs consumed. In contrast, the blood results can identify the specific parent SCs used and blood concentrations are very useful in estimating the levels of impairment based on pharmacological activity. The analytical results from blood are critical in post-mortem investigations.

Simultaneous methods can be a great advantage for forensic toxicology work where saving of effort, time and consumables are very important to obtain targets and meet deadlines.

Chapter 5 - Synthetic Cannabinoids: Case Samples

Prevalence studies of illicit drug use are most often performed by collecting self-reported data. However, these data usually suffer from several limitations. Use of self-reporting alone reduces the reliability of a prevalence study. In addition, the information obtained from a survey is usually insufficient to achieve all the aims of most studies. For example, it is very difficult to know the type of SCs that have been ingested. The type(s) of SCs and the concentration(s) in the body can be obtained by analysis of biological specimens. This information is very useful to the forensic community in different aspects. It may assist clinicians in Emergency Departments in toxidrome cases and evaluating their service. It also may help to understand the trend of SC use in order to address their sale and supply. In addition, it may help to order the priorities for national programmes concerning this issue. For these reasons the analysis of biological specimens in prevalence studies of illicit drug use has increased during the last decades, either replacing the use of questionnaires/interviews, or used as a supplement [289].

Analysis of biological matrices is a necessary part of forensic toxicology to decide whether an individual has been under the influence of any substance during a particular event that might have influenced his or her behaviour (e.g. medico–legal cases). In this project, blood and/or urine samples were used for the prevalence studies of SC use in Scotland and Saudi Arabia.

5.1   Synthetic Cannabinoids in Scotland: Case Samples

5.1.1 Introduction

NPS including SCs are mainly produced in China and India and distributed to the European networks for ongoing supply to Scotland [290]. Measuring the scale of the drug market in Scotland is difficult due to its illicit nature. The evidence base on NPS including SCs, in respect of prevalence, is relatively scarce compared to well-established drugs (e.g heroin) in Scotland [291]. However, existing evidence suggests that use of NPS including SCs is relatively low among the general adult population in Scotland compared with use of traditional illicit drugs [291].

The prevalence of SCs in Scotland in earlier studies has been based on self-report. Currently, there is limited epidemiological research on SC use in Scotland. The Scottish Schools Adolescent Lifestyle and Substance Use Survey (SALSUS) conducted a survey study targeting pupils in Secondary Schools, Years 2 and 4, in 2011 and found approximately 1 and 2% of these 13- and 15-year-olds respectively, had used any NPS [292]. In the most recent SALSUS survey in 2013, 4% of 15-year-olds reported having used one or more NPS at least once in their lifetime: the most commonly used NPS were mephedrone, SCs and salvia [165]. The prevalence of these new drugs among 15-year-olds seems to be an upward trend. Among 13-year-olds, less than 1% reported ever using one or more of the NPS, with the most commonly used NPS in this age group being SCs [165].

According to the 2012/13 Scottish Crime and Justice Survey (SCJS), in 2013, 0.5% of all adults reported using any NPS in Scotland in the year prior to the survey (conducted between April 2012 and March 2013); NPS included in the survey were SCs, khat, salvia divinorum, benzylpiperazine (BZP) and *gamma*-butyrolactone (GBL) [293]. This compares to 5.1% for cannabis, 1.7% for cocaine, and 1.3% for ecstasy. However, they found NPS use to be more prevalent in the 16-24 age group than those aged 25-44 [291]. In 2015, Edinburgh was branded "legal high capital of the UK", according to Members of the Scottish Parliament (MSPs), with approximately 15 head shops selling NPS including SC products across the city [294]. The above studies gave an indication of the prevalence of NPS-use including SCs in Scotland. However, beyond these statistics, we know very little about NPS and SCs. SALSUS is a survey of school pupils, while SCJS targets private households. Those with precarious living arrangements and/or chaotic drug use are likely to be under-represented in these figures [291]. In addition, the reliability of data is problematic due to the illicit nature of drug abuse. For these reasons, they are more an indication of scale rather than true prevalence.

Although the scale of NPS-use in the general population in Scotland appears to be low, the picture is less clear within some subgroups of the general population (e.g. A&E patients prisoners) [290, 291]. In addition, the internet enables NPS distributors to reach new consumers directly, across borders. In 2013, the EMCDDA identified 651 online stores selling NPS including SC products across the EU [295]. The proliferation of online retailers and head shops has facilitated the

emergence of a range of SCs in Scotland. The diversity in the NPS market might cause variation between user groups along this variable.

In response to the NPS 'phenomenon' and to tackle the sale and supply of NPS in Scotland, the Scottish Government established an Expert Review Group in order to review the powers which were currently available. In 2014, they also established an NPS Evidence Group to address existing gaps in knowledge relating to NPS, which included improving information sharing on NPS between different agencies and addressing the sale and supply of NPS [296].

Police Scotland works with EU and UK partners in law enforcement to address the supply sources and transit countries of NPS [290]. Police Scotland also works in partnership with the local communities they serve to monitor and share information about NPS to restrict their supply. Police Scotland's 'day of action on NPS' on 22 August 2014 identified 37 different chemical substances present across hundreds of NPS packets across the country, the majority of these being SCs [290]. On November 2015, Edinburgh City Council seized over £50,000 worth of NPS including SC products as part of a major crackdown on legal highs [297].

The Scottish Drug Forum in Glasgow and Crew in Edinburgh provide up-to-date information on drugs and aim to raise awareness and improve understanding of drug issues. They have raised concerns regarding NPS including SCs misused on the street [52]. Stakeholders across Scotland have raised concerns about the use of NPS and SCs, especially among injecting drug users, adults with mental health issues and vulnerable young people [291]. These groups are unlikely to be captured by targeted online surveys or by national household or school surveys.

In order to evaluate the emerging drugs of SCs in Scotland, a study was carried out among 3 different subpopulations, A&E patients, prisoners and fatalities, to give a better picture of this new phenomenon. Analytical investigations of SCs within these 'at-risk' groups can help in outlining the scale of the problem that is critical to prioritising resources around harm treatment and prevention.

5.1.2 Accident and Emergency Samples, Spring 2014

5.1.2.1   Introduction

Many studies [298-304] have shown that illicit drugs are often found in injury patients in A&E, which makes problematic drug use a significant burden on health organisations. Few prevalence studies of illicit drug use either by blood and urine toxicology or by self-report have been carried out among A&E patients. These studies have also demonstrated large variations in prevalence rates that could be explained by the measures used, time lapse between A&E visits, period of data collection, age range, locale and/or country of research and types of drugs tested [298]. According to these studies, the most prevalent traditional illicit drugs in the USA, Norway and the UK were cannabis, cocaine, opiates and amphetamines. Some studies show a relationship between a positive screen for illicit drug use and demographic characteristics of emergency room patients. For example, Lindenbaum *et al.* [304] found that drug tests were most likely to be positive for males, and in persons aged 21–30 years.

The prevalence of alcohol and drug use among A&E patients was well investigated by blood and urine toxicology and self-report studies. In contrast, NPS including SCs have been the subject of very limited studies. However, there is increasing evidence of the role that NPS play in hospital emergencies in Europe [295]. In July 2015, over 200 persons were hospitalised in Poland following use of "MOCARZ". Some of these cases were in a very serious state of health and one fatal case has been reported [305]. The forensic examination of samples of MOCARZ revealed the presence of SC compounds (e.g. 5F-PB-22). Heath *et al.* [145] reported 2 cases of acute intoxication caused by a SCs in two adolescents presenting to the A&E.

The prevalence of SC use among A&E patients based on self-reporting was evaluated by the Global Drug Survey (GDS). The annual GDS survey runs in November each year for approximately 6 weeks. GDS2014 [306] revealed that SCs were more likely to leave people needing Accident and Emergency Medical Treatment (A&E MT) than any other drug group explored in 2014. They found that compared to traditional cannabis, the use of SC products is associated with a 30 times higher risk of seeking A&E MT. The rates of seeking A&E MT for drugs

of abuse varied widely; in the recent survey GDS2015 [307] which was conducted during November/December 2015 with over 100,000 people, 3.5% of this world population had sought A&E MT following the use of SCs in the last 12 months (Figure 5.1).
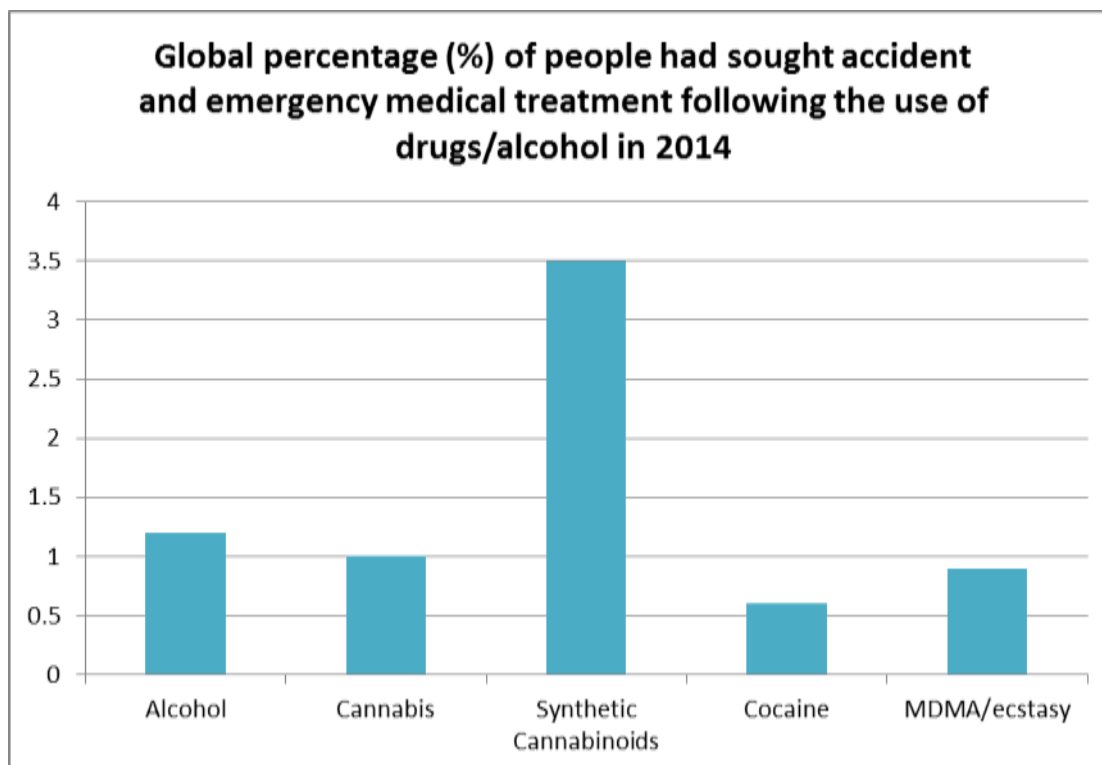
Figure 5.1: Global Percentage of Accident and Emergency Attendees who had Sought Medical Treatment Following the Use of Drugs/alcohol in 2014 (Source of Information: GDS)

Very little medical literature currently exists studying the prevalence of SC use among A&E patients based on the analysis of biological samples. In a recent study, Helander *et al*. [308] monitored the occurrence and trends of NPS including SCs among emergency room patients in Sweden based on blood and urine toxicology. Besides traditional substances such as amphetamines, cannabis and ethanol, many NPS were detected including SCs, synthetic cathinones, ketamine and related substances, piperazines, GABA analogues, opioid-related substances, substituted phenethylamines and hallucinogenic tryptamines. They observed widespread use of many different NPS by mainly adolescents and young adults.

In Scotland, there is lack of prevalence studies among these patients. In order to evaluate the prevalence of SCs among A&E patients in Scotland, a study was carried out in collaboration with Glasgow Royal Infirmary. The present study is

the first prevalence study to be carried out among emergency room patients in Scotland to assess the prevalence of SCs using urine analysis to confirm drug consumption.

5.1.2.2   Aim

The aim of this study was to assess the occurrence and trends of SCs in patients presenting to A&E with suspected acute intoxication. The study took place over a 3 month period (01/05/2014 to 29/07/2014). The prospective study was performed in collaboration with a large emergency department at Glasgow Royal Infirmary, which has approximately 86,000 attendances each year.

5.1.2.3   Ethical Considerations

Advice was obtained from the West of Scotland Research Ethics Committee who considered the study a "service evaluation" " i.e. evaluating the A&E service. All results were reported to the A&E clinician so notes could be put on the patient's record. Consent was waived as the study was considered a service development study and achievement of standard of care. Patients under 16 were excluded from the study.

5.1.2.4   Study Design

Participants were all A&E patients who were suspected of acute intoxication due to drugs either from the case history of the patient or from clinical observations. Urine samples were initially collected by Glasgow Royal Infirmary and stored in additive-free containers. Patient's data and urine samples were encoded with a unique code number to protect the patient's confidentiality. Data analysis was performed using Excel (Microsoft 2010).

On receipt at Forensic Medicine and Science, urine samples were stored at -20°C until analysis. The samples were screened for NPS (SCs were not included) by Lowe *et al.* [309] using LC-HR/MS. The samples were then analysed for of the presence of SCs using the present method.

5.1.2.5   Results

A total of 93 urine samples was collected from 80 patients presenting in the A&E. Case histories, which included gender, age, prescribed drugs, substance(s) used (self-report) and information on whether they were subsequently admitted to hospital or discharged from the A&E, were available for only 75 patients. Forty-nine per cent (37) of the patients were referred by ambulance, 32% (24) by police and 19% (14) by self-referral. 36% (27) of patients required hospital admission whereas 64% (48) were discharged from the emergency department. Seventy-two per cent (54) of the patients were male aged 17 to 55 (median 23) and (28%) 21 were female aged 16 to 47 (median 20). In only 5% (4) of these cases was there any evidence of SC use. They all tested positive for 5F-PB-22 3-carboxyindole with concentrations of 7, 24, 60 and 268 ng/mL. Figure 5.2 shows the MRM ion chromatograms for an authentic urine specimen containing 60 ng/mL 5F-PB-22 3-carboxyindole and its internal standard.
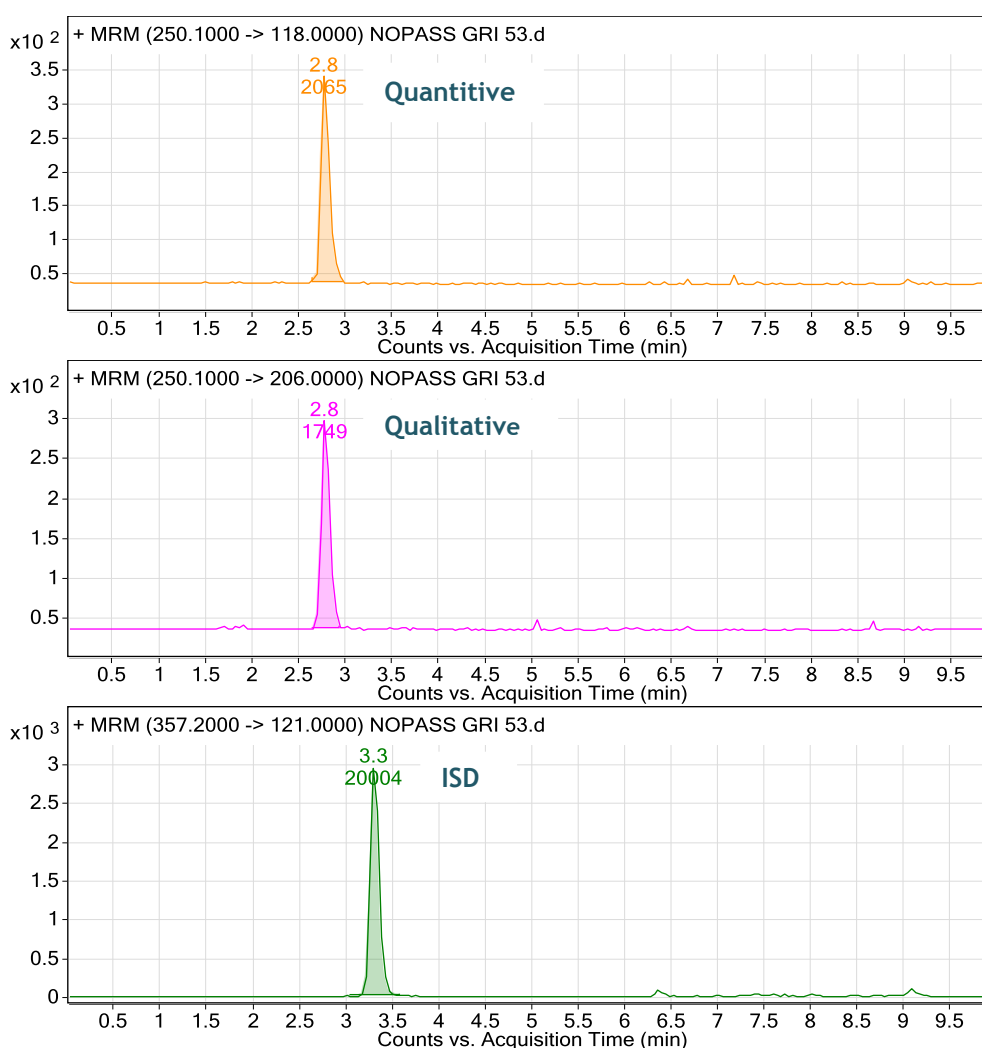
Figure 5.2: LC-MS/MS MRM Chromatograms for 5F-PB-22 3-carboxyindole in an A&E Case Urine Sample (60 ng/mL), and its Internal Standard

Three patients had reported consumption of an NPS (referred to as a legal high in their own terms). Table 5.1 demonstrates the demographic and toxicological data for these cases.

Table 5.1: Demographic and Toxicological Data for 4 SC Metabolite Positive Cases

Case	Sex	Age	Positive SCs	Other Confirmed Drugs*	Conc. ng/mL
1	F	20	5F-PB-22 3-carboxyindole	Citalopram, Methedrone	7
2	F	29	5F-PB-22 3-carboxyindole	-	24
3	F	19	5F-PB-22 3-carboxyindole	-	60
4	M	40	5F-PB-22 3-carboxyindole	Mirtazapine, Methadone, Diazepam, Amitripytline	268

* Tested by Lowe *et al*. [309].

5.1.2.6   Discussion

Analysis and identification of SCs in biological specimens remains an ongoing challenge for forensic toxicologists. Reference standards for metabolites of SCs are not readily available. Therefore, urine screening methods have usually covered a limited panel of these analytes that can cause false negative results for SCs due to the illicit production of newer SCs. Implementing standard screening panels for SCs remains costly and time consuming. For these reasons, targeting the most appropriate substances is the key for the detection of SCs in biological specimens. The Scottish Police Authority kindly provided very useful information about the most significant SCs that are circulating and they are seeing in seizures in Scotland which helped target the analysis.

In the present study, among those 80 patients who were suspected of being exposed to a recreational substance, 4 were confirmed positive for 5F-PB-22 3-carboxyindole. One of these tested positive for another NPS (Methedrone). Two SCs samples were found in combination with other drugs. Diazepam and amitriptyline were illicit but methadone and mirtazapine were prescribed. Both of these cases were found positive for at least one antidepressant drug. Two samples tested positive for only SCs.

In poly-drug intoxications, it is difficult to associate the clinical symptoms with one specific substance. Poly-drug use can be defined as the use of more than

one drug at the same time. Poly-drug detection may suggest the use of SCs for their affordability and availability and not necessarily to avoid the law due to their limited detection in common drug-screening tests.

The presence of only one SC was detected in these cases. However it cannot be excluded that other SCs might have been present in the samples at either extremely low concentrations, or not included in the testing method. It is not surprising that most results of earlier studies vary from the present study regarding the type of SC compounds detected due to the variation in international SC drug scheduling, rapid production of newer SCs, and the targeting of newer 5F-AKB-48 and 5F-PB-22 metabolites.

The results showed a higher SC detection rate in females than males although the data is limited. One patient reported using only ecstasy and it is unclear whether she was not aware she had ingested an SC or if there was deliberate misreporting by this patient. The rest of the cases reported using legal highs and/or SCs. Hospital admission was not required for any of these cases.

The urine samples were screened in the Surveillance of Drugs of Abuse Study (SODAS) for a panel of NPS (SCs were not included) by Lowe *et al*. [309]. They found that a significant percentage (70%, n=56) of patients who were enrolled in the study had samples which were positive for more commonly encountered drugs of abuse, predominantly cocaine, whereas a small percentage (20%, n=16) tested positive for NPS, predominantly the ecstasy (MDMA) "mimics," such as PMA/PMMA, TFMPP and MDAI, and 30% (n=24) tested negative. All NPS samples were found in combination with other drugs.

Gaining insight into the prevalence of these drugs in clinical cases, particularly in patients who presented with a clinical toxidrome suggestive of acute drug intoxication or who reported drug consumption for recreational purposes, is important. The present study had some limitations. It was performed at a single hospital and involved a small number of cases, which may limit its representation of the whole population in Scotland. Due to a lack of reference standards some SCs may not have been detected.

5.1.3 Accident and Emergency Samples, June-December 2015

5.1.3.1   Introduction

This study was an extension to the previous study that targeted A&E patients and the second study was conducted around 1 year after the first over a longer period of time. It is clear that the analysis of biological samples from individuals suspected of using SC products in different periods can give a better picture of which chemicals are ingested in A&E cases. This study was carried out in collaboration with another project dealing with a new SC (MDMB-CHMICA).

In the most recent report [310], during 2010-2015, a total of 42,138 cases of toxic exposure were recorded by 101 participating hospitals in the US. Among these, 456 cases involved SCs. 379 (83.1%) patients were male. 125 (27.4%) occurred in persons aged 13–18 years.

5.1.3.2   Aim

The aim of this study was to assess the trends of SCs in patients presenting to the A&E of Glasgow Royal Infirmary with suspected toxicological ingestion, using the analysis of their blood and/or urine samples. The study took place over a 6-months period in 2015 and compared analysis of their blood and urine samples with collected information about their clinical toxicity, symptoms and associated health hazards.

5.1.3.3   Study Design

Participants were A&E patients who were suspected of acute intoxication due to SCs either from the case history of the patient or based on clinical observations. Unpreserved blood and urine samples were obtained during the clinical management of these A&E patients. Blood and urine samples were transferred to Forensic Medicine and Science (FMS), University of Glasgow, and stored between 2 and 8°C prior to analysis. Case histories including gender, age and circumstances were included with each sample.

The specimens were analysed for SCs using the method detailed in Chapter 4. Due to further intelligence on a new drug, MDMB-CHMICA, they were further analysed as reported by Seywright *et al.* [311].

5.1.3.4 Results

Blood and/or urine samples were analysed from 34 patients out of a total of 98 patients presenting in the A&E of Glasgow Royal Infirmary in the UK, between the 3 June and the 9 December 2015 with suspected toxicological ingestion due to SCs. It was not possible to analyse all the samples for SCs because of the limited sample volume and the need to test them for other drugs. There was no information about the way of referral. 19 cases were confirmed positive for at least one SC. MDMB-CHMICA results are quoted from the paper by Seywright *et al*. [311]. These cases are detailed in Table 5.2.

Only 2 cases out of the 19 cases positive for SCs were female, aged 16 and 20. The majority of positive cases for SCs (17) were males aged 15 to 55 (median 20). The most prevalent substance found in combination with SCs in the samples tested was alcohol (26.3%).

5.1.3.5 Discussion

19 cases out of 34 were found positive for at least one SC. 5F-AKB-48, 5F-PB-22 and MDMB-CHMICA were detected in 9, 3 and 9 cases respectively. Patients reported using several Spice products including "Damnation", "Black Mamba", "Sweet Leaf" and "Vertex". At that time, some of these products were freely available in Scotland in local shops (e.g. newsagents, e-cigarette shops) as well as online [47]. "Damnation", also known as "Exodus Damnation" and "Black Mamba", contained a blend of more than one cannabinoid [52]. During the period (21/06/2014 to 31/03/2015), Crew [52] collected 337 NPS packets, of which 93.8% were picked up from the streets surrounding Crew (in Edinburgh). They found 155 (46%) of the packets were SC compounds, mostly 5F-AKB-48 and 5F-PB-22. According to the listed ingredients, both "Exodus Damnation" and "Black Mamba" contained 5F-AKB-48 and 5F-PB-22. "Vertex" is one of the Spice products family. Online stores sold various "Vertex" brands such as "Space Cadet Edition" and "Pirate Edition". Media and online stores indicated that "Vertex" products contained 5F-AKB-48 and/or AB–CHMINACA. However, the packet list of ingredients in Spice products should not be relied on because their chemical constituents may change over time (See Chapter 2.3.1).

Table 5.2: Details of A&E Cases Positive for SCs

Case	Date Collected	Gender	Age (years)	Sample	SCs detected*	Conc. ng/mL	Circumstances/ Other Substances Present
1	03/06/15	M	25	Blood	5F-AKB-48	2	Ingestion of "Vertex" product.
	04/06/15			Urine	5F-PB-22 3-carboxyindole	20	
2	05/06/15	M	15	Blood	5F-AKB-48	1	Ingestion of "Damnation" product.
	06/06/15			Urine	5F-AKB-48 (*N*-4 OH pentyl), 5F-PB-22 3-carboxyindole	<1, 5	
3	07/06/15	M	20	Blood	5F-AKB-48	1	Ingestion of "Damnation" product.
4	15/06/15	M	23	Blood	5F-AKB-48	3	Ingestion of "Black Mamba" product. Ethanol
5	30/06/15	M	18	Urine	5F-PB-22 3-carboxyindole	<1	Ingestion of "Exodus" product. Ethanol
6	30/06/15	M	55	Blood	5F-PB-22 3-carboxyindole	Present	Ingestion of "Exodus Damnation" product.
				Urine	5F-AKB-48 (*N*-4 OH pentyl), 5F-PB-22 3-carboxyindole	<1, <1	
7	20/08/15	F	20	Blood	MDMB-CHMICA	5	Ingestion of "Sweet Leaf" product. Ethanol and Diazepam
8	28/08/15	M	18	Blood	5F-AKB-48	<0.5	Ingestion of "Exodus Damnation" and "anniliation" products.
9	29/08/15	M	41	Blood	5F-PB-22, 5F-AKB-48	<0.5, <0.5	Ingestion of "Black Mamba" product.

* MDMB-CHMICA tested by Seywright *et al*. [311].

Table 5.2: Details of A&E Cases Positive for SCs (Continued…)

Case	Date Collected	Gender	Age (years)	Sample	Positive SCs*	Conc ng/mL	Circumstances/ Other Substances Present
10	01/09/15	M	41	Blood	MDMB-CHMICA, 5F-AKB-48, 5F-PB22 3-carboxyindole, 5F-PB-22	22, <0.5, Present, <0.5	Ingestion of "Black Mamba" product.
11	01/09/15	M	25	Blood	5F-AKB-48, MDMB-CHMICA	<0.5, <5	Ingestion of "Sweet Leaf" and "Saint Row" products. Ethanol and cannabis
12	22/09/15	M	15	Blood	MDMB-CHMICA	<2	Ingestion of "Red Exodus" product.
13	23/09/15	M	43	Blood	5F-AKB-48	<0.5	Mixed cannabinoid use suggested at ED
14	15/10/15	M	18	Blood	AKB-48 metabolite, 5F-PB-22	<0.5, <0.5	Ingestion of "Exodus Damnation" product.
15	16/10/15	M	15	Blood	MDMB-CHMICA	<2	Ingestion of "Red Exodus" product.
16	18/10/15	M	18	Blood	MDMB-CHMICA	2	Ingestion of "Damnation" product. Ethanol
17	18/10/15	F	16	Blood	MDMB-CHMICA	<1	Ingestion of "Red Exodus" product. Ethanol and cannabis
18	12/11/15	M	24	Blood	MDMB-CHMICA	1	Ingestion of "Obliteration" product. Cannabis, diazepam, desmethyldiazepam, morphine
19	19/11/15	M	20	Blood	MDMB-CHMICA	4	Ingestion of K2 product. Cannabis, diazepam, desmethyldiazepam

* MDMB-CHMICA tested by Seywright *et al*. [311].

The absence of the parent 5F-PB-22 in blood samples in cases 1 and 2 despite the presence of its major metabolite 5F-PB-22 3-carboxyindole in urine, highlights the rapid metabolism of 5F-PB-22 [88] and/or may be due to the instability of the ester bond. The stability of 5F-PB-22 in blood samples was reported in this work in Chapter 4 and the same finding was observed the current study. The elevated temperatures during smoking of products containing 5F-PB-22 might lead to pyrolysis of some percentage of the parent compound [88]. Decomposition as a result of incorrect storage conditions may lead to false negatives as well. This may lead to misinterpretation in forensic settings, when proof of compound intake is required.

The metabolism of 5F-AKB-48 has been identified using human liver microsomal incubation, and found the major metabolites were mono, di and tri-hydroxylated [58]. Only one MRM transition (384.2 > 135.0) was found for the identification and quantification of 5F-AKB-48 as a unique indicator of the parent drug. Figure 5.3 shows an example of 5F-AKB-48 detected in A&E blood case samples. Holm *et al.* [58] and Vikingsson *et al.* [312] also used only one product ion for 5F-AKB-48. In this case, strict identification criteria should be used to avoid misidentification. This is very important in forensic toxicology, particularly for clinicians in the A&E that often needs reliable results to treat patients who have taken these new drugs safely. In addition, law courts rely on toxicological analytical results, therefore the confirmation of identity of a particular drug in a given matrix should be reliable and objective [313]. The following paragraphs discuss the identification criteria which have been used.

Interference occurring from endogenous components or from another drug that shares the same molecular mass is the most undesired problem. Although the presence of interference is rare in LC-MS/MS, which is highly recommended as a confirmatory technique, wherever practical [248], decreasing the HPLC run time to increase the throughput can lead to an increase in the possibility of matrix effects from co-eluting endogenous components. The method has to be able to discriminate between the analytes of interest and all interfering components that could possibly occur in the biological sample.
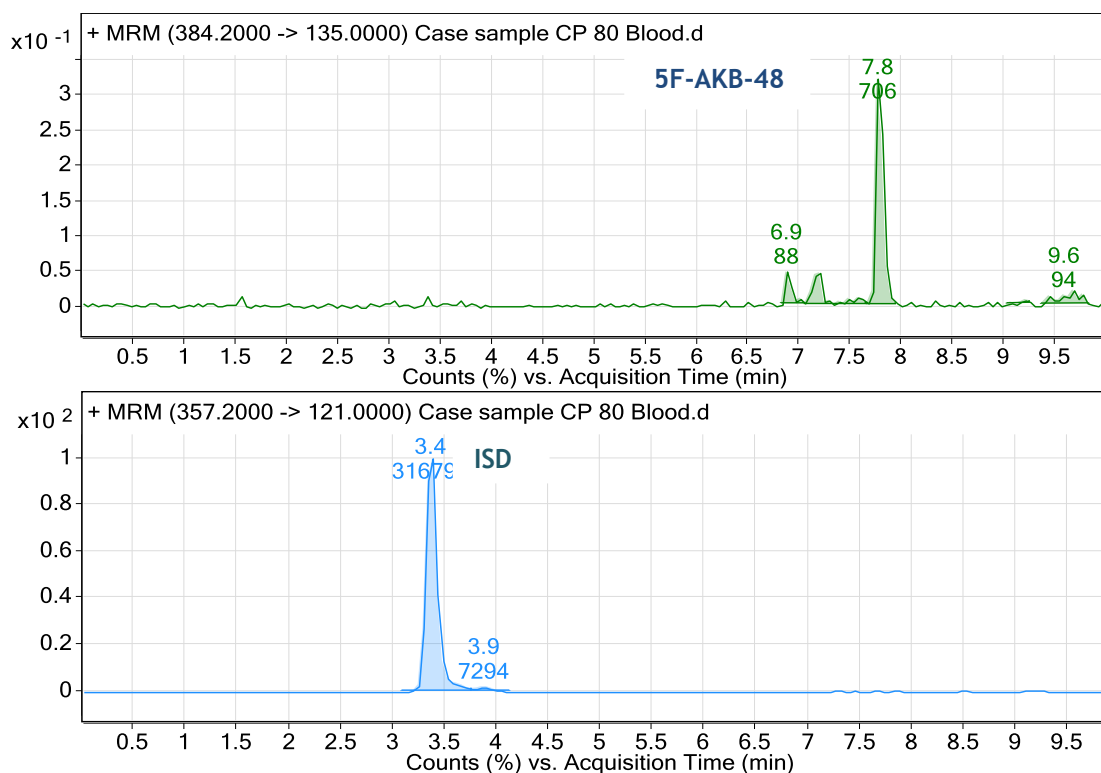
Figure 5.3: LC-MS/MS MRM Chromatograms for 5F-AKB-48 in an A&E Case Blood Sample (1 ng/mL) and its Internal Standard

LC-MS/MS libraries created in-house in most forensic laboratories depend on known certified standards and real cases results [314]. Manual optimisation of LC-MS/MS parameters for an individual analyte is available using an infusion pump. The identification of analytes of interest can be achieved with a certified standard and/or published mass spectra. However, the latter is not recommended since it is possible that the published data is incorrect or that there are differences caused by the type of instrumentation and assay method [314]. It would appear that the creation of a universal LC-MS and LC-MS/MS library of analytes remains a problem because of the differences in mass fragmentation patterns between instruments and also because the spectrum changes according to the parameters used even in the same instrument [315]. Potential reasons for this might because the availability of many types of interface (e.g. ESI, APCI) and mass analyser (e.g. TQ-MS, IT-MS, Q-TOF), and some factors that influence the performance of instruments such as ionisation and retention of analytes due to the use of different columns and mobile phases, leading to differences in fragmentation and spectra as well [315]. Some forensic laboratories have established their own library using in-house LC-MS/MS methods. For example, Dresen *et al.* [316] developed an ESI MS/MS library of 800 compounds including illegal drugs. However, it seems to be very difficult to

continuously involve SCs because of the rapid proliferation of new SCs on the world market.

In forensic and clinical toxicology, the risk of false positives always needs to be considered. Identification criteria proposed by the EU [317] can be used to minimize the risk of errors. The recommendation is that a minimum of three identification points (IPs) should be used in forensic analysis as identification criteria for drug of abuse confirmation using LC-MS/(MS) [317]. IPs are a criterion that can be used to ensure objective and reliable identification of the particular substance [313]. Specifically, they are accumulation points depending on the MS attached to HPLC, whether it is single or tandem mass spectrometry and high or low resolution. In the case of LC-MS/MS, four IPs can be achieved by determining one parent drug with two of its product ions. If the points required cannot be met, a second fragmentation or ionisation technique should be used. However, WADA and SOFT accepted that the identification of analytes can be obtained using only single product ions subject to the uniqueness and resolution of the ion [252, 279].

In the current method, good chromatographic separation was satisfactorily achieved for all compounds (except JWH-250 4-OH pentyl, JWH-250 and AB-FUBINACA which have retention time of 3.5 min, and JWH-073 and JWH-250 which have retention times of 7.0 and 6.9 min respectively) that may provide an additional identification point according to the analyte retention time and the presence of internal standards.

The findings generated from this study suggest that 5F-AKB-48, 5F-PB-22 and MDMB-CHMICA were more toxic and therefore more likely to result in attendance at the A&E in Scotland at that time. It was found that no correlation was detected between the concentration of MDMB-CHMICA in blood and the clinical presentation in these cases [311]. The inclusion of metabolites of MDMB-CHMICA in the analysis can increase the window of detection for the drug that may result in additional positive cases being detected [311].

5.1.4 Scottish Prison Service Samples

5.1.4.1   Introduction

Drugs of abuse are a serious threat to prison security, the safety of prisoners and staff and the health of individual prisoners [318]. Information available from many previous studies is based on self-reported drug use among prisoners, which has a number of methodological limitations due to the nature of the subject (drug use and prison). The prevalence of substance abuse use in prisons in a number of countries has been widely reported and is highly variable [319]. The drugs available in prison can be traditional illegal drugs, NPS or prescription drugs.

For traditional illegal drugs, there are many studies and reports of their prevalence in prison. According to EMCDDA, illegal drug users represent a large proportion of the prison populations in Europe [320]. In Scotland, an alarming amount of illegal drugs permeates prisons. All prisons in Scotland conduct drug testing known Addiction Prevalence Testing (APT) during one month per year, where prisoners released from and admitted to prisons are tested to assess the levels coming into and leaving the institution. The data shows that illegal drugs are still a significant issue in prisons in Scotland. The Scottish Prison Service (SPS) which is an agency of the Scottish Government (established in 1993) carries out an annual APT study in Scottish prisons in November of each year. They tested 1934 samples among the prison population (at reception and liberation) in November 2013 and found 77 and 25% of reception and liberation tests respectively, were positive for traditional illegal drugs (NPS were not included) (See Figure 5.4) [321].

For SCs, the picture of the prevalence among prison populations is still unclear. It seems difficult to determine precisely the prevalence of prisoners using SC products because they are often not tested in the prison programs in most countries like Scotland and Saudi Arabia [322].
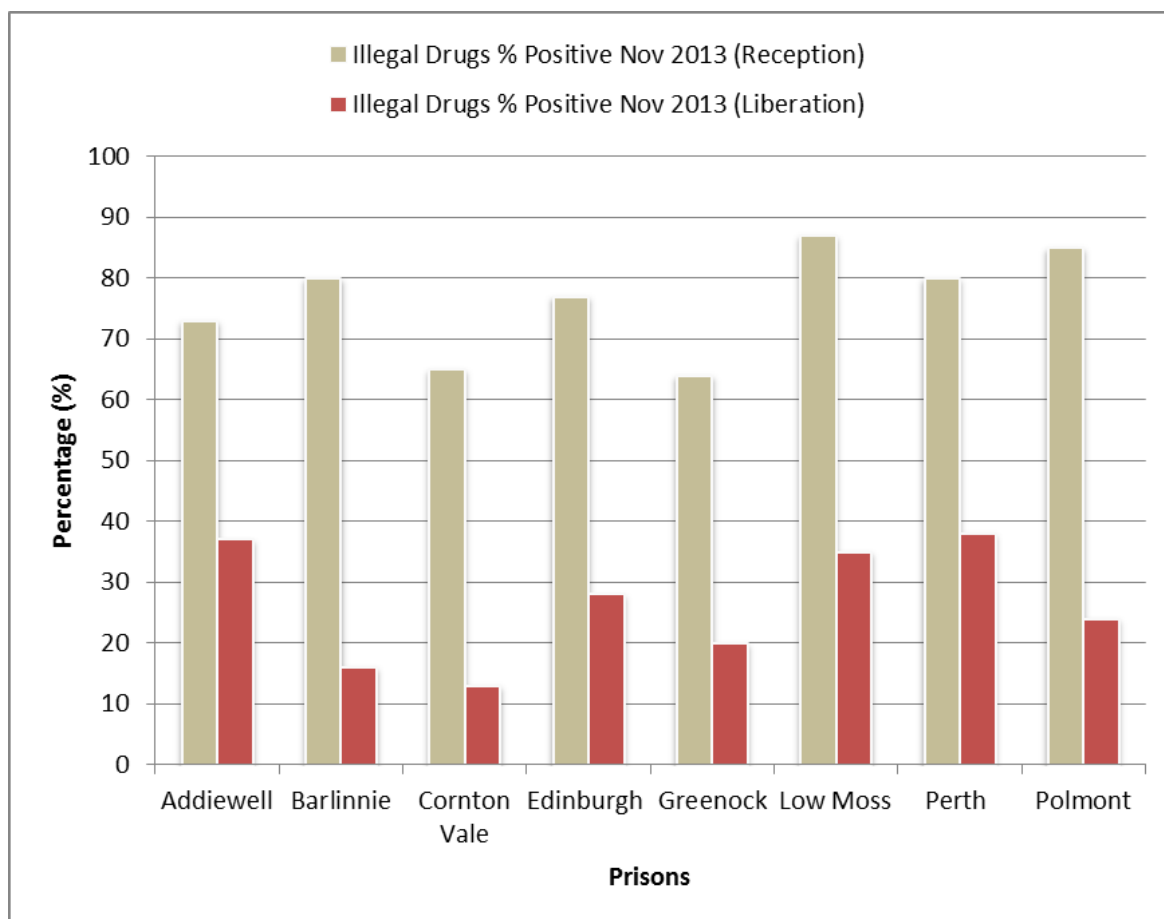
Figure 5.4: SPS Addiction Prevalence Testing Statistics, November 2013
***** These are based on unconfirmed immunoassay screening results for urine samples, which excludes drugs prescribed as part of a treatment programme but include illicit use of prescribed drugs.

In England and Wales, the number of seizures of Spice products in prisons sharply increased from 15 seizures in 2010 to 430 seizures in the first seven months of 2014 [322]. The Prisons and Probation Ombudsmen for England and Wales [323] released a 'learning lessons' bulletin in 2015 and looked at 19 deaths in prisoners where the use of NPS including SCs was suspected between April 2012 and September 2014. The report focused on SCs and related aspects.

In Scotland, the picture of SCs in prisons is unclear. However, there are several media reports suggesting that Spice products are being used by prisoners [324]. It is clear that media reports are frequently inaccurate therefore it needs more scientific work on the prevalence of these new compounds among prisoners using biological toxicology testing and/or self-report.

5.1.4.2   Aims

This study aimed to assess the occurrence of SCs among a prisoner population in Scotland. It also attempted to provide more information on the extent of SCs use in Scotland. This is the first study of the prevalence of SCs to be carried out on a large proportion of prisoners in Scotland using urine analysis to confirm the drug consumption.

5.1.4.3   Study Design

Ethical approval from the West of Scotland Research Ethics Service (WoSRES) was obtained. SPS suggested participating in their annual APT in November of each year when they collect urine samples routinely from all prisoners leaving and arriving in custody. Each prisoner's data and urine sample were completely anonymised using a unique code number to protect the prisoner's confidentiality.

5.1.4.4   Results

Eight out of the 16 prisons in Scotland participated in this study: Edinburgh, Corton Vale, Low Moss, Perth, Barlinnie, Addiwell, Polmont and Greenock and urine samples from newly-admitted and prisoners about to be released were collected over a one month period. It was not possible to include all prisons in Scotland due to logistical reasons of sample transport. Figure 5.5 shows the location of Scotland's prisons and number of collected samples from participating prisons. Participants were all prisoners, male and female, admitted to and released from the selected prisons over a one-month period (November 2013). Urine samples were initially collected in additive-free containers by the SPS to assess the APT of traditional illicit drugs. After the samples had been analysed using an ELISA technique by the SPS, the samples were transferred to FMS and stored at -20°C until analysed.

Demographic Descriptive

A total of 904 urine samples were obtained from SPS. Only the sample numbers and whether they were admission or liberation samples were provided with the samples. The available demographic data on selected prisons is shown in Table 5.3.
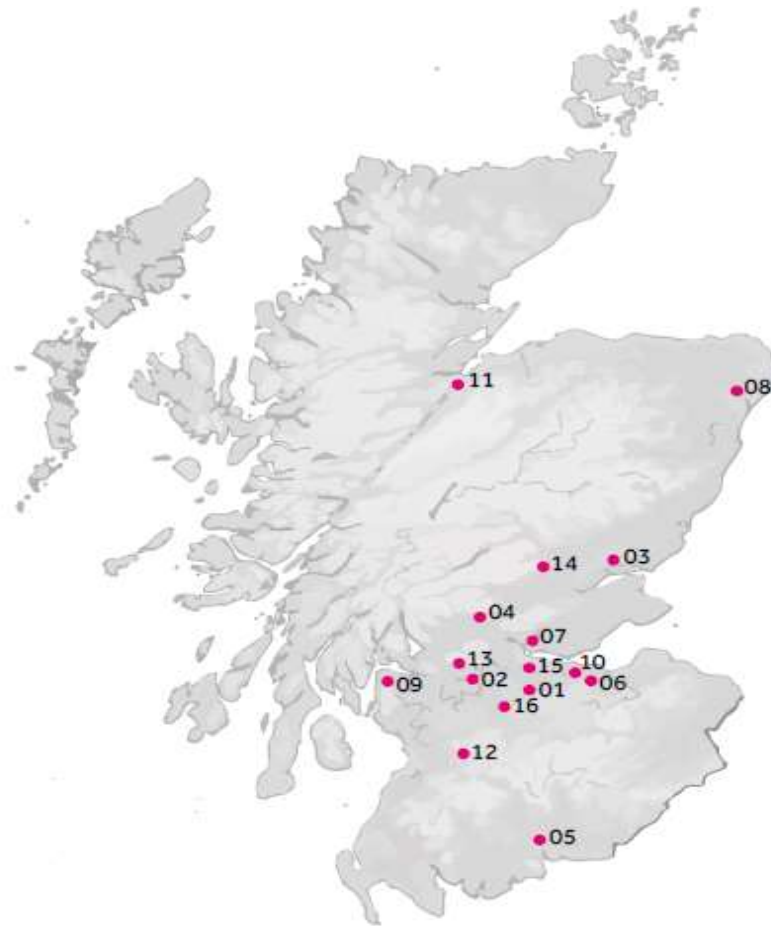
Prison (on the map)	Scotland's Prisons	No. of Collected Samples
1	Addiwell	63
2	Barlinnie	172
3	Castle Huntly	-
4	Cornton Vale	101
5	Dumfries	-
6	Edinburgh	85
7	Glenochil	-
8	Grampian	-
9	Greenock	27
10	Headquarters	-
11	Inverness	-
12	Kilmarnock	-
13	Low Moss	118
14	Perth	187
15	Polmont	151
16	Shotts	-

Figure 5.5: Regional Distribution and Number of Collected Samples from Scottish Participating Prisons [306]

Table 5.3: Demographic Data on Selected Prison. Prison Population Numbers Obtained from SPS [306]

Prison		Average Daily Population		Maximum No.		Age	No. of Collected Samples	A[a]	L[b]	Facility	Area served
		Men	Women	Men	Women						
Addiwell		700	-	700	-	>21	63	33	30	Local	Lanarkshire, West Lothian
Barlinnie		1,303	-	1,433	-	>21	172	106	66	Local	West of Scotland
Cornton Vale	Adult	-	213	-	264	>21	101*	99	11	National	Whole of Scotland
	Young	-	20	-	29	16-21					
Edinburgh		775	89	829	107	>21	85	25	60	Local	Edinburgh, Lothian, Borders, Kirkcaldy, Fife
Greenock		183	52	195	56	>21	27	11	16	Local	West of Scotland
Low Moss		737	-	764	-	>21	118	100	18	Local	North Strathclyde
Perth		643	-	689	-	>21	187	123	64	Local	Angus, Dundee, Perth, Kinross, Fife
Polmont		501	-	546	-	16-21	151	?	?	National	Whole of Scotland
Total		4,842	374	5,156	456		904				

[a] A=Admission. [b] L=Liberation.
* There is no information if this number includes any young subjects

The Use of Synthetic Cannabinoids among Prisoner Population in Scotland

904 urine samples from individuals admitted to/liberated from Scottish prisons over a 1 month period (November 2013) were tested for the presence of SCs. 5F-AKB-48 (N-4 OH pentyl) was detected in 10 cases and the concentration ranged between <1 and 4 ng/mL. Figures 5.6 below shows an example of 5F-AKB-48 (*N*-4 OH pentyl) detected in a prison case sample and its internal standard. Three samples were found positive for 5F-PB-22 3-carboxyindole with concentrations of 5, 7 and 9 ng/mL. All analytes were found alone, except in one case.
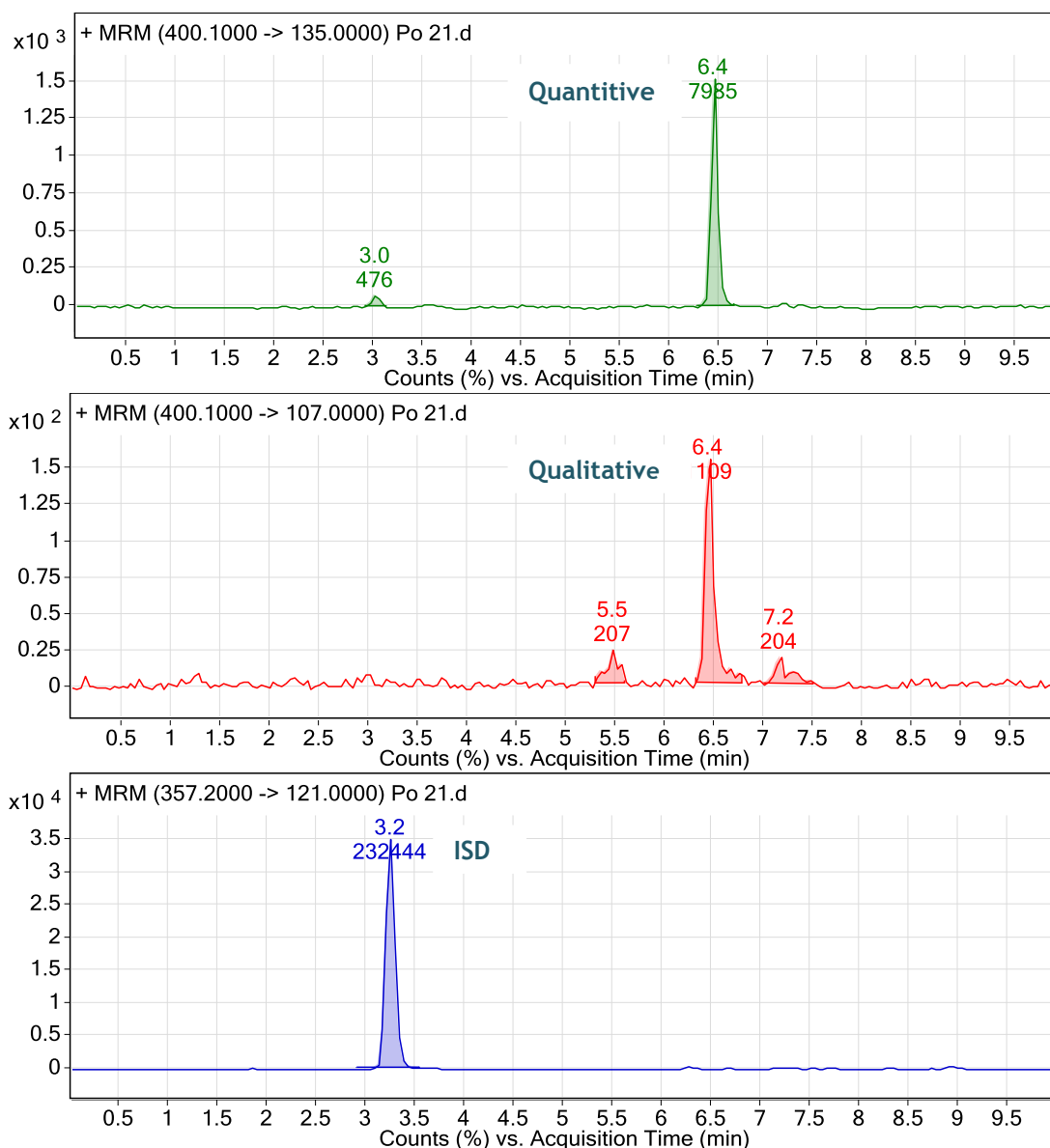
Figure 5.6: LC-MS/MS MRM Chromatograms of 5F-AKB-48 (*N*-4 OH pentyl) Detected in a Prison Case Samples (4 ng/mL), and its Internal Standard

7 samples from Polmont prison were found positive for at least one SC. There is a lack of Admission/liberation information for Polmont samples but all other positive cases were from admission prisoners. The details of the results are found below in Table 5.4.

Table 5.4: Toxicological Data for 12 SC Metabolite Positive Cases

Case	Prison	Positive SCs	Other Screened Drugs*	Conc. ng/mL
1	Polmont	5F-PB-22 3-carboxyindole	-	9
2	Polmont	5F-AKB-48 (*N*-4 OH pentyl)	-	2
3	Polmont	5F-AKB-48 (*N*-4 OH pentyl)	-	2
4	Polmont	5F-AKB-48 (*N*-4 OH pentyl)	-	3
5	Polmont	5F-AKB-48 (*N*-4 OH pentyl)	Buprenorphine, Cannabis	4
6	Polmont	5F-AKB-48 (*N*-4 OH pentyl), 5F-PB-22 3-carboxyindole	Cannabis	2, 7
7	Polmont	5F-PB-22 3-carboxyindole	Benzodiazapines, Cannabis	5
8	Low Moss	5F-AKB-48 (*N*-4 OH pentyl)	Opiates, Benzodiazapines	1
9	Low Moss	5F-AKB-48 (*N*-4 OH pentyl)	Benzodiazapines, Cannabis, Methadone	1
10	Edinburgh	5F-AKB-48 (*N*-4 OH pentyl)	Opiates, Benzodiazapines, Cocaine, Methadone	2
11	Perth	5F-AKB-48 (*N*-4 OH pentyl)	-	2
12	Greenock	5F-AKB-48 (*N*-4 OH pentyl)	Opiates, Benzodiazapines, Cocaine	< 1

* Tested by SPS by ELISA, but not subject to confirmatory analysis.

Illicit Drug Prevalence in Synthetic Cannabinoid Positive Samples

The urine samples were initially tested by the SPS for drugs of abuse including amphetamines, methamphetamines, benzodiazepines, barbiturates, opiates, cannabis, cocaine, buprenorphine and methadone using a dip-stick test. SPS provided the screening results and whether the drug was prescribed or not. All nonprescribed drugs were considered abused by SPS.

SC positive samples were compared with SPS findings to investigate other illicit drugs associated with SCs. Figure 5.7 shows the percentages of illicit drug prevalence in positive SCs samples; details of drugs detected along with SCs are presented in Table 5.4.
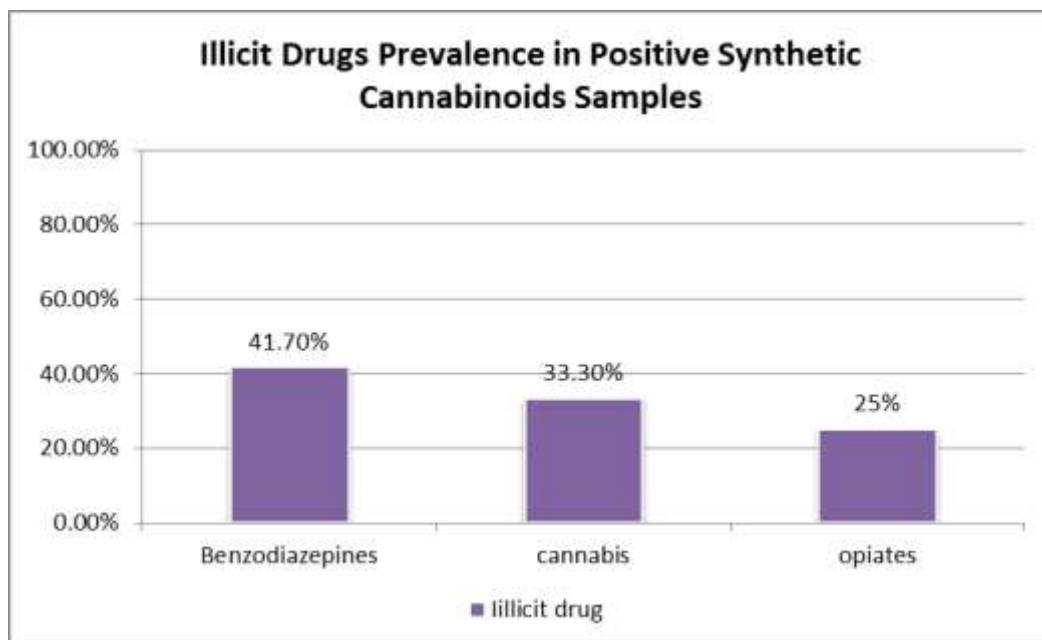
Figure 5.7: Illicit Drug Prevalence Based on an ELISA Test in SC Positive Samples

5.1.4.5   Discussion

The results of the analyses found 12 of the 904 samples to be positive for at least one SC (1.3%). 5F-AKB-48 (*N*-4 OH pentyl) was the most abundant analyte detected in these case samples. It was identified in 10 samples with concentrations ranging between <1 and 4 ng/mL (Figure 5.8).

As mentioned previously, there are many various packets with different brand names which have different SCs. Some packets listed both 5F-AKB-48 and 5F-PB-22 as ingredients (e.g. "Spunout" and "Psyclone clown") but forensic examination of these products by TICTAC confirmed that they contained only 5F-AKB48 [52]. TICTAC is a leading provider of drug information and drug identification to the healthcare and criminal justice sectors located at St. George's University of London.

5F-PB-22 3-carboxyindole was detected in 3 urine samples with concentrations of 5, 7 and 9 ng/mL, respectively.
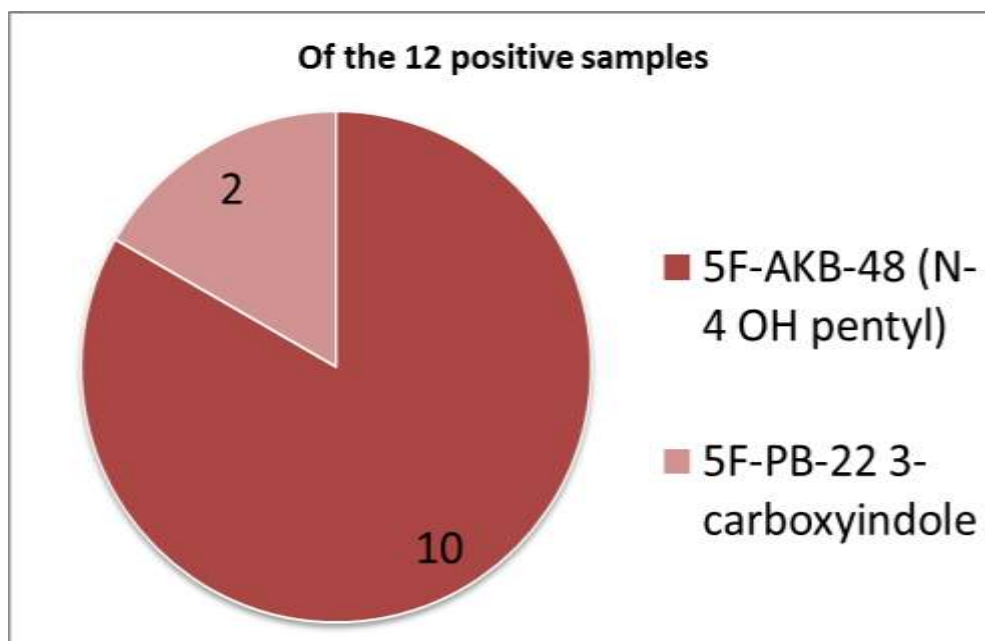
Figure 5.8: The Percentages Prison Case Samples Found to Contain SC Metabolites

Polmont is Scotland's prison for young offenders aged between 16 and 21 years and had the highest proportion of SC positive samples (58.3%) detected in this study. This can be seen as a sign of the high popularity of SCs among adolescents and again supports the previous studies that suggest young people are the primary users of these new substances. A potential reason for this might be because these products are easier for young people to buy than alcohol.

Two (16.7%) of samples positive for SCs were from prisoners at Low Moss Prison. Edinburgh, Perth and Greenock prisons each had one SC-positive sample (8.3%). Edinburgh is a large prison serving Edinburgh, Lothians, Borders and Fife area. Perth is prison receiving offenders predominantly from courts in Perth and Kinross, Fife, Angus and Dundee. Greenock is essentially a male prison but at the time of the study it held a number of female prisoners due to Corton Vale establishment renovation. Greenock prison has been included in the study in spite of its small population as they specifically requested to participate. Barlinnie is the largest prison establishment in Scotland with a capacity of 1,433 prisoners and holds all categories of prisoners. However, no samples tested positive for the selected SCs in this study. Corton Vale is Scotland's only all-female prison and also no SCs were detected in this group.

There is no information on whether Polmont samples were admission or liberation, making the assessment of use of SCs among prisoners at Polmont prison impossible. Although some people believe in tackling the drug problem from the user side, rather than from the supply side, creating a new approach to prevent drugs being smuggled into prisons can help to rid the prison population the scourge of drug addiction.

At the time of this study, some SCs were legal in the community but all forms are banned in prisons. This may increase the price of these products in prison compared to traditional drugs such as cannabis or opiates, which are illegal in both settings. However, these products are still relatively cheap in prisons.

The present study is important to understand the range of drugs that are affecting the prison population. Although the current testing method included only a small panel of SCs, the results reflected the presence of the problem in prisons in Scotland, particularly among young people. Suitable methods for the detection of SC compounds should be added to the Annual Prevalence Testing carried out by SPS. This can elevate the testing capabilities and provide benefits to the prison population and the entire society as well. Clearly there is an increased cost and this needs to be considered.

The present study had several limitations. The major one is that the analytical method was only looking for a limited number of SCs. In addition, there was a lack of information regarding age, sex and medical history provided along with each sample.

5.1.5 Post-mortem Samples

5.1.5.1 Introduction

In post-mortem toxicology, forensic toxicologists assist procurators fiscal, coroners, pathologists, and medical examiners in evaluating contributory aspects in the cause of death. They provide consultations on drug kinetics and interactions, pharmacology, metabolism, drug stability, drug tolerance, and other information supporting the pathologist [12, 13].

Drug-related deaths (DRD) are a complex concept and hence countries have different definitions for DRD cases that may lead to variations in the quality and quantity of mortality data. The following definition is used by the EMCDDA *"deaths caused directly by the consumption of one or more drugs and, generally, occurring shortly after the consumption of the substance(s)"* [326]. At present, national statistics are improving in most European countries and their definitions are becoming relatively similar to the EMCDDA definition.

The latest figures, according to EMCDDA [295], show that the UK had the seventh highest DRD rate in Europe. In 2014, there were 3,346 drug poisoning deaths (involving both legal and illegal drugs) registered in England and Wales [327]. In Northern Ireland, there were 115 DRD in 2013 (the latest figures available) [327].

In Scotland, National Records of Scotland (NRS) produce an annual report on DRD. According to NRS, there were 613 DRD registered in 2014, 86 (16%) more than in 2013 [328]. This is the largest number ever recorded since 1996 when the current series began. Figure 5.9 shows the number of drug-related deaths in Scotland from 1996 to 2014.
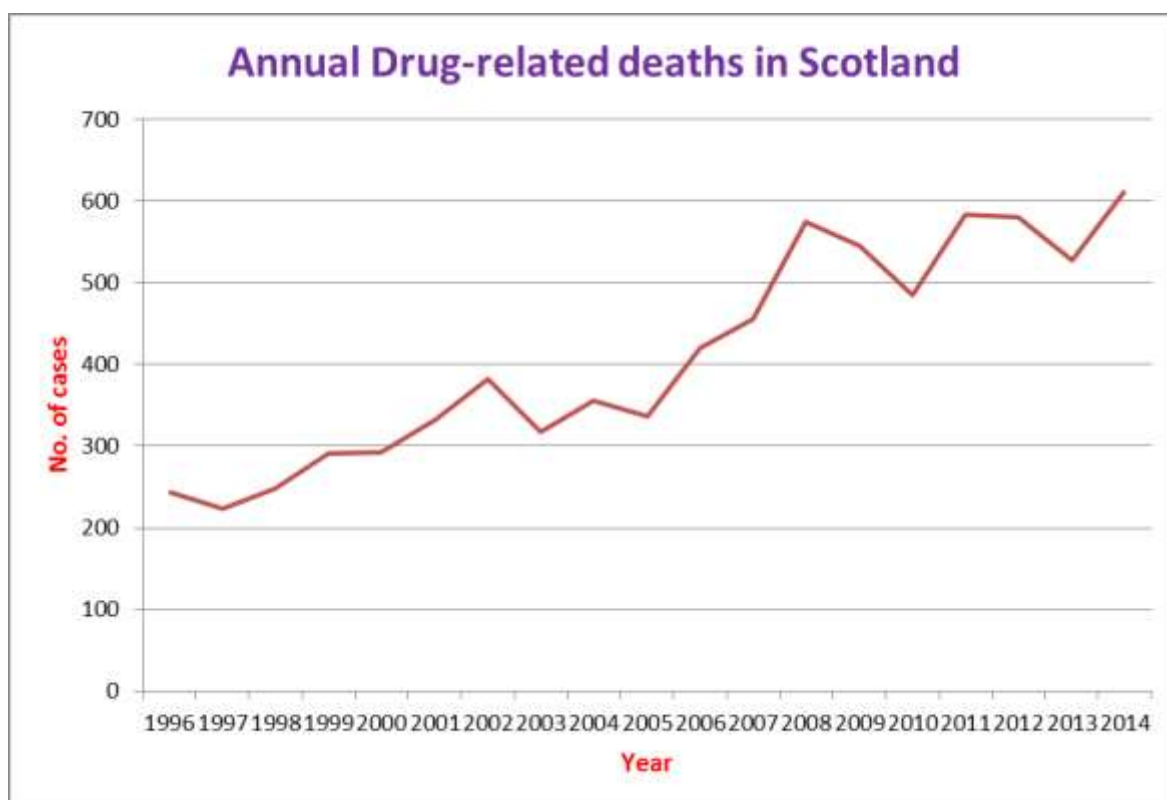
Figure 5.9: Drug-Related Deaths in Scotland 1996–2014 (Source of Information: NRS [328])

The statistics show that males accounted for 74% of DRD in 2014. However, the percentage increase in the number of DRD was greater for females (141%) than for males (50%). They also show that the numbers have fluctuated in the last 5 years before the sharp rise in 2014. However, NRS suggested that the sudden rise may be because the changes in the classification of some drugs in 2014 such as zopiclone and tramadol that would not have been included in previous years. However, direct comparisons between countries in the numbers of DRD should be made with caution due to differences in data collection methods, definition for DRD cases and in the death registration system [295, 326].

NRS uses two terms in their statistics to describe the death associated with NPS; "NPS-related deaths" and "deaths involving NPS". In the case of a death due solely to one of the uncontrolled NPS at the time of death, NRS would not count this as NPS-related deaths. However, it would be counted in the figures for deaths involving NPS. The first Scottish deaths involving NPS were registered in 2009. The number of NPS-related deaths and deaths involving NPS increased rapidly between 2009 and 2014 (Figure 5.10).
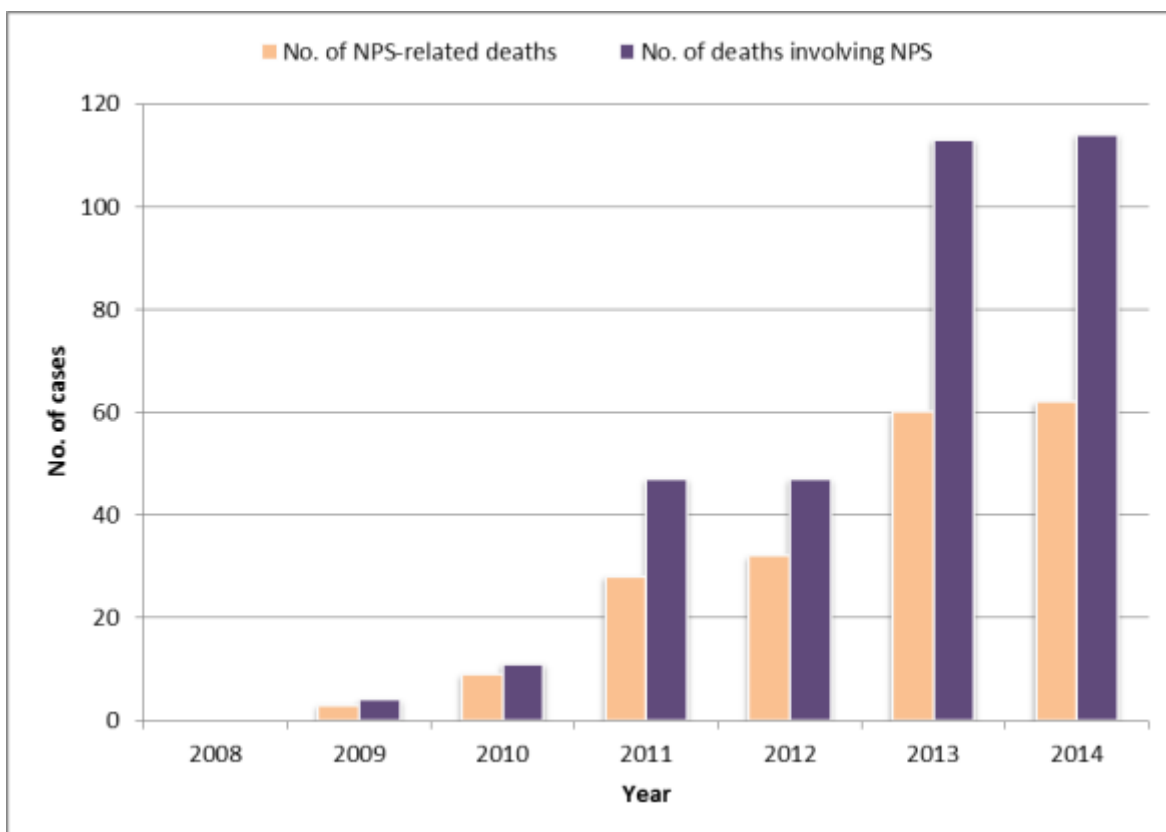
Figure 5.10: NPS-Related Deaths and Deaths Involving NPS in Scotland 2009–2014 (Source of Information: NRS [328])

In 2014, NPS were implicated in 10.1% (62) of DRD registered in Scotland, or potentially contributed to the cause of death. In 40 of the 62 deaths, the only NPS present were benzodiazepine-type NPS (usually phenazepam or etizolam). In 17 cases, other types of NPS were present (e.g. mephedrone, methiopropamine). There were 5 deaths for which both benzodiazepine-type NPS and other types of NPS were present. Only a small proportion of cases (7 out of 62) were believed to have been caused by NPS alone. There were also 52 cases for which NPS were present but were not decided to have contributed to the death.

For SCs, the general feeling was that if they act like cannabis they would not lead to fatalities. However after a string of deaths apparently due to SCs and many reports of serious side effects, more consideration needs to be given to each SC. Some pharmacologic and pharmacokinetic data on earlier generations of SCs are available. However, more recent compounds often have unknown receptor-binding affinity and selectivity that might cause unexpectedly severe toxicity [91].

Recently, a number of cases of SC-related deaths, either on their own or in combination with other drugs, have been reported in publications [3, 110, 146,

148, 154, 329, 330]. In one case [329], a 36 year-old male collapsed at home who was known to have taken "Mary Joy Annihilation". The toxicology report confirmed the presence of 5 different SCs in peripheral blood in addition to 250 ng/mL amphetamine. SCs were considered as a contributory factor in his death due to drug intoxication. Another case [110] describes a 59 year-old male decedent who was found with 3 herbal mixture sachets at his residence. The toxicology analysis showed the presence of MAM-2201 in several of his biological samples and, as no evidence of external injuries or endogenous disease was noted, the use of this drug was determined to have caused his death. In Scotland, only one case of SC-related death has been reported in 2014 by NRS. In this case 5-F-PB-22 was determined to have potentially contributed to the death.

SCs are increasingly tested in post-mortem cases when reported in the case history but some cases do not clearly involve SCs in the case history making the toxicological analysis the only information to confirm if it is a SC-related death.

Many forms of biological matrices are available for drug analysis in post-mortem cases such as blood, urine, hair, bone marrow, stomach contents, liver and many others. Selection of the appropriate specimen is an important step in post-mortem toxicology and often dictated by the case being investigated [331], however the most commonly-used matrices are blood, urine and vitreous humour [331]. Blood can give an indication of levels of impairment in a user and is very useful for indicating most recent use. Blood can provide information about distribution, metabolism, and pharmacokinetics of drugs. Urine samples are less important in interpretation of abused drugs, particularly in post-mortem toxicology. However, they can be useful because drugs can be detected for days, depending on the drug.

5.1.5.2 Aims

This study aimed to better understand the presence of SC in fatalities by comparing the blood concentrations found of these drugs and the symptoms and/or pathological findings.

5.1.5.3 Ethical Approval

Ethics approval was given by the College of Medical, Veterinary and Life Sciences Ethics Committee for Non-Clinical Research Involving Human Subjects at University of Glasgow (See Appendix 5). They reviewed the research proposal and agreed that there was no objection on ethical grounds.

5.1.5.4 Results

4 Samples of blood and urine collected at post-mortem during summer 2015 were analysed for a panel of SCs using the present method as part of the investigation of medico-legal cases involving DRD submitted to FMS, University of Glasgow. Samples were for two post-mortem cases with suspected ingestion of NPS. A case history was obtained with each case.

<u>Case 1</u>

The deceased was a 26 year-old man and had a history of drug abuse which included intravenous heroin and cannabis. He was prescribed methadone. He had started experimenting with legal highs and this had led to erratic behaviour. The day before his death, he presented to A&E with hallucinations, and stated he had been unwell and had taken several legal highs including "Sweet leaf", "Grape Shoot", "Purple G Punch" and "Dark Obsession" and had also taken cannabis. The following afternoon, he was detained under the Mental Health Act and deteriorated before subsequently dying. Only a blood sample (preserved) was available. The blood sample tested positive for 5F-AKB-48 with a concentration of 1 ng/mL.

<u>Case 2</u>

The deceased was a 34 year-old man with a history of heavy cannabis use. As a result of his drug abuse, he started to suffer from mental health problems including schizophrenia, bipolar disorder and depression for the past 16 years. He was prescribed citalopram and risperidone. In the recent past police witnessed him in possession of a bag containing green herbal material believed to be an NPS. In his medical history, he had admitted to taking NPS and it is believed that he had been taking them for some time. Concerns had been raised by his parents regarding his use of SCs known as "Diesel", bath salts known as

"Monkey Dust" and tablets known as "Pink Panthers". He was found lying on his back within the living room evidently dead on arrival of the police. There was evidence of drug use including cigarette papers, overflowing ashtrays with roll up cigarette butts and remnants of a large amount of green herbal products on the ground. Toxicology confirmed the presence of alcohol at concentrations of 11 and 17 mg/dl in preserved blood and urine respectively, in addition to 0.62 and 0.36 ng/mL citalopram and methylthienylpropamine (MPA) respectively in the blood sample (unpreserved). The urine sample tested positive for 5F-AKB-48 (*N*-4 OH pentyl) with a concentration of 18 ng/mL.

5.1.5.5   Discussion

The number of NPS-related deaths is low compared with the number of heroin- and/or morphine-related deaths in Scotland [328]. However, over the past four years there has been an increase in the number of NPS-related deaths, particularly the new benzodiazepines in combination with other respiratory depressants e.g. heroin/methadone. In 2014, NRS reported that 5F-PB-22 was implicated in, or potentially contributed to the one cause of death. However, there are possibly additional deaths related to other SCs which are not recorded due to the difficulties of their detection.

Both cases involved poly-drug use that was compatible with the DRD figures in Scotland in 2014, in which 553 out of 613 involved more than one substance. There are several confounding elements in the interpretation of results of post-mortem toxicology including individual variation, the lack of data examining influences of post-mortem redistribution, the lack of information regarding to a fatal dose of the drug and in cases in which the person had also taken other substances.

The differences in individual pharmacogenetics and drug behaviour of users (including frequency and dose) may affect the length of time that the drug remains in the body and at what concentration, resulting in a wide range of concentrations in fatalities [91, 330]. The redistribution phenomenon refers to the changes in drug concentrations according to the interval and sampling site between death and sample collection [332]. Different pathologists could have different views on whether a specific drug would cause or potentially contribute

to a death. For instance, views can differ on the fatal dose of the drug for the person concerned, or in a case where the person had a poly-drug condition, on the level of impairment that may be caused by the combination of the drugs. Therefore, therapeutic, toxic and lethal concentrations of drugs in biological specimens have been compiled into various books in order to aid interpretation of the result [333].

In contrast, the literature reveals major deficiencies regarding toxic and/or lethal concentrations of most of the SCs. A study by Kronstrand *et al.* [90] presented toxicological findings of some SCs including JWH-018, JWH-250, JWH-201, JWH-203, JWH-210, JWH-122, JWH-081, JWH-019, AM-2201, AM-2233, AM-694, MAM-2201, AB-001, UR-144 and RCS-4 in whole blood from recreational users and from a fatal intoxication. The authors indicated that the median concentrations of the selected SCs were < 0.5 ng/g in whole blood. Recently, Labay *et al.* [153] reported a collection of data for SCs-related death from 25 medical examiner and coroner cases where their presence was analytically determined. They found that some deaths were linked to SCs, either as contributing factors in subjects with pre-existing cardiopulmonary disease or as a cause of death as behavioural toxicity resulting in excited delirium, trauma or accidents. They found that blood concentrations for all the SCs varied widely, ranging from 0.11 to 105 ng/mL, and the manner of death included accident, natural, accident/toxicity and unknown. These findings can be useful to forensic pathologists for comparison purposes to accurately determine cause and manner of death. However, due to the rapid proliferation of SCs on the world market, the data must be updated with the emerging of any new compound.

In this study, in case 1, the blood concentration of 5F-AKB-48 was 1 ng/mL. There is no information regarding the relationship between blood concentrations of 5F-AKB-48 and life-threatening (toxic) and/or lethal case outcomes, making the interpretation of toxicological results for deciding the extent of the contribution of death more challenging. To the author's knowledge, the only publication concerning blood concentrations of 5F-AKB-48 toxicity in recreational users was conducted by the Norwegian Institute of Public Health [316]. The authors found that the concentrations of 5F-AKB-48 in whole blood from 4 DUID cases ranged from 0.9 to 6.5 ng/mL.

In case 2, the urine concentration of AKB-48 (*N*-4 OH pentyl) was 18 ng/mL. Although urine is the most commonly used matrix for toxicological analysis, as it provides evidence of previous drug consumption and parent drugs and/or metabolites are present mostly in higher concentrations than other specimens, the interpretation of drug concentrations detected in urine is usually complex. Importantly, drug concentrations detected in urine cannot reliably determine the amount of drug used, how it was administered, or infer impairment at the time the urine was collected. However, useful findings and indications can be obtained. For instance, a positive result indicates that the specific drug(s) is present and these findings might be correlated to the patient's history and symptom presentation. Individuals who take drugs more regularly will typically have higher concentrations of drugs in their urine [335]. This possibly can lead to longer windows of detection. The nature of the drugs can also play an important role in this issue. Cannabinoid metabolites can persist for extended periods of time due to their high lipophilicity and urine tests can remain positive after discontinuation of use for up to 7 to 10 days in a casual user, 2 to 4 weeks in a heavy user, and months in a chronic heavy user [336].

The causes of death are not available for either case. However, the association of 5F-AKB-48 and deaths seems very challenges because the toxic concentration of 5F-AKB-48 in blood is not yet known, especially in the presence of other substances "poly-drugs".

In comparison with cannabis, SCs seem to exhibit more toxicity due to their increased potency and dose that might cause overdose and serious clinical consequences [37, 337]. Illicit manufacturing of Spice products may lead to the presence of contaminants, impurities, or variability in SC [91] that can cause varying degrees of effect depending on the batch [338]. Unlike opioid drugs, SCs have no available antidote, and treatment of severe adverse effects is largely supportive [153]. In addition, there are no clear symptoms that signal exposure to SCs in someone without a history of SC-product use [153], which might lead to misreporting of the cause of death.

5.2 Synthetic Cannabinoids in Saudi Arabia: Case Samples

5.2.1 Introduction

Despite the suggestion from different sources [61] that the use of NPS including SCs is widespread around the world, there is currently no data on their use in Saudi Arabia. Legislation controlling SCs has been put in place in many countries in a bid to stem the flow. In Saudi Arabia, the Saudi Food and Drug Authority (SFDA) is responsible for the regulation of drugs and medical devices and of chemical and biological substances. The majority of controlled illicit drugs in Saudi Arabia depend on the United Nations Conventions on drugs of 1961, 1971 and 1988. As with most countries, legislation in Saudi Arabia tends to be one step behind the manufacturers of SCs and the only controlled SCs are the first generation compounds including HU-210, JWH-015, oleamide and CP 47,497. Therefore, many SCs still remain legal in Saudi Arabia.

It is necessary to understand the prevalence of illicit drug use and dependence in Saudi Arabia. The true prevalence of illicit drug use in Saudi Arabia is unknown due to the absence of epidemiological population-based studies [9, 339]. Nevertheless, it can be estimated indirectly through indicators like official statistics of illicit drug seizures, illicit drug-related crime statistics, illicit drug-related deaths and treatment data.

According to UNODC [340], in 2012, over 12 tons of amphetamine seizures were reported in the Near and Middle East/South-West Asia, representing 56 per cent of global seizures. The largest seizures were reported in Saudi Arabia, Jordan and the Syrian Arab Republic, in that order. The Saudi Ministry of Interior (MOI) announced the arrest of 17 tons of hashish in 2012 [341]. Illicit drug-related crime includes crimes to obtain money to buy illicit drugs, possession or sale of drugs, driving under the influence of illicit drugs and crimes such as child abuse resulting from illicit drugs [342]. Between 2010 and 2012, 119,000 people have been arrested for illicit drug offences which represent 60% of all crimes in Saudi Arabia [6]. Figures from psychiatric clinics or hospitals show increases in the number of patients seeking treatment for alcohol and drug use disorders in Saudi Arabia [343]. All these indicators clarify the increasing trend of problem drug use

in Saudi Arabia. Social and economic development in Saudi Arabia in the last years might contribute to the increase in drug abusers.

Although there is a lack of official statistics related to drug abuse rates in Saudi Arabia, the prevalence of drug abuse and clinical characteristics and associated socio-demographic among drug abusers in treatment settings have been investigated in several survey studies [343-352]. Conducted over different time periods and regions of Saudi Arabia, these papers provide a useful insight into the prevalence of drug use (Table 5.5). By contrast, the Saudi picture of NPS including SCs is still unclear.

Table 5.5: Prevalence of Drug Abuse among Saudi Patients in Addiction Treatment Settings (Percentage)

No of drug abusers	Year of study	Region	Al	Am	Ca	He	Co	Ba	Be	Ref
485	1992	Jeddah	16.1	5.2	-	43.5	-	8.0	8.7	[324]
116	1995	Dammam	31.2	-	26.0	83.6	-	0.9	0.9	[329]
160	1999	Riyadh	23.8	-	10.6	18.8	-	-	-	[326]
799	2000	Jeddah	18.0	4.0	1.0	70.0	0.1	-	0.5	[325]
423	2000	Al-Qassim	52.0	25.3	4.1	6.7	-	-	-	[327]
302	2001	Jeddah	21.5	-	-	68.2	-	-	-	[330]
101	2008	Jeddah	56.0	70.7	60.0	25.0	2.3	-	8.0	[331]

Al= Alcohol, Am=Amphetamine, Ca=Cannabis, He=Heroin, Co=Cocaine, Ba=Barbiturate, Be=Benzodiazepine

In many countries, when people are applying for employment, especially for safety-sensitive positions (e.g. transport, medicine or armed forces), they have to undergo regular laboratory tests for the detection of illicit substances, known as Workplace Drug Testing (WDT) [353]. Random drug testing could be enforced as well. Forensic toxicologists perform drug screening of human samples (mostly urine) in a programme managed by the guidelines such as EWDTS [354]. The procedures for specimen collection, analysis and interpretation are well-defined to minimise the risk of errors. Current WDT in Saudi Arabia only covers illicit traditional drugs including alcohol, amphetamines, cannabis, cocaine, opiates, benzodiazepines, barbiturates and methadone.

In order to evaluate the prevalence of SCs within WDT in Saudi Arabia, a study was carried out in collaboration with the Security Forces Hospital (SFH), (Riyadh, Saudi Arabia). This study is unique in that no previous publications have studied

SCs among Saudi society to assess their occurrence. Urine samples from workers or people applying for employment over a one-month period were collected. Urine was chosen because it can provide evidence of previous drug consumption and metabolites are present mostly in high concentrations, as well as for its easy sampling compared to blood. Some drugs also remain in the urine for days or longer following their use.

5.2.2 Aim

It is important to understand the range of drugs that are affecting a particular population. The aim of this study was to evaluate the scale of use of SCs in Saudi individuals in WDT during a one-month period (July 2014) using analysis of their urine samples. This study also provided unique information on SCs in Saudi Arabia and this can help for sharing information on the prevalence of SCs in new regions leading to the development of a better understanding of this issue. The prospective study was performed in the Saudi Security Forces Hospital (SFH) which is one of the leading Health Care Providers in Saudi Arabia, founded in 1975.

5.2.3 Ethical Approval

All procedures in this study were conducted according to the guidelines laid down in the College of MVLS Ethics Committee for Non-Clinical Research Involving Human Subjects at the University of Glasgow (See Appendix 6) and they were also approved by the Research Committee at SFH, (Riyadh, Saudi Arabia) (See Appendix 7). Both committees reviewed the research proposal and agreed that there was no objection on ethical grounds.

During the processing of ethical approval, three documents were prepared including the application form for ethical approval, participant information sheet (Appendix 8) and consent form (Appendix 9). The patient information sheet was provided in lay terms (in English and Arabic languages) and in the style of questions and answers. The consent form was also in English and Arabic languages.

5.2.4 Study Design

Participants were Saudi individuals, male and female, presenting at SFH to provide urine samples for WDT as a requirement of their job during a one-month period (July 2014). SFH advised that the urine sampling for both WDT and the research would be at the same time due to the high work load at the hospital site.

The researcher met donors at the toxicology lab in SFH and asked them if they would like to take part in the study and gave them the information sheet to read. Participants were recruited voluntarily and were selected from personnel who already had to undergo regular urine drug screening. Participants were fully briefed regarding the purpose of the study and were allowed to opt out of the study at any time. After a potential participant read the information sheet and agreed to participate they were given the consent form to sign and a plastic container to donate a urine sample. They were asked their age prior to completing the consent form and those under the age of 18 were excluded from this project. Collection of samples was carried out by the researcher under supervision from the hospital administration.

Consent and the urine samples were taken at the same time. The consent form was physically separated from the urine sample in the presence of the subject to maintain the anonymity. The consent forms did not have any number on it which would link it to a number on the sample. The consent forms were stored securely with the researcher until travelling back to the UK where they have been kept with the researcher in a secure cabinet in FMS.

All samples were labelled with a unique number assigned to the individuals at the time consent was given and did not have any dates at all. This made the urine samples completely anonymous, and at no point would they be identified to individuals. Every participant attended on only one occasion and was asked to provide only one urine sample. The urine samples were collected in completely clean containers with pre-prepared serial numbers starting from 1 to 500. These serial numbers were different from the hospital numbers to protect the person's confidentiality. Each sample contained less than 10 mL of human urine. The samples were kept secure in Saudi Arabia by storing in a special freezer at -20°C

in Toxicology lab until the sample shipment. The participant took approximately 3 to 5 minutes to give the sample. The urine collection area was completely suitable for the collection of urine samples and the procedure followed SAMHSA guidelines. The toxicology lab at SFH has accreditation by the Standards Council of Canada, which is a mark of competence and reliability recognised throughout the world.

Urine samples were chilled throughout the transit with dry ice and delivered to FMS, University of Glasgow by CitySprint. The samples were received in an acceptable condition and stored at -20°C until analysis. The packaging process took place in the SFH toxicology lab under supervision of the researcher and lab staff. These measures helped maintain both security and integrity of the samples. Urine samples were stored within the secure unit of FMS in a freezer at -20°C until they were analysed. Only the researcher had access to the data. Figure 5.11 below summarises the study design.
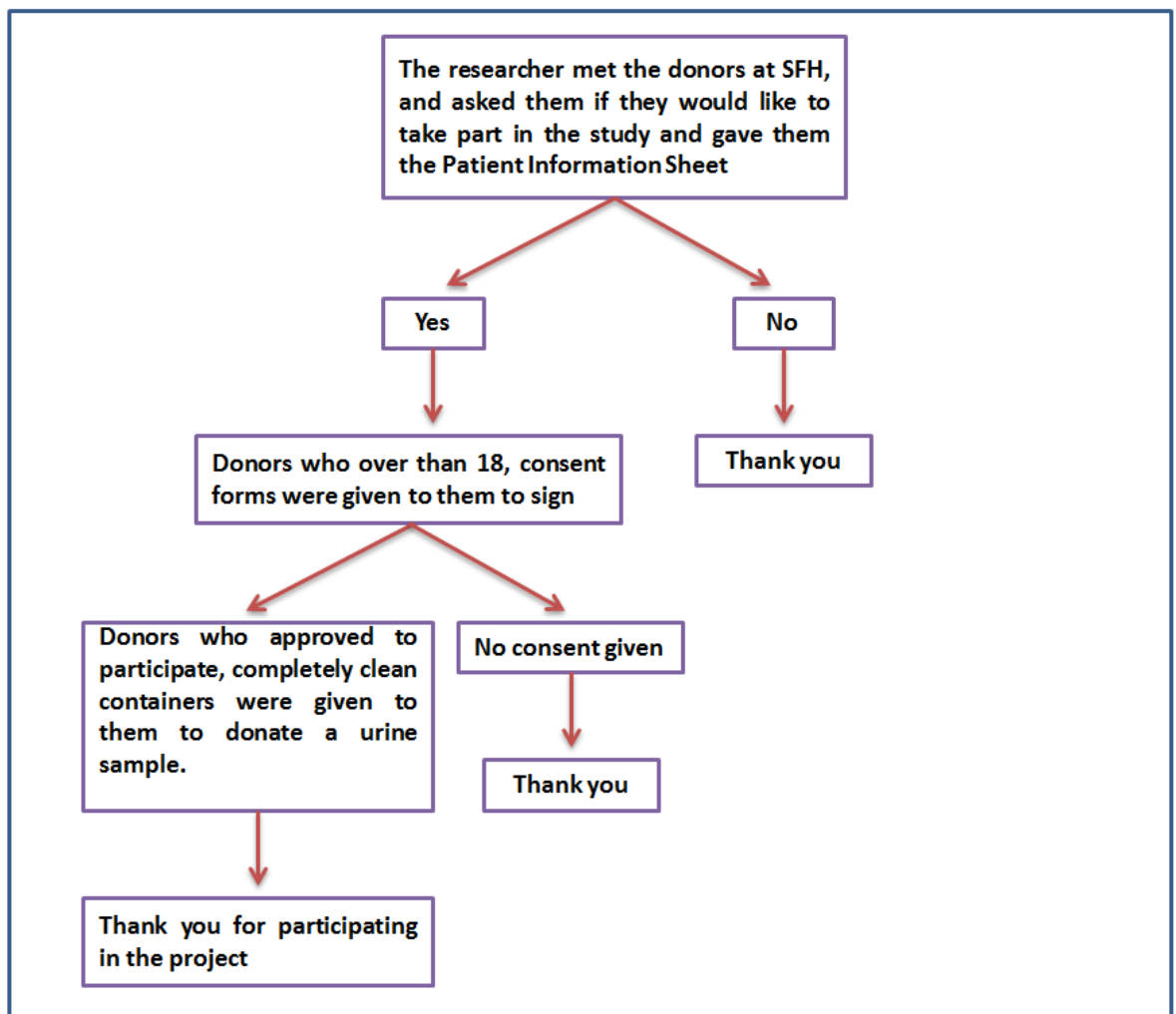
Figure 5.11: Summary of Study Design

5.2.5 Analysis of Urine Samples

Because the picture of use of SCs in Saudi Arabia is largely unknown, it was decided to select as target analytes some of the most prevalent SCs from different generations as follows:

- JWH-018 4-OH pentyl, JWH-073 3-OH butyl and JWH-250 4-OH pentyl (first generation 2008-2009).

- JWH-122 5-OH pentyl, JWH-210 5-OH pentyl and AM-2201 4-OH pentyl (second generation 2010-2011).

- 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole (third generation 2013-2014).

Chemical structures of these compounds can be seen in Figure 2.7. The urine samples were analysed using the method described in Chapter 3.

5.2.6 Results and Discussion

A total of 463 urine samples were collected from personnel who presented to SFH for WDT as a requirement for their job during the period of one month (July 2014). The results of the analysis found 2 of 463 samples to be positive for SCs (0.4%). 5F-PB-22 3-carboxyindole was detected in both of these casas with concentrations of 11 and 51 ng/mL respectively. No other SC drugs or metabolites were found. However it cannot be excluded that other SCs might have been present in the samples at either extremely low concentrations, or not included in the testing method due to a lack of reference standards, which may have caused false negatives. For these reasons, this data should be interpreted with caution.

Although only a few invited participants 2 (4.5%) refused to take part in this study, they may represent a significant number due to the sensitivity of the topic. It was not possible to analyse all WDT samples because the toxicology report that generates by SFH includes alcohol and specific drugs (amphetamine, cannabis, cocaine, barbiturate, benzodiazepine, opiates and methadone) and analyse any further drug needs acceptance from person who is applying for

employment, Moreover, the study was performed at a single institution in one city that may limit how representative it is. The overseas transport of urine samples may also have affected the results with respect to stability of the analytes, although this was mitigated by freezing the samples.

However, these results do indicate the presence of SC use in Saudi Arabia. The findings showed that SCs are a worldwide problem and the Kingdom of Saudi Arabia is no exception. 5F-PB-22 was detected in Scotland and Saudi Arabia. This may be the result of the proliferation of online retailers that enables NPS and SC distributors to reach new consumers across borders. In addition, Saudi Arabia is a big country and its many borders with other countries makes the control of smuggling difficult [339]. Unfortunately, there has been no other study measuring SCs in Saudi Arabia in order to compare with the present one and see the trends of SC use.

Drug misuse and trafficking have been recently recognised and acknowledged by the Saudi government as both a social and law enforcement problem. The GDNC in the Ministry of Interior, the National Committee for Combating Drugs (NCCD) and the Saudi Customs are the principal Saudi authorities dealing with this issue. The Saudi GDNC has 105 branches distributed in all the 13 governorates of Saudi Arabia. In April 2009, the NCCD was founded to coordinate the actions all related agencies. Official responses have become more comprehensive in the country, providing a three-pronged strategy which includes prevention, suppression and rehabilitation strategies to address illicit drug use [6]. However, the emergence of NPS including SCs does not get as much attention from the official authorities in Saudi Arabia as traditional illicit drugs.

Emergency scheduling of SC compounds must be undertaken and updated in Saudi Arabia by SFDA. Nevertheless, SCs could continue to be consumed, as the bans in some countries have proven ineffective in addressing their growing use as has been the case for traditional illicit drugs. The concerned authorities in Saudi Arabia should develop a programme of analytic work to address some of the key knowledge gaps. Awareness campaigns including television programmes, exhibitions and the launching of a website to illustrate the harmful effects of using NPS and SCs, for those who do not yet use NPS and also for those who already use NPS, should be launched as a prevention strategy. Cooperation with

international organisations regarding the tackling of NPS should be improved as a suppression strategy. Suitable methods for the detection of SC compounds should be added to the drug testing policy within Saudi Arabia. It is recommended that a new "Early Warning System" is created in the Arab Gulf in order to mimic the EU (EWS). This can improve the work on the fight against illicit drugs, especially NPS, through sharing information on the trends and appearance of NPS, along with reported harms, from Arab Gulf countries. Before this is set-up, all countries would benefit from accessing information already collated from other countries around the world including those in Europe and the USA.

The present study had some limitations. The major one was the lack of information regarding age, sex and screening results provided along with each sample due to the requirements of ethical approval that not have been given if age and sex were known.

5.3 Conclusions

5F-AKB-48, 5F-PB-22 and MDMB-CHMICA have been identified in Scotland in several cases among 3 subpopulations; A&E patients, prisoners and fatalities during a period from November 2013 to November 2015. 5F-PB-22 3-carboxyindole has been identified in 2 WDT urine samples in Saudi Arabia during the period of one month (July 2014).

The findings give an idea of the extent of use of SCs in Scotland and provide valuable information to the forensic community. They reflect the presence of the problem in Scotland's prisons, particularly among young people, as well as among A&E patients. It seems that 5F-AKB-48 and 5F-PB-22 are most popular in Scotland, or at least during the study period. This was also observed in Wales; according to WEDINOS [44, 338], 5F-PB-22 and 5F-AKB-48 were the most commonly identified of SCs between April 2014 and 31th March 2015. The findings described in this chapter reflect the range and extent of use of SCs in Saudi Arabia and shared an identical trend with Scotland. This is the first study that reports the detection of SCs using human urine toxicology in Saudi Arabia. More information regarding 5F-PB-22, 5F-AKB-48 and MDMB-CHMICA can be found in [56, 58, 59, 88]. The extent of poly-drug ingestion has significant implications

for management of A&E patients as well as for complicated decision-making within DRD.

Forensic communities must monitor the world drug market to ensure that clinical and forensic laboratories are keeping up with the illegal drug suppliers. When new compounds appear in the drug-user marketplace, toxicologists need to respond quickly and identify suitable biomarkers via *in vitro* studies to detect intake. Certified Reference Material suppliers and toxicologists need also to address the synthesis of reference standards for these new compounds. Nowadays, laboratories have gained more experience with these drugs. Therefore, toxicologists should collect larger databases of searchable mass spectra of many SCs and their metabolites and biomarkers that can indicate SC use. However the problem of LC-MS/MS spectra being different between labs and instruments remains.

Chapter 6 - Synthetic Cannabinoids in Saudi Arabia – Survey Study

6.1 Introduction

A survey is defined as a series of questions used to gather information from a sample of a population [355]. The primary purpose of a survey study is to describe characteristics of the population [356]. The description of the whole population is inferred by the findings and interpretations obtained from the sample. In survey studies, researchers ask questions about peoples' characteristics, behaviour and beliefs. Researchers can investigate the associations between different respondents' characteristics and their current attitudes toward some issue. Survey studies typically describe the distributions of variables in a target population but do not make causal inferences [355].

The types of surveys depend on the time frame for data collection and are longitudinal or cross-sectional surveys [355]. A longitudinal survey involves the collection of information at different points in time in order to study changes that occur over a time whereas; a cross-sectional survey involves the collection of information from participants at just one point in time or over a short time period [355].

There are several techniques to obtain information in survey research such as interviews, questionnaires and data mining (e.g. interrogating a database for causes of death). Interviews can be conducted in person (face-to-face) or by telephone. Questionnaires can be handed directly to the participants, completed on the Web, or sent and returned by email or post. Choosing between interviews and questionnaires is an important decision in survey research and it depends on several research aspects. For sensitive topics a questionnaire is generally a better choice because it is more anonymous. It is a type of self-report method which involves questions often in a structured written form. Self-reports have been widely used in forensic toxicology (See Section 2.3.8). For complex topics, interviews are usually preferred because the researcher can explain questions that the participants may not understand.

According to the EMCDDA [295], a number of surveys based on a questionnaire have studied the prevalence of Spice use but their coverage and representativeness is still limited (See Section 2.3.3 for details).

6.2 Aim

The aim of this study was to determine the extent of awareness and usage of Spice products in 3 subpopulations of Saudi Arabia (known drug users, medical professionals and members of the public in and around smoking cafes).

6.3 Ethical Approval

Ethics approval was given by the College of Medical, Veterinary and Life Sciences (MVLS) Ethics Committee for Non-Clinical Research Involving Human Subjects at the University of Glasgow (See Appendix 6) and they were also approved by the General Administration for Research and Studies at the Ministry of Health of Saudi Arabia (See Appendix 10). The letter of approval obtained from the Saudi Ministry of Health was sent to the Al-amal Complex for Mental Health in Riyadh to get permission for this study.

Three documents were prepared during the processing of ethical approval including the application form, a participant information sheet (See Appendix 11) which contains basic information regarding the purpose of the research in lay terms for all participants to read, and a consent form (Appendix 9). The patient information sheet and consent form were in English and Arabic languages.

6.4 Methodology

Survey studies allow the collection of information from a large sample of individuals relatively inexpensively and quickly. However, it is not an easy process. In order to yield valid and reliable information, planning, implementation, and analysis should be conducted carefully. Figure 6.1 shows the process that has been conducted in this study. The cyclical design reflects the use of a pilot survey prior to the main survey.
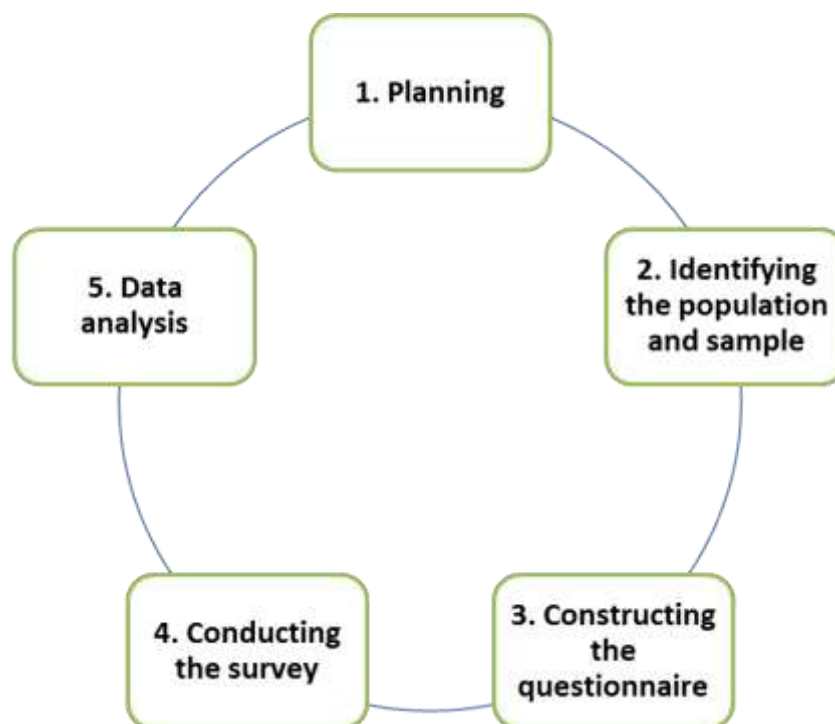
Figure 6.1: The Basic Process of the Study [355]

6.4.1 Planning

The purpose of the study was to explore the knowledge and usage of Spice among three different populations in Saudi Arabia including known drug users, medical professionals and members of the public in and around smoking cafes. A cross-sectional design with self-administered questionnaire was used to collect data. A questionnaire has been chosen because the topic is quite sensitive and the questionnaire can provide more anonymity than other techniques.

6.4.2 Identifying the Population and Sample Size

A population is defined as all individuals of the group that meet the selection criteria to be studied, whereas a sample is a portion of a population [355]. A common goal in choosing the sample is whether the individuals selected are representative of the population. Both the number and 'type' of respondents are important. The two main types of sampling procedures include nonprobability and probability sampling. Nonprobability sampling contains non-random procedures for selecting the subjects of the sample. In this type of sampling, there is no assurance that every member in the population has a chance of being included [355]. Probability sampling is the type of sampling in which every subject has an equal chance of being selected [355]. It includes simple random, stratified, cluster and systematic sampling [355]. Random sampling technique

was applied in the present study to select participants in each group. In this method, individuals of the population were selected in a manner such that everyone had an equal chance of being selected.

In general, the sample size required will be determined by many factors including willingness of people to participate, the budget of the project and the importance of the research. The determination of sample size is an important step of survey study design because inadequate, excessive or inappropriate sample sizes might influence the accuracy and quality of research [357]. Inadequate sample size will not allow reliable insights and excessive sample size will lead to a waste of money and time.

To determine the appropriate sample size, the confidence level and confidence intervals (the level of precision) should be specified dependent on the required accuracy. The level of confidence or risk is the percentage of all probable samples that can be distributed normally about the true value [358]. A confidence level of 95% means that 95 out of 100 samples will be representative of the true population value. A confidence interval or margin of error refers to the positive and negative deviation allowed on survey results for the sample. It is often expressed as: estimate ± margin of error.

After deciding the desired accuracy for the sample data (e.g. 1%, 5%, 10% …), the sample size can be calculated by several methods. A first approach to determine the sample size is to rely on published tables. The second way is to use the same sample size as in similar studies. This approach should review the procedures employed in these studies to avoid repeating any errors. Another approach to calculate the sample size is to use one of several formulas.

6.4.2.1 Medical Professionals

The first target population was Saudi medical professionals including physicians, pharmacists, nurses and medical technologists. This population was selected because of the nature of their work to examine the awareness and use of SCs.

According to the Saudi Commission for Health Specialties, there were a total of 139,309 medical professionals in SA in 2014 [359]. The sample size has been

calculated according to the simplified formula provided by Yamane [341]. A 95% confidence level has been decided. The margin of error on either side of the proportion was estimated to be 5%. The equation is below [341]:

$$n = \frac{N}{1 + N(e)^2}$$
Equation 11

Where N is the population size, n is the sample size and e is the level of precision (confidence intervals).

$$n = \frac{139,309}{1 + 139,309(0.05)^2} = 399$$

The target sample size for the medical professionals group was 399 individuals.

6.4.2.2 Members of the Public in and around Smoking Cafes

The second population of interest for the current study were members of the public in and around smoking cafes or shisha smokers in Saudi Arabia. Shisha smoking is very popular in Saudi Arabia. There are many shisha smoking cafes in Saudi Arabia particularly in the larger cities. Shisha smokers usually spend one to three hours in these cafes mostly in groups. Several articles showed that there is an association between smoking and using illicit drugs [361, 362]. NPS such as SCs are likely to be used by smokers; this project was specifically aimed at reaching this target population. In addition, the majority of shisha smokers in these places are adolescents and this age group has been strongly associated with SC use (See Chapter 2.3.3).

There is no data about Saudi shisha smokers in 2014. However, it was estimated indirectly through the available data. According to the report in 2005 of Saudi Ministry of Health (MOH) in collaboration with the World Health Organization, the prevalence of shisha smoking among the Saudi population is 4.2% [363]. According to the Saudi Central Department of Statistics and Information, Saudi Arabia's population stood at 22.7 million at the end of 2004, of which 16.5 million were Saudis, making up 72.9 percent of the population [364]. Therefore the number of Saudi shisha smokers in 2004/2005 was around 693,000. Using

Equation 11, the target sample size for shisha smokers group was 400 individuals.

6.4.2.3   Known Drug Users

The third target population was known illicit drug users in Saudi Arabia. SCs are likely to be used by illicit drug users for several reasons including trying to evade detection, due to the misconception they are safer, availability, low cost or to experiment with new effects.

As mentioned previously, the number of known drug users in Saudi Arabia is not clear. It is impossible to estimate the size of the population and consequently to calculate the sample size. Therefore, it was considered that the target population was all known drug users in Al-amal hospital in Riyadh giving a sample size of 200 individuals.

6.4.3 Constructing the Questionnaire

6.4.3.1   Preparing the Questionnaire

After planning (defining the problem) and determining the sample group sizes, the next step was the preparation of a questionnaire. Cross-sectional designs usually take less time to complete and require less dedication from research participants compared to longitudinal designs [355]. In order to prepare the questionnaire, an account was created with SurveyMonkey. The questionnaire was in English and Arabic languages and involved an introductory message and the questions. The introductory message was located on the top of the questionnaire form and explained briefly the importance of the respondents' participation and gave a statement guaranteeing confidentiality to encourage people to take part (See Appendix 12).

Four processes were conducted to prepare the questions including deciding, wording, ordering and formatting. These steps might help to maximise the response rate and obtain accurate relevant information for the study.

Several questions that have a key link with the research aims were generated. Depending on the information type, the questions were grouped into 3 sections.

A question to explore whether or not the respondents worked in medical fields was added to confirm that all the participants were relevant to this group (See question 5 in Appendix 12). Three core questions were compiled to obtain primary information. For ethics issues and because of the sensitivity of the topic, the question "Have you ever smoked synthetic cannabis, Spice or k2?" was deleted and replaced by the question "Do any of your friends smoke these drugs?". This question aimed to investigate the general prevalence of Spice usage. However, this limited the information that could be generated from the survey.

General rules for wording the questions have been followed to construct good questions and minimise bias in the questionnaire. Short and simple sentences were generally used. Ambiguous, leading and negative questions were avoided to reduce confusion amongst participants. Closed, multiple choice and open questions were used. A suitable order for the questions was very important to reduce missing data. The particularly sensitive questions were left to the end of the questionnaire to give the participant a chance to feel relaxed. The layout and formatting of the questions and answer choices was important to make it as easy and quick as possible to complete a questionnaire. The questionnaire was fitted on to two pages: the first contained only the demographic section, whereas the second involved the behaviour and the core sections. Plenty of grey space between these three sections and enough room to answer questions were provided. The headings and questions were in bold.

6.4.3.2 Piloting and Evaluating Questionnaire

It is very useful to test the questionnaire and survey methodology on a few participants prior to conducting the main survey. This pilot study is used to detect any flaws in the questionnaire. The questionnaire was piloted with 12 friends and colleagues from the UK and Saudi Arabia..

During the pilot study, three questions were asked to every participant: "Were any questions ambiguous?", "How long did it take to complete?" and "Are there any suggestions?". The feedback from the pilot sample was useful. Based on the results of the pilot study, participants were generally happy inputting their responses. However, the pilot indicated wording in Arabic language of some

questions in the behaviour section could be improved by using common words. Changes in the translation of that question were made to accommodate this situation.

The participants of the pilot sample took approximately 2 to 5 minutes to complete the questionnaire. The general view was that it was feasible to continue the survey. The questionnaire was finalised and printed in two versions; (with page numbers) for the shisha smokers group and (without page numbers) for known drug users group to make the process of data transfer from questionnaire papers to an Excel file easier to organise.

6.4.4 Conducting the Survey

The questionnaires were distributed to participants in Saudi Arabia in two ways, both online and by paper, but they contained the same questions. All the questionnaires were conducted over a 2 month period during December 2014 and January 2015. Any information produced by these individuals was completely anonymous.

6.4.4.1   Online Questionnaire

An online questionnaire (self-administered) was used specifically for the medical professional population. This method was deemed most appropriate for this group for several reasons. Firstly, members of this group were literate and could interpret questions without assistance, secondly, it was relatively easy to identify and access the sample population through the Saudi medical group's emails and finally, self-administered questionnaires can be completed at the respondent's convenience.

The target sample size of the medical professional population was 399 individuals (See Section 6.4.2.1). However, the response rate must be taken into account, particularly for online and postal surveys. Approximately 550 emails were sent to those individuals.

The email included a welcoming message for medical professionals and offered them to partake in the research project by filling out a questionnaire. The email was attached with the web link for the questionnaire (generated by

SurveyMonkey) and also involved information about the purpose of the research project.

6.4.4.2   Paper Questionnaire

The paper survey was aimed at two Saudi populations: shisha smokers and known drug users. Participants in these two groups were identified from engagement with people in public spaces (smoking cafes) and participants at Al-amal hospital in Riyadh (for known drug users). Permission was obtained from cafes and hospital.

The target sample size of the Saudi shisha smoker population was 400 individuals (See 6.4.2.2). Assuming a non-response rate of 20%, 480 questionnaires were distributed to members of the public in and around smoking cafes in Riyadh. The target sample size of the Saudi known drug users was 200 individuals (See 6.4.2.3). However, only 90 questionnaires were distributed to this group due to the restricted facilities of some departments at Al-amal hospital including female and children departments.

Participants were asked if they would like to take part in the study and given the information sheet to read. After a potential participant completed reading the information sheet and agreed to participate, the consent form was supplied for them to sign and the questionnaire was completed. The consent forms were kept separately from the questionnaires to maintain anonymity and kept in a secure place by the researcher. All participants were allowed to opt out of the study at any time. Each participant completed only one questionnaire.

6.4.5 Data Analysis

This part presents different statistical techniques that were used for editing, coding and analysing the data. The choosing of statistical software is very important at the questionnaire design stage. The data was transferred from an Excel file to the Statistical Package for the Social Sciences (SPSS) for analysis. SPSS is a software package used for statistical analysis. In the present study, IBM SPSS Statistic 21 was provided through the University of Glasgow and used to produce statistics of descriptive and frequency for all variables as well as to perform chi-square tests.

Chi-square test (also called x^2) was used to explore relationships between variables. It compares observed frequencies and expected frequencies for categorical variables. Chi-square tests determine whether different kinds of respondents (e.g., male and female) gave significantly different survey responses. A *p*-value will be significant if less than 0.05.

6.4.5.1 Response Rates

In total, 391 questionnaires were returned: 184 questionnaires were completed by medical professionals, 170 by shisha smokers and 37 by known drug users. After collecting all the questionnaires of the three groups, the data was ready to process.

The response rate in survey research is the number of responses divided by the number of people invited to take part in the survey. It is usually expressed in the percentage form. The response rate for self-administered questionnaires is usually low. However, the overall response rates were 33.5%, 35.4% and 41.1% for the medical professionals, shisha smokers and known drug users, respectively and these are considered acceptable rates.

Four of the medical professionals who participated in the survey did not answer more than three-quarters of the questions. It was appropriate to remove those four participants. Subsequently the sample size of medical professional group became 180 participants. The missing data for the rest of all other participants in the three groups was tiny and randomly scattered through the data field accounting for only 1.1%. The sample size of the three groups was sufficient to achieve the aims of the project. The missing data was coded by (999).

6.4.5.2 Editing and Cleaning

Socio-demographic, behaviour and core data from the questionnaire were initially collected and coded. Independent and dependent variables have been used in the present study. Independent variables consist of socio-demographic and behaviour variables. Dependent variables include the data related to the legal highs and Spice knowledge and usage of participants. Coding of the variables and comments is shown in Table 6.1.

Table 6.1: Variables and Coding

Variable	Definition / Question	Coding
Sex	Gender of respondent	Female = 0, Male = 1
Home	Where does the respondent live?	Riyadh = 1, Jeddah = 2, Dammam = 3 Other = 4
Age	Age of respondent	18 – 25 = 1, 26 – 40 = 2, 41 – or over = 3
Education	Educational level of respondent	High school or less = 1, Undergraduate level = 2, Postgraduate level = 3
Behaviour 1	I feel my life is very stressful	Agree = 1, Disagree = 0
Behaviour 2	When I feel upset or down, I am likely looking for medication	Agree = 1, Disagree = 0
Behaviour 3	When I feel upset or down, I am likely looking for a herb	Agree = 1, Disagree = 0
Behaviour 4	I like to try new herb products	Agree = 1, Disagree = 0
Behaviour 5	When I feel upset, I usually ask my friends to give me something could be help	Agree = 1, Disagree = 0
Behaviour 6	I usually buy the herb products that I have seen advertised	Agree = 1, Disagree = 0
Behaviour 7	I usually buy the herb products that my friends recommended to me	Agree = 1, Disagree = 0
Behaviour 8	I like to access the internet	Agree = 1, Disagree = 0
Knowledge 1	Have you heard of new psychoactive substances or legal highs?	Yes = 1, No = 0
Knowledge 2	Have you heard of synthetic cannabis, Spice or K2?	Yes = 1, No = 0
Comments	Where did you hear about "legal highs"/"Spice"?	No comment = 1, On internet = 2, Social media and TV = 3, Lectures = 4, Friends = 5, Literatures = 6
Use	Do any of your friends smoke "Spice" or K2?	Yes = 1, No = 0

6.5 Results and Discussion

6.5.1 General Descriptive

General descriptive statistics were used to describe the data. The study sample included males and females aged 18 years or over. No expatriates were included in this study. The participants were randomly selected from three target populations including medical professionals, members of the public in and around smoking cafes and known drug users in Saudi Arabia, during December 2014 and January 2015. Figure 6.2 below illustrates study sample derivation.
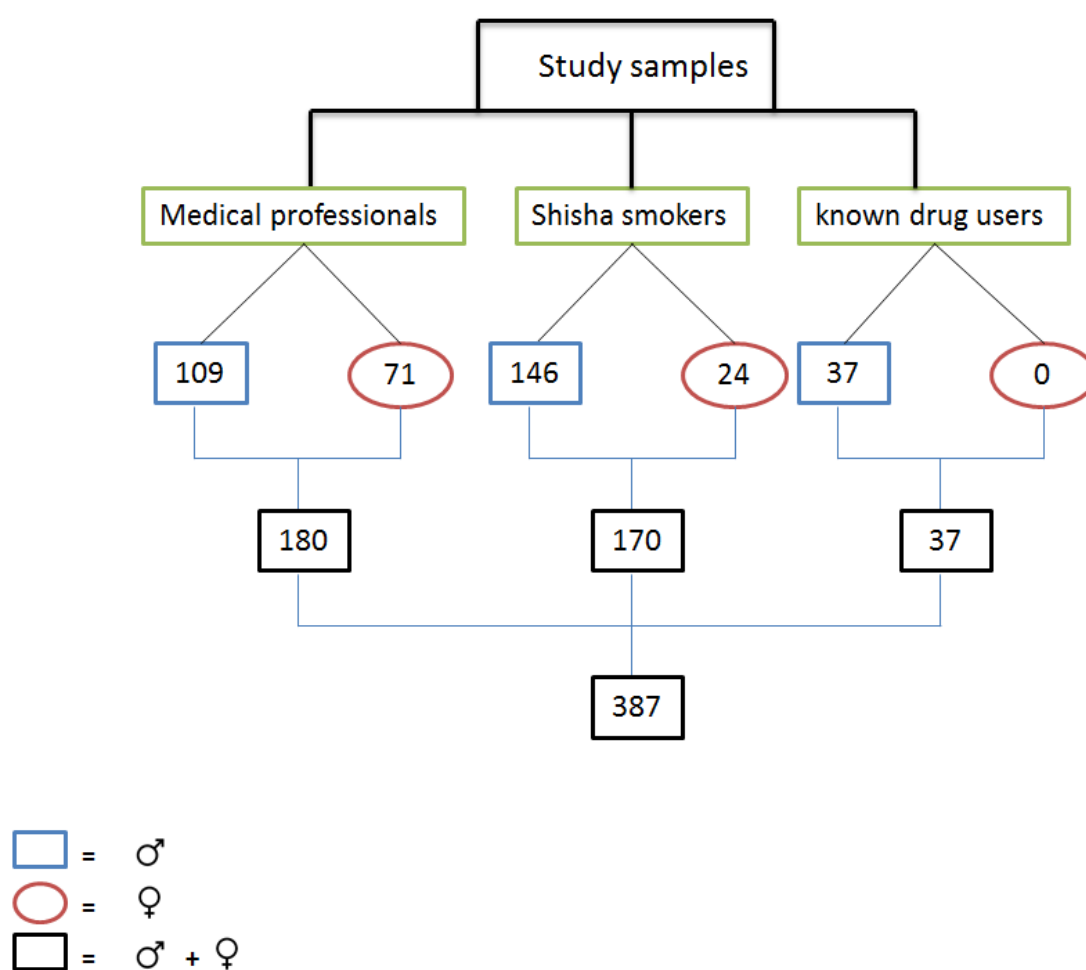
Figure 6.2: Study Sample Derivation

6.5.1.1   Socio-demographic Data

1) Medical Professional Population:

Details of these participants are shown in Table 6.2. The analysis of the population of Saudi medical professionals was based upon a sample of 180 (71

(39%) females, 109 (61%) males). Nearly half (52%) of female participants were between 18-25 years whereas only (5%) of male participants belonged to this age group. This is potentially due to the increased attention of the Saudi government to encourage education for women and open many new medical and health colleges for them in recent years. There were significant differences between the percent of men and women in each age group (Chi-square= 55.7, p <0.0001).

Forty four percent of male participants had postgrad degrees, while only 14% of female participants have this level of education. There were significant differences between the percent of men and women in each education level (Chi-square= 18.6, p <0.0001).

It was shown that almost a third (29%) of male participants was living in Riyadh, whereas approximately 41% of female participants lived in Dammam. About 37% of participants lived outside the main cities of Riyadh, Jeddah and Dammam.

Table 6.2: Socio-demographic Characteristics of Medical Professional Participants (*n*=180)

Characteristic	All (n=180) (%)	Female (n=71) (%)	Male (n=109) (%)	P *
Age groups				<0.0001
18-25 years	42 (23)	37 (52)	5 (5)	
26-40 years	106 (59)	29 (41)	77 (71)	
41 - or over	32 (18)	5 (7)	27 (25)	
Total	180	71 (39)	109 (61)	
Education				<0.0001
High school or less	11 (6)	4 (6)	7 (6)	
Undergraduate level	111 (62)	57 (80)	54 (50)	
Postgraduate level	58 (32)	10 (14)	48 (44)	
Total	180	71 (39)	109 (61)	
Location				0.021
Riyadh	38 (12)	7 (10)	31 (29)	
Jeddah	22 (12)	9 (13)	13 (12)	
Dammam	53 (30)	26 (37)	27 (25)	
Other	65 (37)	29 (41)	36 (34)	
Total	178	71 (40)	107 (60)	

* Value was significantly different between males and females (*P* <0.05) based on chi square analysis

2) Members of the Public in and around Smoking Cafes Population:

Details of these participants are shown in Table 6.3. A total of 170 participants were recruited. The majority were men, 146 (86%), reflecting that the prevalence rate of shisha smoking is generally higher in men in Saudi Arabia [363]. The majority of participants (77%) were between 18 and 25 years. There are no significant differences between the percent of men and women in each age group (Chi-square= 4.8, p =0.092).

The majority of male participants (77%) were educated to high school or less. There were significant differences between the percent of men and women in each education level (Chi-square= 10.5, p =0.005). Most of the participants (68%) were living in Riyadh as this study was carried out in cafes in Riyadh. There were no significant differences between the percent of men and women at each location item (Chi-square= 4.4, p =0.226).

Table 6.3: Socio-demographic Characteristics of Shisha Smoker Participants (n=170)

Characteristics	All (170) (%)	Female (n=24) (%)	Male (n=146) (%)	P *
Age groups				**0.092**
18-25 years	131 (77)	15 (63)	116 (80)	
26-40 years	36 (21)	9 (38)	27 (2)	
41 - or over	3 (2)	0	3 (2)	
Total	170	24 (14)	146 (86)	
Education				**0.005**
High school or less	123 (73)	12 (50)	111 (77)	
Undergraduate level	41 (24)	12 (50)	29 (20)	
Postgraduate level	5 (3)	0	5 (3.4)	
Total	169	24 (14)	145 (86)	
Location				**0.226**
Riyadh	115 (68)	19 (79)	96 (66)	
Jeddah	10 (6)	1 (4.2)	9 (6)	
Dammam	7 (4)	2 (8.3)	5 (3)	
Other	38 (22)	2 (8.3)	36 (25)	
Total	170	24 (14)	146 (86)	

* Value was significantly different between males and females (P <0.05) based on chi square analysis

3) Known Drug User Population:

Details of these participants are shown in Table 6.4. A total of 37 male participants were recruited. On the basis of the instructions given by the Al-amal hospital, female participants were excluded. Nearly half (49%) of participants were aged between 26-40 years. The majority of participants (92%) were educated to only high school. Seventy three percent of those participants lived in Riyadh as the study was conducted in a hospital in Riyadh.

Table 6.4: Socio-demographic Characteristics of Known Drug User Participants (*n*=37)

Characteristics	Male (n=37) (%)
Age groups	
18-25 years	14 (38)
26-40 years	18 (49)
41 - or over	5 (14)
Total	37
Education	
High school or less	34 (92)
Undergraduate level	3 (8)
Postgraduate level	0
Total	37
Location	
Riyadh	27 (73)
Jeddah	4 (11)
Dammam	2 (5)
Other	4 (11)
Total	37

6.5.1.2   Behaviour Data

Detailed in Table 6.5 are the behaviour characteristics of all 3 groups. Nearly half of those participants of medical professional (53%) and known drug user (57%) think their life is very stressful, while only (34%) of those participants of shisha smokers have this feeling. About 40% of medical professional participants were looking for an herb when they are feeling upset, whereas 17% of them are looking for a medication. The same trend can be observed in the other two populations. These findings were expected and compatible with a number of studies that reported a significant percentage of Saudi people using herbs for different reasons [365, 366]. Overall 86%, 76% and 62% of participants of medical professionals, shisha smokers and known drug users, respectively, reported they

liked to access the internet for general use. These results were also expected. A study [367] conducted during 2009 in several areas of Saudi Arabia showed that 84% of Saudi people were found to be internet users.

Table 6.5: Behaviour Characteristics of All 3 Groups (*n*=387)

Characteristics *	Medical professionals All (N=180) (%)	Shisha smokers All(N=170) (%)	Known drug users All (N=37) (%)
I feel my life is very stressful	95 (53)	56 (34)	21 (57)
Total	179	167	37
When I feel upset or down, I am likely looking for medication	30 (17)	60 (36)	12 (33)
Total	178	166	36
When I feel upset or down, I am likely looking for a herb	71 (40)	71 (42)	16 (44)
Total	179	169	36
I like to try new herb products	23 (13)	31 (19)	15 (42)
Total	178	168	36
When I feel upset, I usually ask my friends to give me something could be help	36 (20)	46 (28)	18 (50)
Total	178	163	36
I usually buy the herb products that I have seen advertised	30 (17)	31 (18)	6 (17)
Total	178	169	36
I usually buy the herb products that my friends recommended to me	49 (28)	46 (27)	10 (27)
Total	178	169	37
I like to access the internet	153 (86)	128 (76)	23 (62)
Total	178	169	37

* The analysis based on AGREE answer

6.5.2 The Knowledge and Sources of Information of Legal Highs and Spice in Saudi Arabia

With regards to a Saudi's general awareness of legal highs, 19%, 19% and 22% of those participants of medical professionals, shisha smokers and known drug users, respectively, were aware of the existence of new psychoactive substances or legal high drugs. The awareness of Spice products is slightly less than that of legal highs, with 16%, 11% and 22% of those surveyed of medical professionals, shisha smokers and known drug users, respectively. Figures 6.3 and 6.4 show the results of the knowledge of legal highs and Spice respectively among all three groups. There were no significant differences between women and men

participants in their knowledge of Spice in medical professional and shisha smoker participants (Chi-square= 0.94, *p* = 0.333) and (Chi-square= 1.3, *p* = 0.263), respectively.
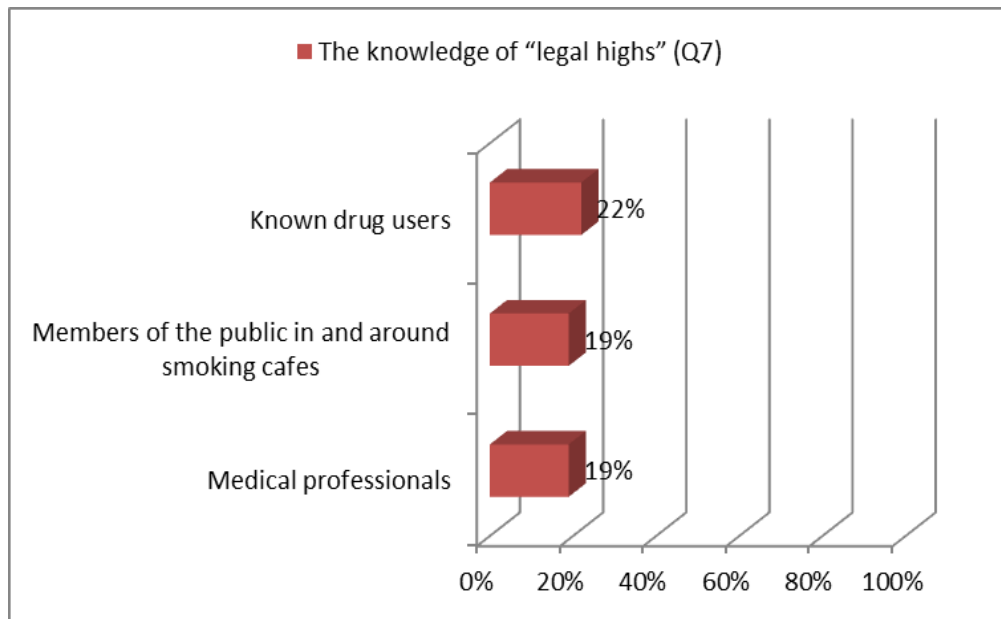
Figure 6.3: The Knowledge of Legal highs (Q7) among All 3 Groups
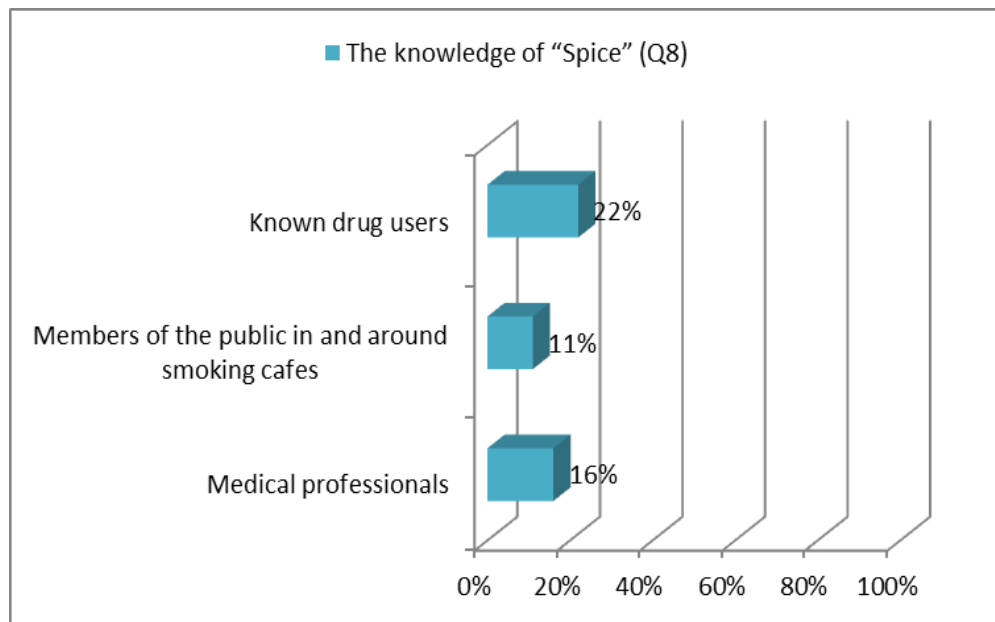
Figure 6.4: The Knowledge of Spice (Q8) among All 3 Groups

Internet, social media and TV, lectures, friends, literatures and at work were the reported means by which respondents became aware of these products. The results are shown in Figure 6.5.
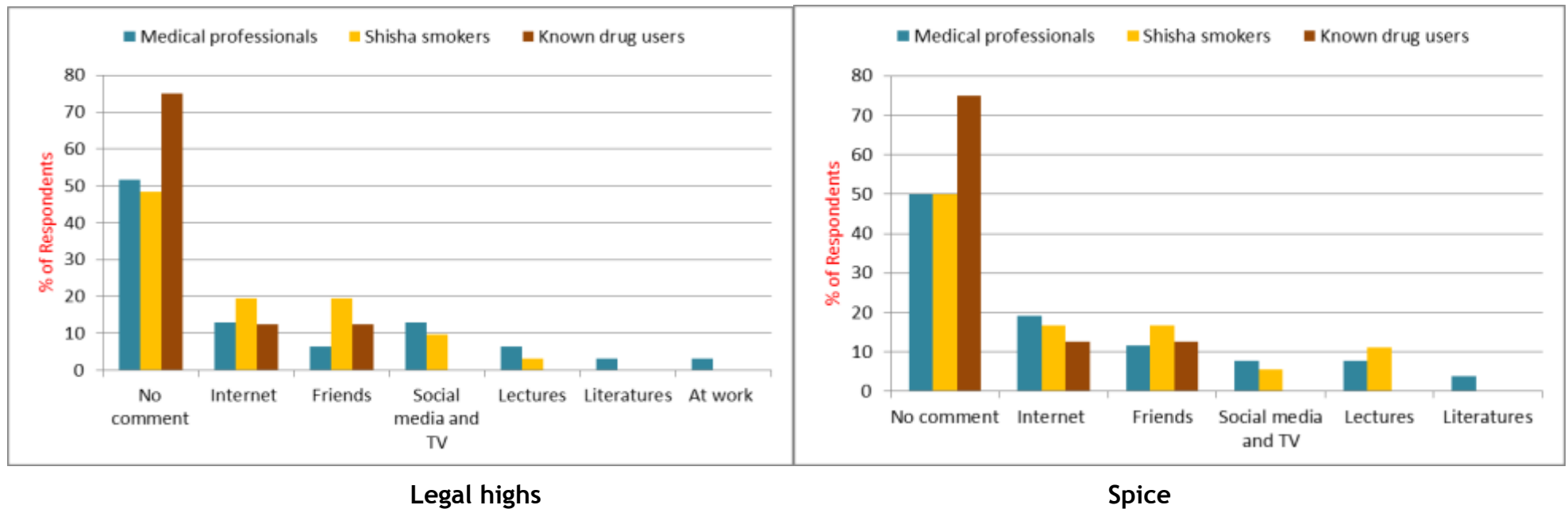
Legal highs   Spice

Figure 6.5: Sources of Information Regarding Legal highs (Q 7) and Spice (Q 8) in All 3 Groups

6.5.3 The Socio-demographic and Behaviour Characteristics of those having Knowledge of Legal Highs and Spice in Saudi Arabia

Table 6.6 summarises the main findings for the socio-demographic and behaviour characteristics that were found among participants having knowledge of legal highs and Spice in all populations. Among the respondents that reported having knowledge of legal highs and Spice, 82% and 83%, respectively, were men. It is not clear which factors might play a role in this result. About 50% and 42% of participants reported having knowledge of legal highs and Spice, respectively, were between 18 and 25 years. 79% and 85% self-reported having knowledge of legal highs and Spice, respectively, like to access the internet.

Table 6.6: Socio-demographic and Behaviour Characteristics Associated with Knowledge of Legal highs and Spice in All Populations

Characteristics *	Legal highs All gender (*n*=70)(%)	Spice All gender (*n*=52)(%)
Socio-demographic characteristics		
Gender		
Female	12 (17)	9 (17)
Male	58 (82)	43 (83)
Age groups		
18-25 years	35 (50)	22 (42)
26-40 years	27 (39)	24 (46)
41 - or over	8 (11)	6 (12)
Education		
High school or less	32 (46)	20 (39)
Undergraduate level	25 (36)	22 (42)
Postgraduate level	13 (19)	10 (19)
Location		
Riyadh	32 (46)	24 (47)
Jeddah	7 (10)	8 (15)
Dammam	14 (20)	7 (14)
Other	17 (24)	13 (25)
**Behaviour characteristics **		
I feel my life is very stressful	21 (30)	22 (42)
I like to access the internet	55 (79)	44 (85)
When I feel upset or down, I am likely looking for a herb	22 (31)	13 (25)
I like to try new herb products	16 (23)	9 (17)
I usually buy the herb products that my friends recommended to me	20 (29)	15 (29)

* The analysis is based on **AGREE** answers

6.5.4 The Use of Spice Products in Saudi Arabia

In sensitive questions, it is usually difficult to obtain truthful answers which may lead to negative responses or even no response. In the present study, the "other people approach" was used to estimate the prevalence of Spice usage in Saudi Arabia. 3.0%, 2.4% and 8.1% of those participants of medical professionals, shisha smokers and known drug users, respectively, reported having friends that have smoked Spice or K2 products in their lifetime (Figure 6.6).

It has been difficult to compare the results obtained from the present survey with other surveys (See 2.3.8) because each researcher or team has used different methods (e.g. definitions, terminologies) and age ranges. However, the present survey can be used to give indications, particularly in the case of lack of prevalence studies of SCs among Saudi people.

In the present study, the overall percentage of usage of Spice products in Saudi Arabia was 4.5%, compared with the most recent findings from the SALSUS survey (4% of 15-year-olds having used one or more NPS and most commonly SC) in 2013; it seems there is no big difference in the prevalence of SC use between Scotland and Saudi Arabia.
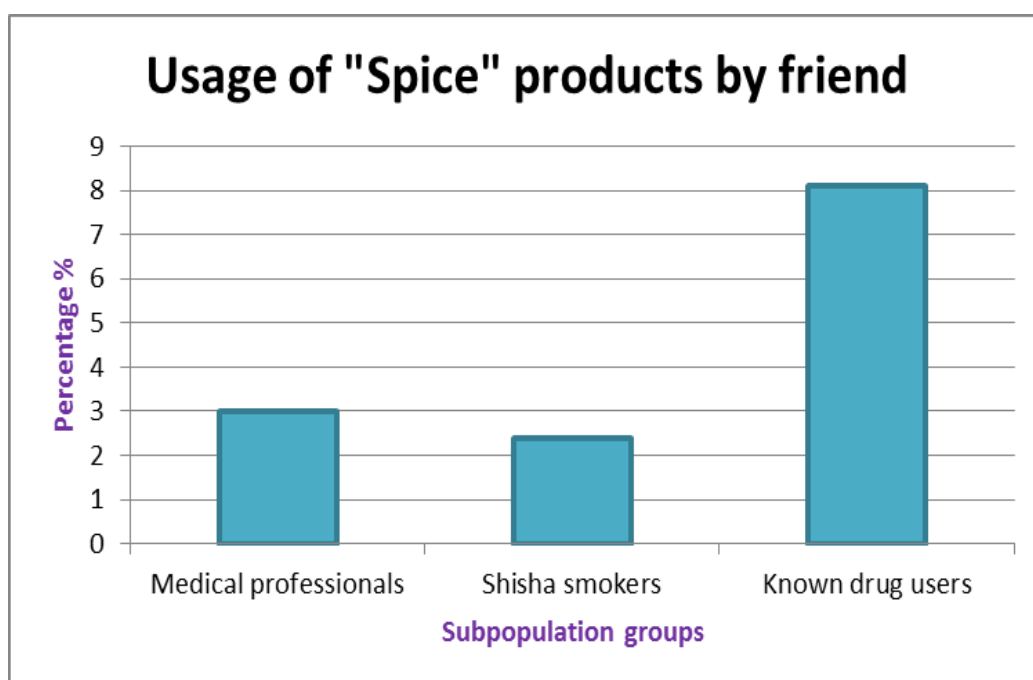
Figure 6.6: Percentage of Respondents who Reported Having Friends that Have Smoked "Spice"

6.5.5 Limitations of the Study

Although great care was taken to ensure that the present study was as accurate as possible, several limitations might have impacted on the results of the study. Firstly, the sample size may not have been fully representative of the whole population. Various factors have affected the achieved sample size including time limitations, willingness of people to participate and the sensitivity of the topic. Furthermore, known drug user population contained only male participants that may also limit the present survey. The elimination of a direct use question prevented examining whether the prevalence of SC use among these groups partly depends on their socio-demographic variables. These limitations could limit the overall generalisability of the results; however, it is sufficient to be statistically viable.

Many aspects should be considered in this situation. These types of surveys are usually conducted by national committees and research centres because they plan the national strategies and have the facilities and resources. As there are no national surveys studying these types of drugs among Saudi society, the present study remains very useful in taking a snapshot of these emerging drugs in the Saudi population.

6.6 Conclusions

The questionnaire prepared was easy to comprehend, complete and return. Participation was not time consuming, taking less than 5 minutes. Associations were analysed in Chi-square statistics to assess statistical significance. The study population consisted primarily of three different populations in Saudi Arabia including known drug users, medical professionals and in members of the public in and around smoking cafes.

This study attempted to understand the significance of SC use among Saudi society. Overall, data obtained from the current study suggests that a significant percentage of the population under examination is not cognisant of the existence of legal highs and/or Spice products. It also suggests that usage of these products among Saudi individuals may be similar to the usage in Scotland. The findings of this study highlight the need to educate potential users of these

drugs and that the internet would provide an effective way of doing this. Whilst the numbers found are low it is important to realise that this is an ever-changing landscape and therefore it would be useful to carry out the same survey on a regular basis (e.g. annually) to see if there is a rising trend of use. Really this study gives baseline knowledge and further studies should build on this.

Chapter 7 - Conclusions

The consumption of NPS, particularly SCs, is a matter of rising concern to society. SCs and their potential role in forensic cases have been investigated in this project. It would appear that the major motivations for SC use are the legal status of these drugs, their lack of detection in common drug-screening tests and their low price level as well as the widespread marketing. Their rapid proliferation on the world market and absence of certified reference materials might resulted in several analytical challenges. It was found that SCs are not ideal compounds for GC-MS analysis in biological samples. LC–MS/MS is the preferred instrument for the detection of SCs in biological samples because it provides better sensitivity.

A simple, cost-effective and accurate LC-MS/MS method for the simultaneous quantification of 10 parent SCs in whole blood and 8 corresponding metabolites in urine has been developed and validated. LLE was an important part of both methods and provided clean extracts and high recoveries of SCs from both blood and urine samples. Moreover, LLE was time-saving and little solvent was required. In addition, using MTBE as solvent and 1 M sodium acetate buffer (pH 5.0) improved the recoveries. Stability studies are a crucial issue in forensic toxicology where loss of analyte from samples could adversely affect the quality of test results. The stability of 5F-AKB-48 (*N*-4 OH pentyl) and 5F-PB-22 3-carboxyindole in methanol/acetonitrile solutions were studied for up to 3 months and were found to be relatively stable, with only the 5F-PB-22 metabolite degrading at room temperature in methanol. Due to the stability issues of SCs in biological samples, it is recommended to keep samples frozen whenever possible and sample extracts should be analysed shortly after extraction.

Selection of the appropriate specimen is an important step in forensic toxicology. In blood, parent compounds of SCs are found at extremely low concentrations therefore methods for analysis of SCs in blood must be very sensitive. Urine is the most commonly collected sample for monitoring recent exposure. SCs have an extensive metabolism, therefore the main target compounds in urine specimens are their hydroxyl- and carboxy-metabolites.

However, the analysis of drugs in urine is complex because it requires knowledge and understanding of the drug's metabolism.

Due to the recurring threat of SCs to public health and their rapidly increasing usage worldwide, the occurrence of SCs in Scotland and Saudi Arabia were evaluated. The prevalence of use of SCs is hard to ascertain because the drug-using population is hidden.

In Scotland, a study using blood and urine analysis was carried out among 3 different subpopulations; A&E patients, prisoners and fatalities. The findings reflected the presence of the problem in A&E patients and prisons in Scotland, particularly among young people. It was noticeable in this research that regulation of SCs does not necessarily reduce DRD or drug related harm. Several cases exhibit poly-drug use that may suggest the use of SCs for their affordability and/or availability and not necessarily to bypass the common drug-screening tests. The deficiencies regarding information on the toxic and/or lethal concentrations of most SCs with/without the presence of poly-ingestion make the interpretation of the cause of death more complicated.

Because the picture of use of SCs in Saudi Arabia is unclear, the most prevalent SCs from different generations have been selected. A study using self-report was conducted among 3 subpopulations in Saudi Arabia (medical professionals, members of the public in and around smoking cafes and known drug users). The study showed a small number of participants are cognisant of the existence of Spice products. A low number of the population under examination has reported they know a friend using Spice products. The results of the analysis found only 2 of 463 samples to be positive for 5F-PB-22 3-carboxyindole.

This project showed a wide range in urine concentrations of SCs (1 – 268 ng/mL) and can be explained by the timing of sample collection, storage, precision of the method, or variability in SC content of Spice products.

Understanding the range and extent of use of SCs that are affecting the populations in Scotland and Saudi Arabia can provide valuable information to the forensic community. The study was carried out over a short period and showed that more recently produced SCs are used.

To address this new phenomenon, drug addiction services, education and police organisations should be proactive in raising awareness around SCs. They should also effectively respond to, engage and treat people using SCs. Suitable urine drug tests for SCs present in Spice need to become more widely available within Scotland and Saudi Arabia, although this will likely always require some kind of mass spectrometric technique as it would be difficult to develop a dip-stick urine assay which would remain current for any length of time.

Chapter 8 – Further Work

Whilst the numbers found in the self-report study are low it is important to realise that this is an ever-changing landscape and therefore it is important to carry out the same survey on a regular basis (e.g. annually) to evaluate the scene with respect to trends of SC use. It would be an advantage to incorporate questions about use of SCs in national surveys in Saudi Arabia like those in Scotland. It would be very useful to introduce a common methodology that enables the data to be comparable between countries.

The present prevalence studies of SCs among A&E patients in Scotland and WDT in Saudi Arabia were conducted at a single institution which limits the representativeness. To obtain more representative figures of the prevalence of SC use, future studies should include a larger sample size from all parts of the country and should be conducted in Scotland and Saudi Arabia. Accurate data regarding sex, age and other drug positives should be collected with each biological sample to generate a better understanding of the users of SC. It is also very useful to combine use of drug testing and questionnaires to obtain more accurate data.

The current method tested only a small panel of SCs due to lack of reference standards which may have caused false negatives. SCs are constantly being modified and rapidly becoming widely available; therefore forensic laboratories should update their scope for detecting the most prevalent compounds at a specific time and/or use HRMS to look for unknowns. Stability of SCs, especially UR-144 and 5F-PB-22 in blood, requires further investigation using different methods of preservation under different storage conditions.

Studying the relationship between drug effects and the blood concentrations of SCs may shed further light on the association between cannabinoids and DRD although this is clearly difficult with poly-drug use. Publishing of blood concentrations found and their clinical effects should be encouraged in order to aid interpretation of positive results in any setting.

Awareness among health organisations should be raised regarding these drugs and their risk to individuals, especially young people. With more research and

better understanding of the problem and its extent, the response can be improved and be more effective.

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Appendix 1: Bioanalytical Procedures for Determination of SCs and Metabolites in Blood, Serum or Plasma Samples

SCs	Matrix	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
(25 SCs): JWH-018, JWH-073, JWH-081, JWH-203, JWH-210, JWH-250, JWH-251, JWH-007, JWH-015, JWH-019, JWH-030, JWH-302, JWH-398, CP47, 497, CP47, 497 C8-homolog, HU-210, AM-694, AM-1241, RCS-4, RCS-4 2- and 3-methoxy homolog, RCS-8, RCS-4-C-4-homolog, WIN48 098, WIN55 212-2 mesylate	Whole blood	LLE	LC-MS/MS-ESI+/-	LOQ = 0.5 and 5	[190]
(29 SCs): JWH-018, JWH-019, JWH-020, JWH-073, JWH-122, JWH-147, JWH-200, JWH-210, JWH-250, JWH-251, JWH-398, JWH-007, JWH-203, JWH-015, JWH-073-methyl, JWH-081, JWH-098, AM-1220, AM-1241, AM-2201, AM-2233, MAM-2201, AB-001, AM-694, RCS-4, RCS-4 ortho, RCS-8, UR-144, WIN55 212-2	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[90]
(2 SCs): JWH-018, JWH-073	Whole blood	LLE	LC-MS/MS-ESI+	0.01 ; 0.05	[154]
(4 SCs): JWH-018, JWH-073, JWH-019, JWH-250	Whole blood	LLE	LC-MS/MS-ESI+	0.006 ; 0.016	[191]
(20 SCs and 7 metabolites): AM-2201, AM-2201-*N*-4-OH-pentyl, JWH-018, JWH-210, JWH-203, JWH-250, JWH-251, JWH-081, JWH-122, JWH-122-*N*-5-OHpentyl, JWH-018-*N*-4- and 5-OH-pentyl, JWH-147, JWH-200, JWH-200-4-OHindole, JWH-201, JWH-302, JWH-398, JWH-412, MAM-2201, RCS-4, RCS-4 ortho isomer, RCS-4-*N*-5- OH-pentyl, RCS-4- *N*-COOH, RCS-8, WIN48 098, WIN55 212	Whole blood	PPT	LC-MS/MS-ESI+	Not specified	[146]
(18 SCs): 5F-PB-22, JWH-018, JWH-073, JWH-019, JWH-122, JWH-210, JWH-250, JWH-015, JWH-081, AM-2201, Cl-2201, MAM-2201, UR-144, XLR-11, BB-22, PB-22, AB-PINACA, ADB-PINACA,	Whole blood	LLE	LC-MS/MS	Not specified	[3]
(13 SCs): JWH-018, JWH-073, JWH-081, JWH-019, JWH-122, JWH-200, JWH-210, JWH-250, JWH-175, AM-2201, AM-694, RCS-4, RCS-8	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[253]
(18 SCs): JWH-018, JWH-073, JWH-081, JWH-250, JWH-251, JWH-122, JWH-200, JWH-210, JWH-015, JWH-019, JWH-020, CP47, 497, HU-210, AM-694, AM-2201, RCS-4, RCS-4-C4, RCS-8, WIN55,212-2	Whole blood	LLE	LC-MS/MS-ESI+	Not specified	[167]

SCs	Matrix	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
UR-144 and pyrolysis product [1- (1-pentyl-1H-indol-3-yl)-3-methyl-2-(propan-2yl)but-3-en-1-one]	Whole Blood	LLE	LC-MS/MS-ESI+	0.15 ; 0.5	[200]
(10 SCs): JWH-018, JWH-073, JWH-081, JWH-250, JWH-015, JWH-200, JWH-019, JWH-020, WIN55 212-2, methanandamide	Serum	LLE	LC-MS/MS-ESI	0.1 ; 0.1 - 0.6	[192]
(15 SCs): JWH-018, JWH-073, JWH-081, JWH-019, JWH-122, JWH-200, JWH-210, JWH-250, JWH-251, JWH-203, JWH-307, JWH-015, AM-1220, AM-2201, AM-694	Serum	LLE	LC-MS/MS-ESI+/-	0.02 - 0.4 ; 0.05 - 0.5	[193]
JWH-018	Serum	LLE	LC-MS/MS-ESI+	0.07 ; 0.21	[149]
(30 SCs): JWH-018, JWH-018 adamantyl derivative, JWH-073, JWH-081, JWH-122, JWH-122 5-fluoropentyl derivative, JWH-200, JWH-210, JWH-250, JWH-251, JWH-015, JWH-019, JWH-020, JWH-203, JWH-307, JWH-387, JWH-398, JWH-007, AM-694, AM-1220, AM-2201, AM-2233, MAM-2201, methanandamide, CRA-13, RCS-4, RCS-4 ortho isomer, RCS-8, WIN 48 098, WIN 55 212-2	Serum	LLE	LC-MS/MS-ESI+	0.01 - 2.0 ; 0.1 - 2.0	[152]
(46 SCs): JWH-018, JWH-073, JWH-081, JWH-122, JWH-182, JWH-200, JWH-007, JWH-015, JWH-019, JWH-210, JWH-250, JWH-251, JWH-020, JWH-022, JWH-203, JWH-307, JWH-370, JWH-387, JWH-398, JWH-412, AM-694, AM-1220, AM-1220 azepane isomer, AM-2201, APICA, AM-1248, AM-2232, AM-2233, MAM-2201, Methanandamide, AB-001, Cannabipiperidie- thanone, CRA-13, RCS-4, RCS-4-C4, RCS-4 ortho isomer, RCS-8, STS-135, UR- 144, UR-144 isomer, XLR-11, XLR-11 isomer, WIN48 098, WIN55 212-2, AKB48, 5F-AKB-48	Serum	LLE	LC-TOF/MS-ESI+	LOD = 0.1 - 0.5	[194]
(5 SCs): JWH-122, PB-22, AM-2233, BB-22, 5-F PB-22	Plasma	LLE	LC-HR-MS	Not specified	[195]
(20 SCs): JWH-018, JWH-073, JWH-081, JWH-122, JWH-200, JWH-007, JWH-015, JWH-016, JWH-018-6-MeO, JWH-098, JWH-210, JWH-250, JWH-022, JWH-203, AM-694, AM-2201, AM-1241, RCS-4, RCS-8, WIN 48 098	Blood, serum	SPE	LC-TOF/MS-ESI+	Not specified	[210]

Appendix 2: Bioanalytical Procedures for Determination of SCs and Metabolites in Urine Samples

SCs	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
(5 metabolites of JWH-018): JWH-018 *N*-5-OH-pentyl, 5-OHindole, *N*-COOH, *N*-dealkylated-5-OH-indole and 2-OH-naphthoyl	Hydrolysis, LLE	LC-MS/MS-ESI+	0.1 ; 0.5 Only JWH-018-*N*-OH-pentyl & COOH	[129]
Metabolites of JWH-018 and AM-2201	Hydrolysis, LLE	LC-HR/MS	Not specified	[82]
(3 metabolites of JWH-018): *N*-5-OH-pentyl, *N*-COOH, 6-OH-indole	Hydrolysis, LLE	LC-MS/MS-ESI+	0.01 - 0.1 ; 1	[130]
(10 metabolites of 7 SCs): JWH-018 *N*-5-OH-pentyl, -COOH, JWH-073 *N*-4-OH-butyl, -COOH, JWH-250 5-OH-indole, *N*-COOH, JWH-122 *N*-5-OH-pentyl, JWH-200 5-OH-indole, JWH-019 5-OH-indole, RCS-4 *N*-5- OH-pentyl	Hydrolysis, LLE	LC-MS/MS-ESI+	LOQ = 0.1	[151]
JWH-018 and its metabolites	Hydrolysis, LLE	LC-MS/MS heated-ESI	Not specified	[79]
(JWH-018 and 7 metabolites): JWH-018 *N*-5-OH-pentyl, *N*-COOH, 2-, 4-,5-,6- and -7-OH-indole **(JWH-073 and 6 metabolites):** JWH-073 *N*-4-OHbutyl, *N*-COOH, 4-, 5-, 6- and -7-OH-indole	LLE/SPE	LC-MS/MS-ESI+	LOD = 1	[166]
Metabolites of UR-144	Hydrolysis	GC-MS, LC-MS/MS	Not specified	[236]
CP47, 497	Dilution	LC-MS/MS-ESI-	10 ; 20	[187]
(JWH-018 and 6 metabolites): JWH-018 *N*-5-OH-pentyl, –COOH and 4-, 5-, 6-, 7-OH-indole **(JWH-073 and 6 metabolites):** JWH-073 *N*-4-OH-butyl, -COOH and 4-,5-,6-,7-OH-indole	Hydrolysis, LLE	LC-MS/MS-ESI+	< 2 ; 1.8- 10.8	[131]
(3 metabolites of JWH-018): JWH-018 *N*-4- and 5-OH-pentyl and COOH **(3 metabolites of JWH-073):** JWH-073 *N*-3- and 4-OH-butyl and COOH.	Hydrolysis, SPE	LC-MS/MS-ESI+	0.1 ; 0.1	[262]
JWH-018 and 19 metabolites (mainly hydroxylated and *N*-dealkylatedmetabolites)	Hydrolysis, LLE	LC-MS/MS-ESI+	LOD = 0.1	[120]

SCs	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
AB-001 and 7 metabolites	LLE	GC-MS	Not specified	[196]
(4 metabolites of JWH-018): JWH-018 *N*-COOH, 6-OH-indole, *N*-4- and 5 OH-pentyl **(4 metabolites of JWH-073):** JWH-073 *N*-COOH, 6-OH-indole, *N*-3- and 4-OH-butyl	Dilution, Hydrolysis, SPE	ELISA, LC-MS/MS-ESI+	0.025 – 0.1 ; 2.5	[211]
(UR-144 and 5 metabolites): despentyl-OH-UR-144, di- OH-UR-144, despentyl-UR-144, dehydrated OH-UR-144, OH-UR-144 **(AM2201 and 7 metabolites):** JWH-018 *N*-(5-OH-pentyl), *N*-(5-OH-pentyl) dihydrodiol-JWH-018, di-OH-AM-2201, dihydrodiol-AM-2201, OH-AM-2201,despentyl-AM-2201, JWH-018 *N*-COOH	Hydrolysis, LLE	LC-MS/MS-ESI+	Not specified	[127]
RCS-4 and its metabolites	Hydrolysis, LLE,	GC-MS	Not specified	[197]
(8 metabolites of 5 SCs): JWH-018 *N*-4- and 5-OH-pentyl, JWH-073 *N*-3- and 4-OH-butyl, JWH-250 *N*-4-OHpentyl, JWH-019 *N*-5- and 6-OH-hexyl, AM-2201 *N*-4-OH-pentyl	Hydrolysis, LLE	ELISA, LC-MS/MS-ESI+	Not specified	[198]
5F-AKB-48 and its metabolites	Hydrolysis, LLE	LC-HR-MS	Not specified	[58]
(37 metabolites of 17 SCs): JWH-018 *N*-COOH, -6-OH-indole, *N*-4- and 5 OH-pentyl, JWH-073 *N*-COOH, 6-OH-indole, *N*-3- and 4-OH-butyl, JWH-250 *N*-COOH, *N*-4- and 5 OH-pentyl, JWH-122 *N*-4- and 5 OH-pentyl, JWH-019 *N*-6 OH-pentyl, JWH-210 *N*-4- and 5 OH-pentyl, JWH-081 *N*-5 OH-pentyl , JWH-398 *N*-4- and 5 OH-pentyl, JWH-203 *N*-COOH, *N*-4- and 5 OH-pentyl, AM-2201-6-OH-indole, *N*-4 OH-pentyl, MAM-2201 *N*-COOH, *N*-4 OH-pentyl, UR-144 *N*-COOH, *N*-4- and 5 OH-pentyl, XLR-11 *N*-4 OH-pentyl, AB-PINACA *N*-COOH, *N*-4 OH-pentyl, 5F-AB-PINACA *N*-4 OH-pentyl, AKB48 *N*-COOH, *N*-4- and 5 OH-pentyl, 5F-AKB48 *N*-4 OH-pentyl	Hydrolysis, SPE	LC-MS/MS-ESI+	LOD = 0.1 - 1	[256]
Major metabolites of JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, RCS-4	Hydrolysis, LLE	LC-MS/MS-ESI+, LC-TOF/MS-ESI+	Not specified	[114]
(UR-144 and 7 metabolites): UR-144 *N*-COOH, di-OH-UR-144, UR-144 *N*-5-OH-pentyl- β-Gluc, despentyl-UR-144, despentyl-OH-UR-144, dehydrated-OH-UR-144, OH-UR-144	Hydrolysis, LLE	LC-TOF-MS-ESI+	0.15 ; 0.5	[200]

SCs	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
(4 metabolites of JWH-018): JWH-018 *N*-4- and 5- OH-pentyl, *N*-COOH, methyl ester **(2 metabolites of JWH-073):** JWH-073 *N*-3-OH-butyl, *N*-COOH JWH-072 *N*-COOH	Hydrolysis	LC-MS/MS-ESI+	LOQ = 4	[257]
(47 metabolites of 21 SCs): JWH-018 *N*-OH-pentyl, *N*-COOH, 5- and 6-OH-indole, JWH-073 *N*-OH-butyl, 5- and 6-OH-indole, NCOOH, JWH-122 *N*-OHpentyl, *N*-COOH, JWH-250 5-OH-indole, *N*-OH-pentyl, *N*-COOH, JWH-210 *N*-COOH, *N*-OH-pentyl, JWH-200 5- and 6-OH-indole, JWH-398 *N*-OH-pentyl, *N*-COOH, JWH-019 5-OH-indole, -OH-hexyl, JWH-081 *N* OH-pentyl, AM-2201 6-OH-indole, *N*-OH-pentyl, MAM-2201 *N*-OH-pentyl, RCS-4 *N*-OH-pentyl, *N*-COOH, M9, UR-144 *N*-OH-pentyl, *N*-COOH, degradant-COOH, XLR-11 *N*-OH-pentyl, 6-OH-indole, ADB-PINACA *N*-OH-pentyl, AKB48 *N*-OH-pentyl, *N*-COOH, 5F-AKB48 *N*-OH-pentyl, 5F-AB-PINACA *N*-OH-pentyl, AB-PINACA *N*-OH-pentyl, -COOH, PB-22 *N*-OH-pentyl, *N*-COOH, *N*-OH-pentyl-3-COOH-indole, *N*-COOH-3-COOHindole, 3-COOH-indole, 5F-PB-22 3-COOH indole	Dilution, Hydrolysis, SLE+	LC-TOF/MS-ESI+	LOD = 0.25 - 20	[245]
(35 SCs and 19 metabolites): JWH-018, JWH-018 *N*-4- and 5-OH-pentyl, *N*-COOH, -4-, 5-, 6- and 7-OH-indole, 6-methoxy-indole, -1-methyl-hexyl, JWH-073, JWH-073 *N*-3- and 4-OH-butyl, -4-, 5-, 6-, and 7-OH-indole, *N*-COOH, 2- and 3-methyl homology, JWH-122, JWH-122 *N*-5-OH-pentyl, JWH-200, JWH-200 4-OH-indole, JWH-250, JWH-251, JWH-081, JWH-147, JWH-007, JWH-015, JWH-019, JWH-201, JWH-210, JWH-203, JWH-302, JWH-398, JWH-412, CP47, 497,CP47, 497-C8, CP55,940, HU-210, AM-694, AM-1220, AM-1220 azepane isomer, AM-2201, MAM-2201, RCS-4, RCS-4 ortho isomer, *N*-5-OH-pentyl, *N*-COOH, RCS-8, AB-0101, WIN48 098, WIN55 212	Hydrolysis, SPE	LC-TOF-MS-ESI+	Not specified	[212]
22 SC and 37 metabolites	No sample preparation	Biochip array technology immunoassay	Not specified	[285]
JWH-018 *N*-COOH (calibrator)	No sample preparation	ELISA	Not specified	[220]

SCs	Sample Prep	Detection Mode	LOD (ng/mL) ; LOQ (ng/mL)	Ref
(9 SC and 20 metabolites): JWH-018, JWH-018 *N*-5-OH-pentyl, 5- and 6-OH-indole, *N*-COOH, JWH-073, JWH-073 *N*-4-OH-butyl, *N*-COOH, 5- and 6-OH-indole, JWH-250 *N*-4- and 5-OH-pentyl, 5-OH-indole, JWH-200 5- and 6-OH-indole, JWH-081, JWH-081 *N*-5-OH-pentyl, JWH-210, JWH-210 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-122, JWH-122 *N*-5-OH-pentyl, AM-2201, AM-2201 6-OH-indole, *N*-4-OH-pentyl, MAM2201, RCS-4, RCS-4 *N*-5-OH-pentyl, *N*-COOH	PPT, Hydrolysis	LC-MS/MS-ESI+	LOD = 0.5 - 10	[259]
(5 SC and 6 metabolites): JWH-018, JWH-018 *N*-4- and 5-OHpentyl, 4- and 5-OH-indole, JWH-073, JWH-073 *N*-3- and 4-OH-butyl, JWH-250, HU-210, AM-2201	Hydrolysis, SPE	LC-MS/MS-ESI+	0.01 - 0.5 ; 0.05 - 5	[213]
(20 SC and 33 metabolites): JWH-018, JWH-018 *N*-OH-pentyl, *N*-COOH, 5- and 6-OH-indole, JWH-073, JWH-073 *N*- OH-butyl, *N*-COOH, 5- and 6-OH-indole, JWH-019, JWH-019 5-OH-indole, -*N*-OH-hexyl, JWH-250 *N*-OH-pentyl, *N*-COOH, -5-OH-indole, JWH-081, JWH-081 *N*-OH-pentyl, JWH-210, JWH-210 *N*-OH-pentyl, *N*-COOH, 5-OH-indole, JWH-200 5- and 6-OH-indole, JWH-122, JWH-122 *N*-OH-pentyl, JWH-398, JWH-398 *N*-OH-pentyl, *N*-COOH, JWH-203, CP47, 497-C7, CP47, 497-C7-OH, CP47, 497-C8, CP47, 497-C8-OH dimethyloctyl, HU-210, AM-694, AM-2201, AM-2201 6-OH-indole, *N*-OH-pentyl, MAM-2201, MAM-2201 *N*-OH-pentyl, *N*-COOH, UR-144 *N*-OH-pentyl, *N*-COOH, RCS-4, RCS-4 *N*-OH-pentyl, M9, M10, *N*-COOH	Dilution, Hydrolysis, SLE	LC-MS/MS-ESI+/-	0.05 - 1 ; 0.1 - 1	[260]
(38 metabolites of 12 SCs): JWH-018 *N*-4- and 5-OH-pentyl, *N*-COOH, 5-, 6- and 7-OH-indole, JWH-073 *N*-3- and 4-OH-butyl, *N*-COOH, 5-, 6- and 7-OH-indole, JWH-250 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-019 *N*-5- and 6-OH-hexyl, JWH-210 *N*-4- and 5-OH-pentyl, *N*-COOH, JWH-122 *N*-4-OH-pentyl, *N*-5-OH-pentyl, JWH-081 *N*-5-OH-pentyl, *N*-COOH, JWH-398 *N*-4- and 5-OH-pentyl, *N*-COOH, AM-2201 *N*-4-OHpentyl, 6- and 7-OH-indole, MAM-2201 *N*-4-OHpentyl, *N*-COOH, UR-144 4- and 5-OH-pentyl, *N*-COOH, RCS-4 4- and 5-OH-pentyl, *N*-COOH	SALLE	HEIA, LC-TOF/MS-ESI+	Not specified	[222]
(8 metabolites of 3 SCs): JWH-018 *N*-4 and 5-OH-pentyl, *N*-COOH, *N*-5-OH-pentyl β-Gluc, 6-OH-indole, JWH-073 *N*-COOH, AM-2201 *N*-4-OH-pentyl, 6-OH-indole	Hydrolysis, SPE	LC-MS/MS-ESI+	0.1 ; 2.5	[83]
(2 metabolites of JWH-018): JWH-018 *N*-4-OH-pentyl, *N*-COOH, **(2 metabolites of JWH-073):** JWH-073 *N*-3-OH-butyl, *N*-COOH	Hydrolysis, LLE	LC-MS/MS-ESI+	LOQ = 4	[199]

Appendix 3: Bioanalytical Procedures for Determination of SCs and Metabolites in Oral Fluid Samples

SCs	Sample Prep	Detection Mode	LOD (ng/mL); LOQ (ng/mL)	Ref
(10 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, HU-210, CP47,497, nabilone, AM-694	Dilution	LC-MS/MS-ESI+	LOD = 1 - 20	[267]
(10 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-081, HU-210, CP47,497, CP47,497 (C8), AM-2201, RCS-4	Dilution	ELISA, LC-MS/MS-ESI+	0.1 ; 0.25	[218]
(28 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-122, JWH-019, JWH-210, JWH-007, JWH-015, JWH-387, JWH-398, JWH-251, JWH-307, JWH-081, JWH-203, JWH-020, JWH-412, AM-694, AM-1220, AM-2233, AM-2201, MAM-2201, methanandamide, RCS-4, RCS-8, RCS-4 ortho isomer, WIN48, 098, WIN55 212-2	PPT	LC-MS/MS-ESI+	0.015 - 0.9 ; 0.15 - 3	[189]
(30 SCs): JWH-018, JWH-018 adamantyl derivative, JWH-073, JWH-250, JWH-081, JWH-122, JWH-007, JWH-015, JWH-387, JWH-398, JWH-251, JWH-307, JWH-210, JWH-200, JWH-203, JWH-019, JWH-020, AM-2201, AM-2233, AM-694, AM-1220, CRA-13, AB-001, MAM-2201, methanandamide, RCS-8, RCS-4, RCS-4 ortho isomer, WIN48 098, WIN55 212-2	LLE	LC-MS/MS-ESI+	0.015 - 0.9 ; 0.15 - 30	[201]
(18 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, JWH-015, JWH-020, JWH-210, JWH-081, JWH-251, HU-210, AM-2201, AM-694, RCS- 4, RCS-4-C4, RCS-8, WIN55 212-2	LLE	LC-MS/MS-ESI+	LOD = 0.05 - 1.2	[202]
(UR-144 and 2 metabolites): UR-144 4-OH-pentyl, pyrolysis product of UR-144 **(XLR-11 and 2 metabolites):** XLR-11 4-OHpentyl and pyrolysis product of XLR-11	SPE	LC-MS/MS-ESI+	0.35 - 1.93 ; 5	[126]
(6 SCs): JWH-018, JWH-073, JWH-250, HU-210, CP47,497, CP47,497 (C8)	SPE	LC-MS/MS-ESI+/-	LOQ = 0.5	[214]
(7 SCs): JWH-018, JWH-073, JWH-250, JWH-200, HU-211, CP47,497, CP47,497 (C8)	SPE	LC-MS/MS-ESI+/-	0.025 - 1.0 ; 0.1 - 2.5	[215]

Appendix 4: Bioanalytical Procedures for Determination of SCs and Metabolites in Hair Samples

SCs	Sample Prep	Detection Mode	LOD (pg/mg); LOQ (pg/mg)	Ref
(18 SCs): JWH-018, JWH-073, JWH-018-*N*-NCOOH, JWH-250, JWH-200, JWH-122, JWH-210, JWH-081, JWH-015, JWH-020, JWH-019, JWH-203, JWH-007, HU-210, CP47,497, AM-2201, AM-694,WIN55 212-2	Washing, Digestion	LC-MS/MS-ESI+	LOQ = 500	[268]
(JWH-018 and 3 metabolites): JWH-018 *N*-4- and 5-OH-pentyl, *N*-NCOOH **(JWH-073 and 3 metabolites):** JWH-073 *N*-3- and -4-OHbutyl, *N*-COOH	Washing, LLE	LC-MS/MS-ESI+	LOD = 0.05	[203]
(5 SC and 11 metabolites): JWH-018, JWH-073, JWH-122, AM2201, MAM2201, JWH-018 *N*-4- and 5-OH-pentyl, *N*-NCOOH, JWH-073 *N*-3- and -4-OHbutyl, *N*-COOH, JWH-122 *N*-5-OH-pentyl, *N*-COOH, AM-2201 *N*-4-OH-pentyl, 6-OH-indole, MAM-2201 *N*-4-OHpentyl	Washing, LLE	LC-MS/MS-ESI+	0.05 ; 0.1	[204]
(23 SCs): JWH-018, JWH-073, JWH-250, JWH-122, JWH-019, JWH-200, JWH-015, JWH-020, JWH-210, JWH-081, JWH-251, JWH-307, JWH-007, JWH-398, JWH-203, HU-210, AM-694, AM-2201, AM-1220, RCS- 4, RCS-8, WIN55 212-2, WIN48 098	Washing, Digestion, LLE	LC-MS/MS-ESI+	0.2 – 1.3 ; 0.7 – 4.3 except (HU-210 = 80 LOQ)	[205]
(8 SCs): JWH-018, JWH-073, JWH-250, JWH-200, JWH-122, JWH-210, JWH-081, AM-694	Base hydrolysis, LLE	LC-TOF-MS-ESI+	LOD = 10	[206]
(5 SCs): JWH-018, JWH-073, JWH-250, JWH-200, HU-210	Washing, digestion, LLE	LC-MS/MS-ESI+	0.02 – 0.18 ; 0.07 - 18	[207]

Appendix 5: Ethical Approval for Post-mortem Samples

University of Glasgow

Dear Dr Torrance,

29/4/16

MVLS College Ethics Committee

Project Title: Analysing background case information in deaths involving synthetic cannabinoids
Project No: 200150101

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, subject to the following conditions:

- Project end date: End October 2016
- 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

Clinical Reader

Jesse Dawson
MD, FRCP, BSc (hons), MBChB (hons)
Clinical Reader / Honorary Consultant

College of Medicine, Veterinary & Life Sciences
Institute of Cardiovascular and Medical Sciences
Western Infirmary
Glasgow
G11 6NT
jesse.dawson@glasgow.gla.ac.uk
Tel – 0141 2116395 or page 4824

The University of Glasgow, charity number SC004401

Appendix 6: Ethical Approval (from University of Glasgow) to Collect Urine Samples and Questionnaires from Saudi People

University of Glasgow | College of Medical, Veterinary & Life Sciences

15th October 2014

Dear Dr Hazel Torrance and Abdulaziz Aldlgan

MVLS College Ethics Committee

Project Title: Is Synthetic Cannabis a Problem in Saudi Arabia?
Project No: 200130158

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. They are happy therefore to approve the project, subject to the following conditions

- Project end date: November 2016

- 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

Prof. Andrew C. Rankin
Deputy Chair, College Ethics Committee

Andrew C. Rankin
Professor of Medical Cardiology
BHF Glasgow Cardiovascular Research Centre
College of Medical, Veterinary & Life Sciences
University of Glasgow, G12 8TA
Tel: 0141 211 4833
Email: andrew.rankin@glasgow.ac.uk

Appendix 7: Ethical Approval (from SFH) to Collect Urine Samples from Saudi People

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: 27 March 2014

Ethics approval

Reference Number:	14-128-01
Project Title:	Are synthetic cannabinoids a problem in Saudi Arabia
Researcher:	Abdulaziz Abdulmohsen Aldigan
Supervisor:	Dr. Hazel J. Torrance – University of Glasgow
Approval Date:	20 Mar 14
Expiry Date	20 Mar 16

The research committee in Security Forces Hospital is constituted and functions in accordance with the National Committee of Bio Ethics (NCBE) in Saudi Arabia. The Committee has reviewed the research proposal for compliance with national requirements and approval of this project is conditional upon your continuing compliance with this document.

I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the documents submitted with your letter dated on 25.11.2013. As evidence of continuing compliance, The research committee in Security Forces Hospital requires that the researcher immediately report:
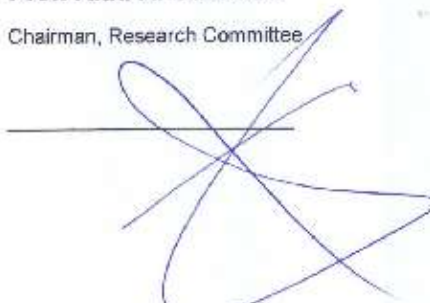
○ Proposed changes to the protocol including changes to the investigators involved.

With the Committee's best wishes for the success of this project.

Thank you and best regards

Prof. Riad A. Sulimani

Chairman, Research Committee

Appendix 8: Participant Information Sheet for Urine Samples Study in Saudi Arabia

University | College of Medical,
of Glasgow | Veterinary & Life Sciences

PARTICIPANT INFORMATION SHEET
معلومات حول الدراسة

Is Synthetic Cannabis a Problem in Saudi Arabia?
هل الحشيش الصناعي يمثل مشكلة بالسعودية؟

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.

أنت مدعو للمشاركة في دراسة بحثية نظمت بواسطة جامعة جلاسكو. قبل أن نقرر, من المهم بالنسبة لك أن نعرف لماذا يُجرى هذا البحث وماذا سوف سيترتب على مشاركتك في هذه الدراسة. يرجى أخذ الوقت الكافي لقراءة المعلومات التالية بعناية ومناقشتها مع الباحثين إذا كنت ترغب في ذلك. الرجاء السؤال إذا كان كنت ترغب في مزيد من المعلومات.

What is the purpose of the Study? ما هو الغرض من الدراسة؟
In recent years, the using of new psychoactive substances (NPS) including synthetic cannabinoids has been increasing around the world. Synthetic cannabis has been sold as herbal mixtures 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 altered mood and perception to psychosis.[1,2,3]
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 cannabinoids use in Saudi Arabia.

في السنوات الأخيرة، إزداد استخدام المواد الجديدة ذات التأثير النفسي مثل الحشيش الصناعي في عدد من دول العالم. الحشيش الصناعي يباع على انه خلائط عشبية وبخور وأصبحت متاحة على نطاق واسع على شبكة الانترنت. بعض الناس قد يظن أن هذه المواد هي آمنة. في الحقيقة، هذه المواد تتسبب بمجموعة واسعة من الأضرار على الجسم وخاصة على الجهاز العصبي المركزي. لهذا السبب، فإن الغرض من هذا المشروع هو تطوير طرق تحليل للكشف عن هذه المواد في البول باستخدام طرق الكروماتوغراف الغازي وأيضا لتعطينا فكرة عن انتشار المواد المخدرة الاصطناعية في المملكة العربية السعودية.

Why have I been chosen? لماذا تم اختياري؟

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.

الناس الذين يجب أن يخضعوا لفحوصات دورية للكشف عن المخدرات في البول يعتبرون فئة جيدة ليتم استخدامها في هذا المشروع. تحليل هذه العينات هو خطوة هامة في عملية التحقق من صحة الطريقة المخبرية.

Do I have to take part? هل يجب أن أشارك؟

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.

الأمر متروك لك لتقرر ما إذا رغبت في المشاركة من عدمها في هذا المشروع. وإذا قررت المشاركة ستعطى لك ورقة المعلومات هذه ويطلب منك التوقيع على استمارة الموافقة.

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 for synthetic cannabinoids. You will not be given any personal results from the tests.

What I have to do? ماذا سيحدث لي إذا شاركت؟

To participate in the study, you are asked to consent freely to donate a urine sample.

لتشارك في الدراسة، سيطلب منك الموافقة وتقديم عينة بول. سوف تحصل على عينة بلاستيكية للتبرع بعينة البول مرة واحدة.

What are the possible disadvantages and risks of taking part?
ما هي العيوب والمخاطر المحتملة على من يشارك؟

No disadvantage will be found. Urine sample collection is very simple and non-invasive.
لا توجد أية عيوب. جمع عينات البول هو بسيط جدا.

What are the possible benefits of taking part?
ما هي الفوائد المحتملة على من يشارك؟

This research will provide an extremely important tool to assess the popularity of synthetic cannabinoids and in turn can help in assessing the importance of providing education on the use of synthetic cannabinoids.
لا نستطيع أن نعدك بأن الدراسة سوف تساعدك مباشرة ولكن هذا البحث سوف يسهم للغاية في مجال السموم الشرعية وعلوم المختبرات ومشاركتكم محل تقدير بشكل كبير.

Will my taking part in this study be kept confidential?
هل ستبقى مشاركتي في هذه الدراسة سرية؟

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 incuding the hospital. Your anonymised results

along with all other participants will be published in peer-reviewed papers and presented at international conferences.

نعم. وسوف نتابع جميع المراحل الأخلاقية والقانونية كما وسيتم التعامل مع جميع المعلومات بسرية تامة. اؤكد لكم ايضا انه لن يتم نقاسم نتائجك مع أي شخص اخر.

What will happen to the results of the research study?
ماذا سيحدث لنتائج هذه الدراسة البحثية؟

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.
نتائج تحليل العينات ستبقى مجهولة، و أية منشورات علمية ناتجة عنها لن تحتوي على أية معلومات شخصية يمكن من خلالها التعرف على اصحاب العينات. لن تصلك أية نتائج من هذه الاختبارات.

Who is organising and funding the research study? من ينظم ويمول البحث؟

This research is funded by Saudi Arabia Government.

جامعة غلاسكو تنظم البحث وتقوم الحكومة السعودية بتمويل الدراسة.
من سيراجع هذه الدراسة؟ **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.
جامعة غلاسكو تقوم بتنظيم هذا البحث ومراجعته

Contact for Further Information

For any further information concerning this project please contact Abdulaziz Aldlgan at the

Abdulaziz.Aldlgan@formed.gla.ac.uk

الباحث: عبدالعزيز عبدالمحسن الدلقان

البريد الإلكتروني: Abdulaziz.Aldlgan@formed.gla.ac.uk

Thank you for participating in the project.

شكراً لكم على قراءة ورقة المعلومات يرجى الاحتفاظ بنسخة كمرجع

References:
[1] S. M. R. Gurney, K.S.S., S. L. Kacinko, B. C. Presley, B. K. Logan, *Pharmacology, Toxicology, and Adverse Effects of Synthetic Cannabinoid Drugs*.

[2] Seely, K.A., et al., *Spice drugs are more than harmless herbal blends: A review of the pharmacology and toxicology of synthetic cannabinoids*. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2012. 39(2): p. 234-243.

3 Forrester M, Kleinschmidt K, Schwarz E, Young A: Synthetic cannabinoid exposures reported to Texas poison centers; J Addict Dis 30:351; 2011.

College of MVLS 3 January 2013 (lh)
Ethics Committee

Appendix 9: Consent Form for Survey and Urine Samples Studies in Saudi Arabia

University of Glasgow | College of Medical, Veterinary & Life Sciences

Centre Number:
Project Number:
Subject Identification Number for this trial:

CONSENT FORM
نموذج إقرار

Title of Project: Is Synthetic Cannabis a Problem in Saudi Arabia?

Name of Researcher(s): ABDULAZIZ ABDULMOHSEN ALDLGAN

عنوان المشروع: هل الحشيش الصناعي مشكلة بالمملكة العربية السعودية؟
الباحث: عبدالعزيز عبدالمحسن الدلقان

Please initial box

I confirm that I have read and understand the information sheet dated _____
(version _____) for the above study and have had the opportunity to ask questions.

أقر بقراءة وفهم جميع ما ورد في ورقة المعلومات المتعلقة بالمشاركة في الدراسة المذكورة أعلاه, كما اتيحت لي الفرصة لطرح الاسئلة.

I understand that my participation is voluntary and that I am free to withdraw at
any time, without giving any reason, without my legal rights being affected.

أعلم أن مشاركتي تطوعية ولي الحق في الانسحاب من الدراسة في أي وقت ومن دون توضيح الاسباب, كما ان مشاركتي ليس لها تأثير على حقوقي القانونية.

I agree to take part in the above study.. أوافق على المشاركة في الدراسة المشار لها اعلاه..

I am over the age of 18   18 انا عمري فوق

Name of subject اسم المشارك	Date	Signature

Name of Person taking consent (if different from researcher) اسم الشخص الذي أخذ الموافقة (إذا كان غير الباحث)	Date	Signature

Researcher الباحث	Date	Signature

(1 copy for subject; 1 copy for researcher)
نسخة للمشارك ونسخة للباحث

College of MVLS Ethics Committee   1   January 2013 (lh)

Appendix 10: Ethical Approval to Collect Questionnaires from Saudi Known Drug Users

Kingdom of Saudi Arabia
Ministry of Health
King Fahad Medical City
(162)

المملكة العربية السعودية
وزارة الصحة
مدينه الملك فهد الطبية
(١٦٢)

King Fahad Medical City

IRB Registration Number with KACST, KSA: H-01-R-012
IRB Registration Number with OHRP/NIH, USA: IRB00008644
Approval Number Federal Wide Assurance NIH, USA: FWA00018774

December 23, 2014
IRB Log Number: 14-272E
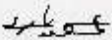Department: External
Category of Approval: EXEMPT

Dear AbdulAziz Abdulmohsen A. Aldigan:

I am pleased to inform you that your submission dated October 28, 2014 for the study titled **'Are Synthetic Cannabinoids a Problem in Saudi Arabia'** was reviewed and was approved. Please note that this approval is from the research ethics perspective only. You will still need to get permission from the head of department or unit in KFMC or an external institution to commence data collection.

We wish you well as you proceed with the study and request you to keep the IRB informed of the progress on a regular basis, using the IRB log number shown above.

If you have any further questions feel free to contact me.

Sincerely yours,

Prof. Omar H. Kasule
Chairman Institutional Review Board–IRB.
King Fahd Medical City, Riyadh, KSA.
Tel: + 966 1 288 9999 Ext. 17540
E-mail: okasule@kfmc.med.sa

Appendix 11: Participant Information Sheet for Survey Study

University of Glasgow | College of Medical, Veterinary & Life Sciences

PARTICIPANT INFORMATION SHEET
معلومات حول الدراسة

Is Synthetic Cannabis a Problem in Saudi Arabia?
هل الحشيش الصناعي يمثل مشكلة بالسعودية؟

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.

أنت مدعو للمشاركة في دراسة بحثية نظمت بواسطة جامعة جلاسكو. قبل أن تقرر، من المهم بالنسبة لك أن تعرف لماذا يُجري هذا البحث وماذا سوف سيترتب على مشاركتك في هذه الدراسة. يرجى أخذ الوقت الكافي لقراءة المعلومات التالية بعناية ومناقشتها مع الباحثين إذا كنت ترغب في ذلك. الرجاء السؤال إذا كان ترغب في مزيد من المعلومات.

What is the purpose of the Study? ما هو الغرض من الدراسة؟

In recent years, the using of new psychoactive substances (NPS) including synthetic cannabinoids has been increasing around the world. The purpose of this study is to assess the current knowledge and awareness of synthetic cannabinoids within the Saudi Arabian population.

في السنوات الأخيرة، إزداد استخدام المواد الجديدة ذات التأثير النفسي مثل الحشيش الصناعي في عدد من دول العالم. الغرض من هذه الدراسة هو لتقدير مدى معرفة ووعي المجتمع السعودي بهذه المواد الجديدة (الحشيش الصناعي)

Why have I been chosen? لماذا تم اختياري؟

We are targeting people who are likely to be aware of synthetic cannabinoids either through their lifestyle or professional work.

الناس الذين نعتقد ان لديهم العلم والوعي بهذه المواد الجديدة سواء لنمط حياتهم اليومية او طبيعة عملهم يعتبرون فئة جيدة ليتم استخدامها في هذا المشروع.

Do I have to take part? هل يجب أن أشارك؟
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.

College of MVLS   1   January 2013 (lh)
Ethics Committee

الأمر متروك لك لتقرر ما إذا رغبت في المشاركة من عدمها في هذا المشروع. وإذا قررت المشاركة ستعطى لك ورقة المعلومات هذه ويطلب منك التوقيع على استمارة الموافقة.

What will happen to me if I take part? ماذا سيحدث لي إذا شاركت؟

You will be asked to fill out a questionnaire which will take no more than 5 minutes after you have given consent

سيطلب منك تعبئة استبيان لن يأخذ أكثر من 5 دقائق من وقتك بعد الأقرار بالموافقة

What I have to do? ماذا على أن أفعل؟

To participate in the study, you are asked to consent freely to fill out a questionnaire providing some information about yourself.

لتشارك في الدراسة، عليك تعبئة استبيان يحتوي معلومات عن نفسك

What are the possible disadvantages and risks of taking part?
ما هي العيوب والمخاطر المحتملة على من يشارك؟

No disadvantage will be found.

لا توجد أية عيوب.

What are the possible benefits of taking part?
ما هي الفوائد المحتملة على من يشارك؟

This research will provide an extremely important tool to assess the popularity of synthetic cannabinoids and in turn can help in assessing the importance of providing education on the use of synthetic cannabinoids.

لا نستطيع أن نعدك بأن الدراسة سوف تساعدك مباشرة ولكن هذا البحث سوف يسهم للغاية في مجال السموم الشرعية وعلوم المختبرات ومشاركتكم محل تقدير بشكل كبير.

Will my taking part in this study be kept confidential?
هل ستبقى مشاركتي في هذه الدراسة سرية؟

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

نعم وسوف نتابع جميع المراحل الأخلاقية والقانونية كما وسيتم التعامل مع جميع المعلومات بسرية تامة.

What will happen to the results of the research study?
ماذا سيحدث لنتائج هذه الدراسة البحثية؟

This research will help us assess whether synthetic cannabinoids are being used in Saudi Arabia currently. The information that you provide will be completely anonymous and the original raw data will not be shared out with the research group. The results of this study will be published in peer-reviewed journals and presented at international conferences.

هذا البحث سوف يساعدنا لمعرفة ما اذا كانت هذه المواد تستخدم بالسعودية. نتائج الدراسة ستبقى مجهولة و أية منشورات علمية ناتجة عنها لن تحتوي على أية معلومات شخصية يمكن من خلالها التعرف على اصحاب الاستبيان. لن تصلك أية نتائج من هذه الاختبارات. نتائج الاختبارات سوف تنشر في المجلات الطبية والمؤتمرات الدولية

Who is organising and funding the research study? من ينظم ويمول البحث؟

This research is funded by Saudi Arabia Government.

جامعة غلاسكو تنظم البحث وتقوم الحكومة السعودية بتمويل الدراسة.

Who has reviewed the Study? من سيراجع هذه الدراسة؟

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.

جامعة غلاسكو تقوم بتنظيم هذا البحث ومراجعته

Contact for Further Information: معلومات التواصل للحصول على مزيد من المعلومات

For any further information concerning this project please contact Abdulaziz Aldlgan at the Abdulaziz.Aldlgan@formed.gla.ac.uk

الباحث:

عبدالعزيز عبدالمحسن الدلقان

البريد الإلكتروني:

Abdulaziz.Aldlgan@formed.gla.ac.uk

Thank you for participating in the project.

شكراً لكم على قراءة ورقة المعلومات يرجى الاحتفاظ بنسخة كمرجع

Appendix 12: Questionnaire Form

General questionnaire

Dear Sir Madam,

I am Abdulaziz Aldlgan, a PhD student in Forensic Toxicology at the Glasgow University, in the United Kingdom. I need your inputs for the studying and I can assure you that your participation in the study will remain confidential and I have already undergone stringent vetting for ethics at the university.

Sincerely

Abdulaziz Aldlgan
Abdulaziz.Aldlgan@formed.gla.ac.uk
aziz5aziz@hotmail.com

السلام عليكم ورحمة الله وبركاته

أنا عبدالعزيز الدلقان، طالب دكتوراه بجامعة جلاسكو بالمملكة المتحدة في قسم السموم الجنائية،الرجاء تعبئة هذا الأستبيان ويمكنني أن أؤكد لكم أن مشاركتكم في هذه الدراسة سوف تظل سرية، ولقد مرت بالفعل بأجراءات تدقيق صارمة في الجامعة
وتقبلوا تحياتي

1. Please tick your gender من فضلك ضع علامة صح على نوع جنسك

- ⭕ Male (ذكر)
- ⭕ Female (أنثى)

2. What city do you currently live in? ما هي المدينة التي تعيش بها بالوقت الحالي؟

- ⭕ Riyadh الرياض
- ⭕ Jeddah جدة
- ⭕ Dammam الدمام
- ⭕ Other أخرى

3. Please tick your age
من فضلك ضع علامة صح على فئتك العمرية

- ⭕ 18-25 years
- ⭕ 26-40 years
- ⭕ 41- or over

4. Education level?
المستوى التعليمي؟

- ⭕ High school or less(المرحلة الثانوية او اقل)
- ⭕ Undergraduate level (Diploma and university degree)(المرحلة الجامعة)
- ⭕ Postgraduate level (Master and PhD degree)(مستوى الدراسات العليا ماجستير ودكتوراه)

5. Do you work in one of the medical fields? هل تعمل بأحد المجالات الطبية؟

- ⭕ Yes (نعم)
- ⭕ No (لا)

General questionnaire

6. Please indicate your response to the following statements من فضلك قم بأختيار ما يناسبك في الحالات التالية

	موافق Agree	غير موافق Disagree
I feel my life is very stressful اشعر بان حياتي ملينة بالتوتر	○	○
When I feel upset or down, I am likely looking for medication عندما اشعر بالارهاق حاليا ما ابحث عن دواء	○	○
When I feel upset or down, I am likely looking for a herb عندما اشعر بالارهاق حاليا ما ابحث عن أعشاب مهدئة	○	○
I like to try new herb products حاليا ما اود تجربة الأعشاب الجديدة	○	○
When I feel upset, I usually ask my friends to give me something could be help عندما اشعر بالارهاق حاليا ما اسأل اصدقائي لإعطائي ما يخفف امالي	○	○
I usually buy the herb products that I have seen advertised احيانا اشتري المنتجات العشبية من محلات العطارة حسب الإعلانات السوقية	○	○
I usually buy the herb products that my friends recommended to me احيانا اشتري المنتجات العشبية من محلات العطارة حسب توصية اصدقائي	○	○
I like to access the internet احب ان استخدم الانترنت	○	○

7. Have you heard of new psychoactive substances or legal highs? هل سبق ان سمعت عن المخدرات النفسية الجديدة؟

○ Yes (نعم)

○ No (لا)

If yes, where did you hear about it? أنا كانت إجابتك بنعم كيف سمعت بها

[   ]

8. Have you heard of synthetic cannabis, spice and k2?? هل سبق ان سمعت عن الحشيش الصناعي؟

○ Yes (نعم)

○ No (لا)

If yes, where did you hear about it? أنا كانت إجابتك بنعم كيف سمعت به

[   ]

9. Do any of your friends smoke these drugs? هل يوجد من اصدقائك من سبق له تدخين الحشيش الصناعي؟

○ Yes (نعم)

○ No (لا)

Validation of a Liquid Chromatography—Tandem Mass Spectrometry method for the Detection and Quantitation of 9 Synthetic Cannabinoid Metabolites in Urine

Abdulaziz A. Aldlgan and Hazel J. Torrance
Forensic Medicine and Science, University of Glasgow, Glasgow, UK

Abstract

Synthetic cannabinoids (SCs) are one of the new groups of drugs, "Legal highs", which are substances created and used to avoid the law and mimic the effect of traditional drugs. Recently the use of SCs has increased at an unprecedented pace around the world. Several methods have been published recently for the analysis of SCs in different biological matrices. However, comprehensive methods for urine analysis covering the most recent substances 5-F-AKB48 (N-4 OH pentyl) and 5-F-PB-22 3-carboxyindole are lacking. SCs have an extensive metabolism, therefore the unchanged compounds usually are not found in urine after consumption. A simple, cost-effective and accurate liquid chromatography tandem mass spectrometric (LC-MS/MS) method for the simultaneous quantification of JWH-018 5-OH pentyl, JWH-018 4-OH pentyl, JWH-073 3-OH butyl, AM-2201 4-OH pentyl, JWH-250 4-OH pentyl, JWH-122 5-OH pentyl, JWH-210 5-OH pentyl, 5-F-AKB48(N-4 OH pentyl) and 5-F-PB-22 3-carboxyindole in urine has been developed and validated. Drug-free urine samples spiked with various SC metabolites were buffered, hydrolyzed, and extracted with a liquid-liquid extraction technique and separated on a Phenomenex Gemini C18 (150 x 2.0 mm, 5μm) column. Gradient elution was employed using a mobile phase consisting of A: 2M ammonium acetate and formic acid 0.1% in deionised water and B: 2M ammonium acetate and formic acid 0.1% in methanol. All SCs were detected and quantified within 11.5 min without endogenous interferences. The correlation coefficient (R^2) was > 0.996 for all SCs with precision < 14 and accuracy ranging from 87 to 110% for all analytes at concentrations of 5, 40 and 75 ng/mL. The limit of detection ranged between 0.01 and 0.25 ng/mL and, the lower limit of quantification for each compound was determined to be 1.0 ng/mL. The recovery ranged from 77 to 108 % and the matrix effect was acceptable and ranged from 81 to 108 % for all analytes at concentrations of 5, 50 and 100 ng/mL.

➢ Oral presentation
Presented at the 2nd Scottish Student Forensic Research Symposium, the University of Dundee, **Dundee, UK (March 2015**

➢ Three Minute Thesis competition

Presented at the Scientific Society for Saudi Students in the UK, the University of Leeds, **Leeds, UK (June 2015).**

Application of a Validated LC-MS/MS Method for the Simultaneous Analysis of 9 Synthetic Cannabinoid Metabolites to Hospital Admission Urine Samples

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Introduction

Recently the use of cannabimimetics (more commonly known as synthetic cannabinoids) has increased at an unprecedented rate around the world. As a result the importance for the analysis of synthetic cannabinoids in human matrices is evident and continues to be increasingly challenging since these compounds are constantly being modified and are rapidly becoming available. They have extensive metabolism pathways; therefore the main target compounds in urine specimens are their hydroxyl and carboxy metabolites, which is important to recognise when establishing clinical and forensic toxicology screening and confirmatory methods.

Aims

The aim of the study was to apply a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the detection and quantitation of JWH-018 5-OH pentyl, JWH-018 4-OH pentyl, JWH-073 3-OH butyl, AM-2201 4-OH pentyl, JWH-250 4-OH pentyl, JWH-122 5-OH pentyl and JWH-210 5-OH pentyl, and the most recent substances 5-F-AKB48 (N-4 OH pentyl) and 5-F-PB-22 3-carboxyindole in urine samples from individuals admitted to Accident and Emergency, Glasgow Royal Infirmary, UK.

Methods

The urine samples were buffered with 1M sodium acetate (pH 5.5) before being hydrolysed with β-glucuronidase. Liquid-liquid extraction (methyl-tert-butyl-ether) was used to isolate and concentrate 9 synthetic cannabinoid metabolites from urine. Chromatographic separation (run-time 11.5 mins) was achieved using a Phenomenex Gemini C18 (150 x 2.0 mm, 5μm) column and a gradient elution system consisting of two mobile phases; 2mM ammonium acetate and 0.1% formic acid in de-ionised water and in methanol. Agilent LC-MS/MS equipped with an electrospray ionisation interface, operated in positive polarity, was used for analysis. Multiple Reaction Monitoring (MRM) mode was used to monitor 2 ion transitions (quantifier and qualifier) for each analyte and 1 transition was used for the internal standard.

Results

All synthetic cannabinoids were detected and quantified without endogenous interferences. The correlation coefficient (R^2) was ≥ 0.996 for all analytes, with precision < 14% and accuracy ranging from 87 to 110% for all analytes at concentrations of 5, 40 and 75 ng/mL. The limit of detection ranged between 0.01 and 0.25 ng/mL and, the lower limit of quantification for each compound was determined to be 1.0 ng/mL. The recovery ranged from 77 to 108 % and the matrix effects were acceptable, ranging from 81 to 108 % for all analytes at

concentrations of 5, 50 and 100 ng/mL. The presented method was successfully applied to 93 authentic urine admission samples collected at Glasgow Royal Infirmary, UK. Four (4.3%) samples were found positive for 5-F-PB-22 3-carboxyindole with concentrations of 6.6, 23.7, 60.3 and 267.7 ng/mL.

Conclusions

A simple, cost-effective and accurate LC-MS/MS method for the simultaneous quantification of 9 synthetic cannabinoid metabolites in urine has been developed and validated. The method has been successfully verified using 93 authentic hospital case samples.

Synthetic Cannabinoids, Urine, LC/MS-MS

➢ Poster

Presented at the 53rd The International Association of Forensic Toxicologists meeting, **Florence, Italy (August-September 2015).**