University of Glasgow

Synthetic Cannabinoid Receptor Agonists in Scottish Sub-Populations

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

Alice Seywright
BSc (Hons) MSc MRSC

Forensic Medicine and Science
(School of Medicine, Dentistry and Nursing)

March 2020

Acknowledgements

Thanks, first and foremost, must go to my supervisors, Dr. Hazel Torrance and Dr. Fiona Wylie, for their help, guidance and support in conducting this research, obtaining samples and reviewing this thesis.

The input from Denise McKeown was invaluable to the method development aspect of this work and my great thanks go also to her for this, and her general support.

I would not have the opportunity to undertake this PhD were it not for Dr. Gail Cooper, who not only supported me through my MSc, but invited me back to the department of Forensic Medicine and Science to work and conduct this research.

It is entirely possible I would have binned my work and given up entirely if it wasn't for the fact that Lauren O'Connor was more often than not experiencing the same frustrations and challenges as I was. Thanks to Lauren for listening to my rants and for all her help and support throughout this… ~~ordeal~~ *journey*. Thanks also to Claire Parks for all her technical support, tending to QTrap1, and generally keeping me sane over the past few years.

I genuinely would not have been able to complete this PhD without Fraser, most likely as I would have succumbed to hunger and rage. My dinner was always on the table when I got in; he has given hours of his life to proof reading and my practice presentations; and his overall support was immense. He certainly knows much more about SCRAs than the average Maths Teacher. Thank you.

For general and miscellaneous advice and support, you cannot get much better than my dad, Ian, and mother-in-law Lindsay. They know where a semi-colon goes and exactly what word it is I know exists but cannot immediately place. My dad has also had the unenviable task of raising me in less than ideal circumstances and I owe him more than just thanks.

No acknowledgements section is complete without mention of Tchai and Edinson. Their resigned acceptance of hugs, endured without struggle or scratching, got me through many a bad day of failed extractions and instrument breakdowns.

“Were there none who were discontented with what they have, the world would never reach anything better.”

– Florence Nightingale

“Life shrinks and expands in proportion to one’s courage”

– Anaïs Nin

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 _____

Table of Contents

List of Figures

List of Tables

List of Publications

Seywright A, Torrance HJ, Wylie FM, McKeown DA, Lowe DJ, Stevenson R. Analysis and clinical findings of cases positive for the novel synthetic cannabinoid receptor agonist MDMB-CHMICA. Clinical Toxicology. 2016 Sep 13;54(8):632-7

List of Abbreviations

Δ9-THC	Δ9-tetrahydrocannabinol
°C	Degrees centigrade
µg	Microgramme
µL	Microlitre
µm	Micrometre
$\bar{\chi}$	Mean value in a data set
σ	Standard deviation in a data set
%CV	Relative standard deviation in a data set
2-AG	2-arachidonoylglycerol
5F-AB-PICA	N-(1-Carbamoyl-2-methyl-propyl)-1-(5-fluoropentyl)indole-3-carboxamide
5F-AB-PINACA	N-((1S)-1-(Aminocarbonyl)-2-methylpropyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
5F-ADB-PINACA	N-(1-Amino-3,3-dimethyl-1-oxo-2-butanyl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide
5F-AKB48	(N-(Adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide)
5F-CUMYL-PINACA	1-(5-fluoropentyl)-N-(2-phenylpropan-2-yl)indazole-3-carboxamide
5F-MDMB-PINACA	Methyl (S)-2-(1-(5-fluoropentyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate
5F-MMB-PINACA	2-(1-(5-Fluoropentyl)-1H-indazole-3-ylcarbonylamino)-3-methylbutanoic acid methyl ester
5F-NPB-22	1-(5-Fluoropentyl)-1H-indazole-3-carboxylic acid 8-quinolinyl ester
5F-PB-22	1-(5-Fluoropentyl)-1H-indole-3-carboxylic acid 8-quinolinyl ester
5F-SDB-005	1-(5-fluoropentyl)-N-phenyl-1H-indole-3-carboxamide
$5HT2_A$	5-hydroxy-tryptamine receptor 2_A
6-MAM	6-monoacetylmorphine
AA	Ayrshire and Arran

AAPT	Annual Addictions Prevalence Testing
AB-CHFUPYCA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-3-(4-fluorophenyl)-1H-pyrazole-5-carboxamide
AB-CHMINACA	N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide
AB-CHMINACA metabolite 1A	(S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-((4-hydroxycyclohexyl)methyl)-1H-indazole-3-carboxamide
AB-CHMINACA metabolite 2	N-[[1-(cyclohexylmethyl)-1H-indazol-3-yl]carbonyl]-L-valine
AB-FUBICA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indole-3-carboxamide
AB-FUBINACA	N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]indazole-3-carboxamide
AB-FUBINACA-d_4	N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]indazole-3-carboxamide deuterated on the indazole group
AB-FUBINACA metabolite 2B	4-amino-3-(1-(4-fluorobenzyl)-1H-indazole-3-carboxamido)-2-methyl-4-oxobutanoic acid
AB-FUBINACA valine metabolite	N-[[1-[(4-fluorophenyl)methyl]-1H-indazol-3-yl]carbonyl]-L-valine
AB-PICA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indole-3-carboxamide
AB-PINACA	(S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide
ACN	Acetonitrile
AMB-PINACA	Methyl (1-pentyl-1H-indazole-3-carbonyl) valinate
ACMD	Advisory Council for the Misuse of Drugs
AKB48	N-(1-adamantyl)-1-pentylindazole-3-carboxamide
AKB48-d_9	N-(1-adamantyl)-1-pentylindazole-3-carboxamide deuterated on the pentyl chain
AKB48 N5OH pentyl	1-(5-hydroxypentyl)-N-tricyclo[3.3.1.13,7]dec-1-yl-1H-indazole-3-carboxamide
AKB48 N5OH pentyl-d_4	1-(5-hydroxypentyl)-N-tricyclo[3.3.1.13,7]dec-1-yl-1H-indazole-3-carboxamide deuterated on the pentyl chain
AM1248	1-adamantyl-[1-[(1-methylpiperidin-2-yl)methyl]indol-3-yl]methanone

AM2201	[1-(5-fluoropentyl)indol-3-yl]-naphthalen-1-ylmethanone
AM2201-d_5	[1-(5-fluoropentyl)indol-3-yl]-naphthalen-1-ylmethanone deuterated on the indole group
APICA	N-(1-adamantyl)-1-pentylindole-3-carboxamide
APINACA	See entry for AKB48
BB-22	Quinolin-8-yl 1-(cyclohexylmethyl)indole-3-carboxylate
BHB	β-hydroxybutyrate
BCE	Before the Common Era
Cal	Calibrator standard
CB_1	Cannabinoid Receptor type-1
CB_2	Cannabinoid Receptor type-2
CBD	Cannabidiol
CE	Collision energy
CEP	Cell entrance potential
cm	Centimetre
CNS	Central Nervous System
CP-47,497	2-[(1R,3S)-3-hydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol
CP-55,940	2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol
CRM	Certified reference material
CSEW	Crime Survey England and Wales
CUMYL-PeGACLONE	2,5-dihydro-2-(1-methyl-1-phenylethyl)-5-pentyl-1H-pyrido[4,3-b]indol-1-one
CVS	Cardiovascular system
CXP	Cell exit potential
DEWS	Drugs Early Warning System
DG	Dumfries and Galloway
DOA	Drugs of abuse
DP	Declustering potential
EC	Effective concentration

ED	Emergency department
EDND	European Database for New Drugs
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EP	Entrance potential
ESI	Electrospray Ionisation
EWS	Early Warning System
EU	European Union
eV	Electron volts
FD	Forensic Directorate
FEWS	Forensic Early Warning System
FMS	Department of Forensic Medicine and Science, University of Glasgow
FUB-PB-22	Quinolin-8-yl 1-[(4-fluorophenyl)methyl]indole-3-carboxylate
FUBIMINA	(1-(5-fluoropentyl)-1H-benzo[d]imidazol-2-yl)(naphthalen-1-yl)methanone
g	Gramme
GABA	Gamma Aminobutyric Acid
GC-MS	Gas chromatography – mass spectrometry
GCS	Glasgow Coma Scale
GGC	Greater Glasgow and Clyde
GDC	Glasgow Drugs Court
GRI	Glasgow Royal Infirmary
h	Hour(s)
H_2O	Water
HLM	Human liver microsomes
HMIP	Her Majesty's Inspectorate of Prisons
HMP	Her Majesty's Prison
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry

HU-210	(6aR,10aR)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-6a,7,10,10a-tetrahydrobenzo[c]chromen-1-ol
I.S.	Internal standard
IUPAC	International Union of Pure and Applied Chemistry
JWH-018	Naphthalen-1-yl-(1-pentylindol-3-yl)methanone
JWH-072	Naphthalen-1-yl-(1-propylindol-3-yl)methanone
JWH-200-d_5	[1-(2-morpholin-4-ylethyl)indol-3-yl]-naphthalen-1-ylmethanone deuterated on the indole group
JHW-250	2-(2-methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone
K_i	Inhibition constant
kg	Kilogramme
L	Litre
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-TOF-MS	Liquid chromatography-time of flight-mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
M	Molar
MAB-CHMINACA	N-[(2S)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide
MAB-CHMINACA metabolite 1	N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-((4-hydroxycyclohexyl)methyl)-1H-indazole-3-carboxamide
MAM2201	[1-(5-fluoropentyl)indol-3-yl]-(4-methylnaphthalen-1-yl)methanone
MAO	Monoamine oxidase
MDA	Misuse of Drugs Act 1971
MDA*	Methylenedioxymethamphetamine
MDEA	Methylenedioxyethylamphetamine
MDMA	Methylenedioxymethylamphetamine

MDMB-CHMICA	Methyl (2S)-2-[[1-(cyclohexylmethyl)indole-3-carbonyl]amino]-3,3-dimethylbutanoate
MDMB-CHMINACA	Methyl (2S)-2-[[1-(cyclohexylmethyl)indazole-3-carbonyl]amino] -3,3-dimethylbutanoate
MDMB-PINACA	N-[[1-pentyl-1H-indazol-3-yl]carbonyl]-3-methyl-L-valine methyl ester
ME	Matrix effects
MeOH	Methanol
mg	Milligramme
MHRA	Medicines and Healthcare Products Regulations Agency
Min	Minute(s)
mL	Millilitre
mM	Millimolar
mm	Millimetre
MMB2201	Methyl (2S)-2-[[1-(5-fluoropentyl)indole-3-carbonyl]amino]-3-methylbutanoate
MMB-CHMICA	Methyl 2-[[1-(cyclohexylmethyl)indole-3-carbonyl]amino]-3-methylbutanoate
MMB-FUBINACA	Methyl-(2S)-2-[[1-[(4-fluorophenyl)methyl]indazole-3-carbonyl]amino]-3-methylbutanoate
MP	Mobile phase
MRM	Multi-reaction monitoring
MS	Mass Spectrometry, metabolic system (relating to PSS)
ms	Millisecond(s)
MVLS	Medical, Veterinary and Life Sciences
MW	Molecular weight
m/z	Mass-to-charge ratio
n	Number of samples in a data set
NEG	Negative
ng	Nanogramme
NHS	National Health Service

nM	Nano-molar
NM2201	Naphthalen-1-yl 1-(5-fluoropentyl)indole-3-carboxylate
NMDA	*N*-methyl-D-aspartic acid
No.	Number
NPS	Novel Psychoactive Substance(s)
PAR	Peak area ratio
PB-22	Quinolin-8-yl 1-pentylindole-3-carboxylate
PB-22-d_9	Quinolin-8-yl 1-pentylindole-3-carboxylate deuterated on the pentyl group
PE	Process efficiency
POCT	Point of care test
POS	Positive
PP	Protein precipitation
PSA	Psychoactive Substances Act 2016
PSS	Poison Severity Score
QC	Quality control
QL1	Qualifier ion 1
QL2	Qualifier ion 2
QT	Quantifier ion
R	Correlation coefficient
RCS-4	(4-methoxyphenyl)-(1-pentylindol-3-yl)methanone
Rec.	Recovery
REC	Research Ethics Committee
RP	Reverse phase
RT	Room temperature; 16 - 24 °C
SALSUS	Scottish Schools Adolescent Lifestyle and Substance Use Survey
SCRA	Synthetic Cannabinoid Receptor Agonist
SCOTSS	Society of Chief Officers of Trading Standards in Scotland

SNBTS	Scottish National Blood Transfusion Service
SNR	Signal-to-noise ratio
SPS	Scottish Prison Service
STS-135	N-(1-adamantyl)-1-(5-fluoropentyl)indole-3-carboxamide
$t_{1/2}$	Half-life
*t*BME	*tertiary*-Butyl Methyl Ether
Temp.	Temperature
THJ-2201	[1-(5-fluoropentyl)indazol-3-yl]-naphthalen-1-ylmethanone
t_R	Retention time
TSS	Trading Standards Scotland
UG	University of Glasgow
UK	United Kingdom
UNODC	United Nations Office of Drugs and Crime
UR-144	(1-pentylindol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone
US	United States of America
V	Volts
Vol.	Volume
WEDINOS	Welsh Emerging Drugs and Identification of Novel Substances Project
WIN-55,212-2	Methanesulfonic acid;[(11R)-2-methyl-11-(morpholin-4-ylmethyl)-9-oxa-1-azatricyclo[6.3.1.04,12]dodeca-2,4(12),5,7-tetraen-3-yl]-naphthalen-1-ylmethanone
XIC	Extracted ion chromatogram
XLR-11	[1-(5-fluoropentyl)indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)methanone
y.o.	Years old (referring to age)
YPBAS	Young Persons' Behaviour and Attitudes Survey

Abstract

The term Synthetic Cannabinoid Receptor Agonists (SCRAs) describes a group of hundreds of compounds which are not derived from the Cannabis plant but bind at the cannabinoid receptors. These compounds have been available for recreational use since the late 2000s and have been linked to a variety of adverse effects and death. Due to the number of compounds available, and their novel nature, controlling the manufacture, sale and possession of SCRAs has proved challenging under current legislative structure. The introduction of the Psychoactive Substances Act 2016 brought under control the manufacture, distribution and possession in a custodial facility of any SCRA which had not already been controlled by the Misuse of Drugs Act 1971.

Whilst clarification has been brought to the legal status of these drugs, what remains largely unknown is the scale of use within Scotland, and different sub-populations.

Simple and quick protocols were developed for the extraction of 40 SCRAs (comprising parent compounds and metabolites) from blood and urine. Sensitive Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) methods were developed to detect and quantify the most commonly encountered compounds at realistic blood and urine concentrations. Depending on the timing of cohort sample receipt, one of these methods was then applied to cohorts of individuals from various sub-populations within Scotland. Optimised methods for detection and quantitation in blood and urine then underwent validation.

Overall, in 1177 cases tested, SCRA prevalence was found to be low, relative to the prevalence of more ‘traditional’ drugs of abuse such as opiates/opioids or benzodiazepines. The detection of SCRAs was highest in the cohort of individuals presenting at an Emergency Department (ED) with suspected drug toxicity, with 56% of cases tested positive. Second highest was the cohort of deceased individuals undergoing post-mortem (PM) examination, with SCRAs found in 11% of cases tested. It should be noted, though, that samples from both these cohorts were only tested if SCRA use had been suspected. Samples collected from individuals undergoing admission to or liberation from Scottish Prison Service (SPS) facilities were found to contain SCRAs at a rate of 3% for all samples. All of the positive samples in this cohort were admission samples (except one which was not labeled admission or liberation), thus 5% of admission samples were

positive for SCRAs. Out of 73 samples collected from individuals under the jurisdiction of the Glasgow Drug Court (GDC), only 1 sample was positive (1.4%). All 95 samples collected from individuals being treated by the NHS Greater Glasgow and Clyde Forensic Directorate (FD) were negative for all SCRAs included in the panel.

These results indicate that SCRAs are having negative effects on the health of users and that they are being used by the offending community, both of which have been reported in mainstream media. Another suspected aspect of SCRA use was the intention of avoiding detection by mandatory drug tests. Both the GDC and FD cohorts were aware of their required compliance with drug abstinence and mandatory drug testing regime, but the low findings of SCRAs in these groups suggest this is not the case.

It is acknowledged that the numbers of individuals tested in the cohorts were relatively low, and that the studies were not a true calculation of prevalence. In addition to this, not all SCRAs were included in the analytical method, and those not included would not be identified in samples. Nonetheless, important information was gained about the scale and nature of SCRA use within Scotland.

1. Introduction

1.1. Background

The term synthetic cannabinoid receptor agonist (SCRA) refers to any exogenous compound not present in the natural cannabis plant which exhibits an agonistic action on the cannabinoid receptors in the human body. While there is limited information on the activity of SCRA metabolites on these receptors, for simplicity in this thesis, the term SCRA will also include metabolites. In order to understand the existence, pharmacology, toxicology and abuse potential of these compounds, it is first necessary to discuss the cannabinoid receptors, phytocannabinoids and endocannabinoids.

1.2. Cannabinoid Receptors

Cannabinoid receptors are classical G-protein coupled receptors and can be separated into cannabinoid receptor type-1 (CB_1) and cannabinoid receptor type-2 (CB_2), although there is some evidence for a cannabinoid receptor type-3 (1-5). They were discovered, initially in rat brain and then human brain, in 1990 (CB_1) and 1993 (CB_2) (3). CB_1 receptors are located primarily within the central nervous system (CNS) and are therefore responsible for the psychoactive effects of cannabinoids, such as changes in perception and memory, anxiety and paranoia (1-4, 6-9). Activation of these receptors mainly cause inhibitory responses, such as a reduction in neurotransmitter release (acetylcholine, glutamate, dopamine), hypothermia, analgesia, cataplexy and suppression of locomotion (the latter 4 known as the 'cannabinoid-tetrad') (1, 2, 4, 6). The relatively low concentration of CB_1 receptors in the brain stem, medulla and thalamus may explain why even high concentrations of cannabinoids do not tend to be considered a threat to life (6). CB_2 receptors are located more peripherally, primarily within the immune system, although are present within the CNS, and are thought to play a role in the modulation of pain and inflammation (1-4, 6, 8, 9). Due to the effects of their agonists including analgesia and the reduction of inflammation and nausea, the cannabinoid receptors elicited significant interest in their potential therapeutic value.

1.3. Phytocannabinoids

The first steps to the development and abuse of SCRAs were taken thousands of years ago with the use of the cannabis plant for its pharmacological properties. Possibly the earliest written record of cannabis use in medicine dates back to *ca.* 2350 BCE in Egypt, with the psychoactive effects having been noted in Sanskrit, Hindu and Chinese writings from *ca*. 10 CE (6, 10).

Although not isolated or characterized at the time, the compounds these cultures were exploiting were the phytocannabinoids, present naturally in cannabis plant material. The term phytocannabinoids refers to a group of over 60 compounds unique to the cannabis genus (11). These can be sub-divided into 10 classes, including the Δ-9-tetrahydrocannabinol (Δ9-THC) class and cannabidiol (CBD) class containing their respective namesake compounds (11). Δ9-THC and CBD are arguably the most relevant phytocannabinoids when discussing SCRAs due to their actions on the cannabinoid receptors in the human body.

Δ9-THC (Figure 1, left) is the main psychoactive component in cannabis and the synthesis of this was first reported in 1965 by Raphael Mechoulam (6, 8, 11-14). By acting predominantly as a partial agonist at the CB_1 receptor with an inhibition constant (K_i) value in the low nanomolar range, and binding at the CB_2 receptor, Δ9-THC is responsible for the “high” felt after cannabis use (1, 2, 4, 7, 8, 11, 13). It has been hypothesized that the increasing content of Δ9-THC in cannabis causes an increase in the schizotype psychotic effects when smoked, and thus that Δ9-THC may cause these effects (7, 9, 15). Strains of cannabis, such as *sinsemilla* (translates as “without seed”) and skunk, which are developed to contain high levels of Δ9-THC, cannot also produce high CBD levels, so these are low as a result (7).

The structure of CBD (Figure 1, right) was elucidated in 1963 and reports of pharmacological aspects of the compound were reported from the 1970s (16). The affinity of CBD as an agonist at both CB_1 and CB_2 is significantly less than that of Δ9-THC, within the micromolar range, but it acts as an antagonist at both receptors in the low nanomolar concentration range (4). CBD has been found to possess anticonvulsant, antipsychotic and antiemetic properties, as well as producing analgesia without the effects on memory produced by Δ9-THC (4, 6, 9, 16, 17).

Currently, a mixture of Δ9-THC and CBD is approved in the UK as treatment for musculoskeletal disorders such as multiple sclerosis. After some confusion as to whether CBD qualified as a medicine under the Medicines and Healthcare Products Regulations Agency (MHRA), the agency issued an opinion stating that CBD was a medicine and required a license to be legally sold (18, 19).

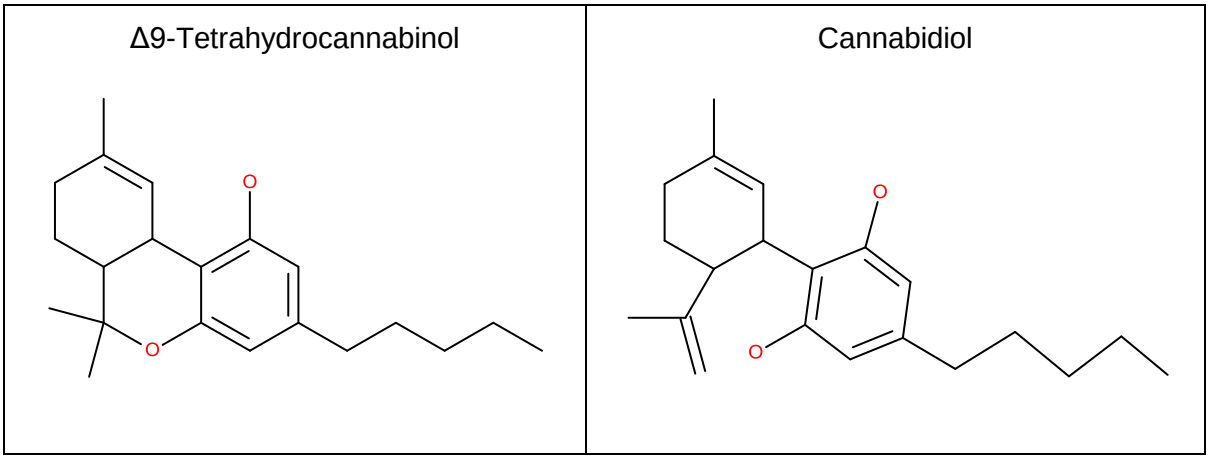


Figure 1 – Structural Formulae of Δ9-tetrahydrocannabinol (left) and cannabidiol (right)

1.4. Endocannabinoids

The two endogenous cannabinoid receptor ligands of most interest in this context are N-arachidonoylethanolamide (anandamide, from the Sanskrit word for “bliss”; Figure 2, left) and 2-arachidonoylglycerol (2-AG; Figure 2, right) (2, 7). These appear to be produced post-synaptically for use as neurotransmitters when required, and are eliminated *via* reuptake and hydrolysis by fatty acid amide hydrolases and other enzymes (2, 7, 9).

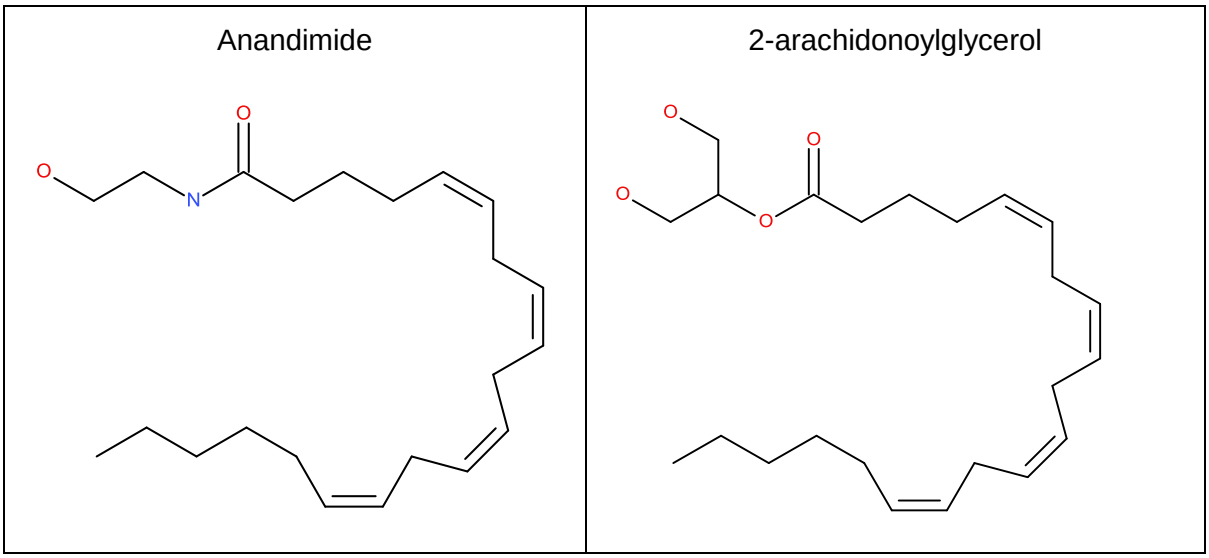


Figure 2 – Structural formulae for anandimide (left) and 2-arachidonoylglycerol (right)

Like Δ9-THC, anandamide acts as a partial agonist at the CB_1 receptor, with limited activity at CB_2 (2, 4). The potency and duration of action of the latter is less than the former (6). However, 2-AG has agonistic activity at both cannabinoid receptors which is higher than that of anandamide, with higher affinity for CB_1 than CB_2 (2, 4, 20). Both anandamide and 2-AG play a role in the prevention and healing of inflammation-induced pain, but the mechanism through which this is induced remains unclear (6). In an attempt to clarify the role of endo- and phytocannabinoids in pain and immune modulation, compounds similar to those produced in nature were synthesized (17, 21).

1.5. Synthetic Cannabinoid Receptor Agonists

Research into the cannabinoid receptors and their ligands has been ongoing since the 19th century, but the synthesis of novel cannabinoid receptor agonists began around 1940, with the work of Roger Adams in the US and Alan Todd in the UK (22). The first compounds were synthesized in attempts to produce pure forms of the naturally occurring active components of cannabis, but in doing so synthetic analogues of compounds such as Δ9-THC (*e.g.* parahexyl (3-hexyl-6,6,9-trimethyl-7,8,9,10-tetrahydrobenzo[c]chromen-1-ol)) were produced (22). During the years that followed, completely novel SCRAs were developed and patented by the likes of John W. Huffman (the JWH- series of compounds), researchers at the Hebrew University (the HU- series of compounds), and Pfizer (the CP- series of

compounds) (3, 5, 13, 21-23). Structure-activity relationship and receptor binding studies were undertaken using these novel compounds with the intention of further elucidating the role of the cannabinoid receptors and probing the potential of this system in medical therapeutics (5, 24). Given the number of SCRAs produced in pursuit of these goals, it is not surprising that great variation exists in the affinities and actions of these compounds at the CB_1 and CB_2 receptors. Table 1 provides the binding affinities at the cannabinoid receptors for selected original SCRAs, with those of Δ9-THC given for reference.

Table 1 – Binding affinities of selected original synthetic cannabinoid receptor agonists, with that of Δ9-tetrahydrocannabinol for reference

Compound	K_i (nM)		Reference
	CB_1	**CB_2**	
Δ9-THC	41 ± 2	36 ± 10	(5)
WIN-55,212-2	1.9 ± 0.1	0.28 ± 0.16	
JWH-018	9 ± 5	2.9 ± 2.6	
JWH-072	1050 ± 55	170 ± 54	

The inhibition constant (K_i) provides a measure of the receptor binding of a compound, traditionally *via* determination of the concentration of the compound required to inhibit a specified enzyme. This can be calculated by plotting the inverse rate of a substrate-enzyme reaction at different concentrations of enzyme against the concentration of the inhibiting compound. Where these lines converge is $-K_i$. The lower the K_i value, the higher the affinity of binding of that compound. The degree of binding affinity does not, however, equate to the potency of action of that compound.

There has been no evidenced authorisation of SCRAs for medicinal therapies within Europe, and the recreational use of them is a relatively new phenomenon (21). It is thought that SCRAs have been available for abuse since around 2004, but that the use of them has shifted more into the mainstream since around 2008 (9, 13, 17, 25-30). The first compounds to be detected were JWH-018, HU-210 and CP-47,497, found to be ingredients in 'K2', described on the packaging as 'herbal incense, not for consumption' (9, 25, 31). 'Spice' products were also among the first to contain SCRAs (29, 32, 33). These first series of SCRAs (JWH-, HU-, and CP-) came to be known as '1st generation' SCRAs due to their presence in the first wave of SCRA products (21). Figure 3 shows the Google Trends UK data for worldwide searches of terms related to SCRAs from January 1st 2004 to

January 1st 2019. This illustrates the varying interest in these compounds. 'Spice Gold' searches were on the rise first – around August 2005 – before peaking around October 2008 and declining since then. 'Spice drug' and 'K2 drug' have similar trends, increasing around February 2009 before gradually decreasing from around November 2012. They then both have various spikes in interest from around April 2015 onwards to the end of the search period. The terms 'fake weed' and 'synthetic weed' show similar trends to each other, with their popularity increasing from around March 2010, before peaking around June 2012 and gradually decreasing from then.

While it is acknowledged that the search terms selected will affect the data, these terms were chosen as they tend to be commonly used to describe SCRAs in the non-scientific community. It is also accepted that the number of Google searches does not necessarily correlate with prevalence of use, but is used to illustrate the interest in the drugs.

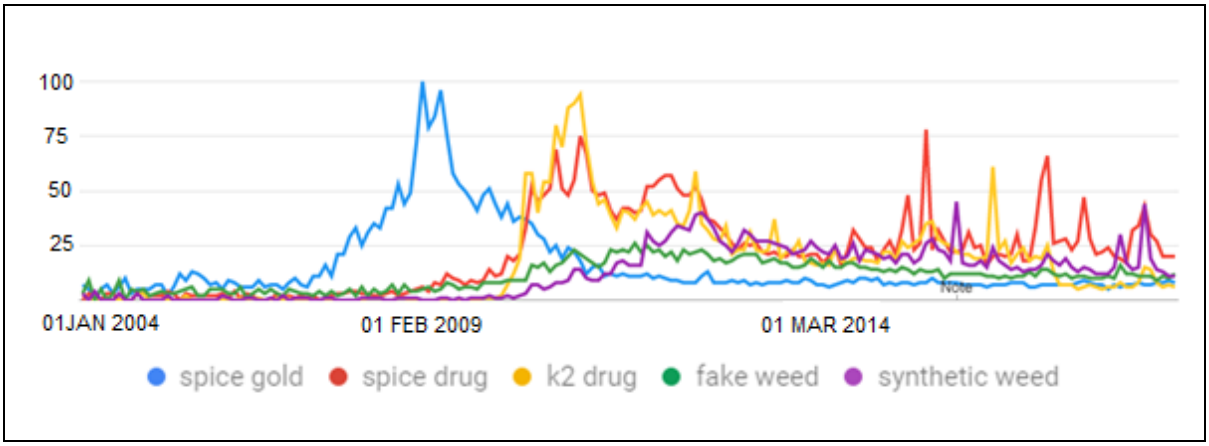


Figure 3 – Google Trends UK plot showing the popularity of selected Synthetic Cannabinoid Receptor Agonist-related search terms over time

It has been hypothesised that the rise of SCRAs could be down to a number of factors including:

- A lack of a co-ordinated international response to the emerging compounds and their abuse;
- Their potential for simple structural adaptation, circumventing legislation, *e.g.* addition of a terminal fluorine; and
- The reputation smoking has of being straightforward, relatively safe and common (34).

The first point is interesting as, due to the uncontrolled nature of the psychoactive compounds in the SCRA products, there was little monitoring by national or international agencies regarding their manufacture, importation or distribution. Indeed, SCRA products were sold openly online and in shops specialising in smoking paraphernalia (so-called 'head shops'). It appears that confusion regarding which agencies' jurisdiction monitoring or control of such compounds fall under (*i.e.* legislators, medicine regulatory, public health, trading standards) may have contributed to this (21).

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) began monitoring SCRAs in 2008, issuing a report in 2009 with the intention of clarifying the nature, availability and potential harms of SCRAs (21). Similarly, the Advisory Council for the Misuse of Drugs (ACMD) in the UK sent a report to the Home Secretary in July 2009 outlining the situation closer to home (33). Both concluded that, although there was a lack of comprehensive understanding regarding many aspects of SCRAs, their availability, popularity and evolution should be monitored with potential legal reforms considered. In what may now be considered an underestimation, the ACMD report surmised that the potential for harm of SCRAs may be "comparable" to those of herbal cannabis (33).

In 2009, an amendment was made to the Misuse of Drugs Act 1971 (MDA) to classify the SCRAs WIN 55,212-2, HU-243, CP 50,556-1, HU-210, nabilone and any compounds produced *via* specified structural derivations of these drugs as Class B drugs. These were placed in Schedule 1, as they had no acknowledged medicinal use, with the exception of nabilone, which was placed in Schedule 2 (35). This was the first legal acknowledgement of the potential for harm of SCRAs by the UK Government.

A second report to the Home Secretary from the ACMD in 2012 highlighted the concern at the rise in SCRAs available and their popularity, and suggested further legislative control (36). The compounds discussed in this report - the so-called '2nd generation' SCRAs - included AM2201, MAM-2201, RCS-4, and UR-144. The outcome of this, which came into effect in 2013, was the extension of the generic definitions introduced to MDA in 2009, covering, among others, structural derivatives of:

- Specified naphthoylindoles, specified adamantoylindoles 3-phenylacetylindole, and 3-benzoylindole by specified substitution at the nitrogen atom of the indole ring; and
- Naphthoylpyrroles by specified substitution at the nitrogen atom of the pyrrole ring;

The substitutions specified included, but were not limited to, alkyl, haloalkyl, alkenyl, hydroxyalkyl and cycloalkylmethyl (36). This amendment to MDA brought under control the likes of AM2201, RCS-4, UR-144 and MAM-2201 as Class B drugs. These compounds were also placed into schedule 1, as the ACMD concluded they had no recognized medical use (36).

In a third report to the Home Office in 2014, on the subject of '3rd generation' SCRAs, the ACMD acknowledged the futility in repeatedly updating MDA with generic definitions given the relative speed and ease with which illicit chemists can synthesize uncontrolled variations (37). This report compiled information from the Home Office Forensic Early Warning Systems (FEWS) and the Drug Early Warning System (DEWS) to identify the most commonly encountered SCRAs. Both these systems highlighted the prevalence of 5F-AKB48 and 5F-PB-22, their non-fluorinated analogues, AB-FUBINACA and AB-PINACA as being identified in products seized from 'head shops' (37). The legislative recommendation from this report proposed a change in tack intended to prevent easy circumvention from illicit chemists, by taking the SCRA JWH-018 as a base and splitting it into 4 components – the 'ring', the 'link', the 'core' and the 'tail' (26, 37). Compounds comprising functional groups specified for each of the 4 components, along with named modifications or substitutions were placed under Class B of MDA in Schedule 1 in November 2016, with the exception of certain named therapeutic drugs which remained prescription-only (37).

The repeated revision to MDA's coverage of SCRAs is reflective of the struggle faced internationally against the spread of these drugs since their emergence. Between 2009 and 2018 cumulatively, the group of NPS drugs with the highest number of new compounds notified to the EMCDDA Early Warning System (EWS) for the first time has been SCRAs (38-40). This data is presented in Figure 4 and illustrates the highly novel nature of SCRA compounds.

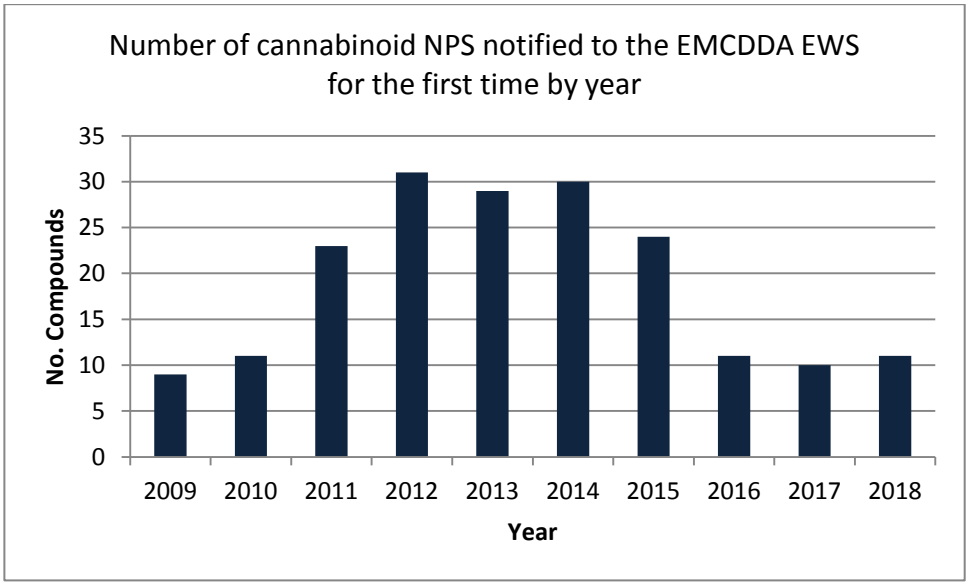


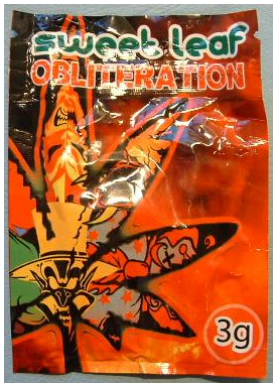
Figure 4 – European Monitoring Centre for Drugs and Drug Addiction Early Warning System data showing numbers of cannabinoid-type Novel Psychoactive Substances notified for the first time by year, 2009 - 2018 (38-41)

As of 26th May 2016, any SCRAs not controlled under MDA by the previous amendments are potentially covered by the Psychoactive Substances Act 2016 (PSA). This Act prohibits any compound not controlled under MDA which is "capable of producing a psychoactive effect" and which is not exempted from the Act (42). In compounds where psychoactivity has not already been established, this is assessed by determining whether the compound in question binds to either CB_1, GABA, $5HT2_A$, NMDA, μ-opioid or MAO transporter receptors present in the central nervous system and, in doing so, activates the receptor and elicits a biological response (43). In November 2018 a House of Commons debate took place discussing the reclassification of SCRAs from Class B to Class A. The ACMD were asked, informally, to produce a report on the motion, outlining their recommendations, by July of the next year (44, 45). As of August 2019 no such report had been released, however the request highlights the continued concern over the dangers of SCRAs.

Prior to the implementation of the PSA, a joint operation between Trading Standards Scotland (TSS), the Society of Chief Officers of Trading Standards in Scotland (SCOTSS) and other contributors cracked down on the sale of Novel Psychoactive Substances (NPS), including SCRAs, in shops in Scotland (46). Operation Alexander, as it was named, was based on the premise that the products on sale had been linked to adverse reactions, and The General Product

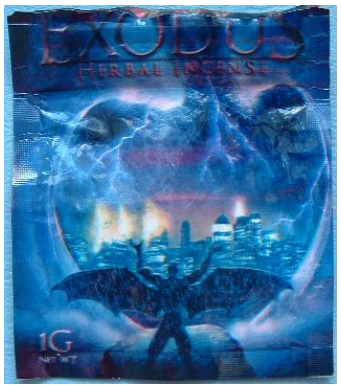
Safety Rules 2005 were therefore implemented to prohibit their sale. In total, 7,323 products with an estimated value of £146,460 were seized, although the report does not specify what proportion of these were SCRAs (46). This multiagency operation is another example of the battle waged against SCRAs.

Examples of the types of products seized during Operation Alexander are given in Figure 5. Similar products have been received by the Welsh Emerging Drugs and Identification of Novel Substances (WEDINOS) project (47). This is a service, run by Public Health Wales, whereby users can send their drugs in and have the contents analytically confirmed. SCRA packaging tends to be <10cm in any dimension (for smaller amounts), and square or rectangular in shape, with colourful artwork and alluring branding. They are often marked as ‘not for human consumption’, ‘herbal incense’, or with the ‘18’ certification symbol; and are available in sizes from 1g to kilogramme amounts. Prices vary based on the product and size, but tend to be around £10-13 for 1g, with bigger quantities carrying reductions per gramme (48-50).



Sweet Leaf Obliteration

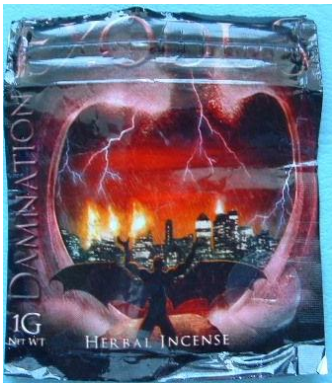
Cherry Bomb



Exodus Herbal Incense

Blueberry Blitz

Insane Joker Limited Edition



Exodus Damnation Herbal Incense

Figure 5 – Examples of synthetic cannabinoid receptor agonist product packaging showing colourful designs © WEDINOS (47)

The products generally comprise dried plant material which has the SCRA compound or compounds sprayed onto it with a volatile solvent, although powders without plant material and liquid for e-cigarettes have also been seized (1, 13, 37). The original SCRA products used plants which had reputations for psychoactive properties, such as white and blue water lily (*Nymphaea alba* and *N. caerulea*) and

marshmallow (*Althaea officinalis*) (21, 27, 33). More recently, plant materials used have been considered psychoactively inert, such as damiana (*Turner diffusa*) and members of the mint and thyme genera, although little is known about the pharmacology of smoking such material (26, 51).

It has been reported that the typical lifecycle of an SCRA product from its initial appearance on the market to its decline in popularity is around 6 – 9 months (31). This is potentially a result of updates to the legislation, prohibiting specific compounds, and has contributed to the exponential rise in SCRA products identified by various agencies. This has also made it incredibly challenging for forensic chemists and toxicologists to maintain current and fit-for-purpose methods of analysis, not least through the lack of commercially available reference standards. At the time of writing (July 2019) the number of compounds under the cannabinoids heading in the EMCDDA European Database for New Drugs (EDND) is around 190, however other sources have stated the number of SCRAs as over 400 (51, 52). While it is understandably difficult for manufacturers of certified reference materials (CRMs) to produce every SCRA potentially available to users, it is essential that agencies are able to confirm analytically which SCRAs are available and involved in adverse reactions. Dissemination of information on prevalence and potential harms of SCRAs is undertaken internationally by agencies such as the United Nations Office on Drugs and Crime (UNODC) and the EMCDDA. Within the UK, WEDINOS, Scottish Drug Forum and the UK Focal Point provide invaluable information regarding drugs available to users, and user-reported effects of these drugs. There is, however, a lack of real-time intelligence regarding the availability of analytically confirmed compounds in Scotland.

WEDINOS compiles (approximately) quarterly and annual reports highlighting developments and the current state of the drugs market from its perspective, including the top 10 most identified substances. Information concerning SCRAs garnered from these quarterly reports, beginning in late 2013 and titled PHILTRE, is presented in Figure 6. From this it is clear to see the main offenders between 2013 and 2016 are 5F-AKB48 and 5F-PB-22, and the rise of MDMB-CHMICA is documented from 2015. 5F-MDMB-PINACA is mentioned from the beginning of 2016 and MMB-FUBINACA from the end of 2016.

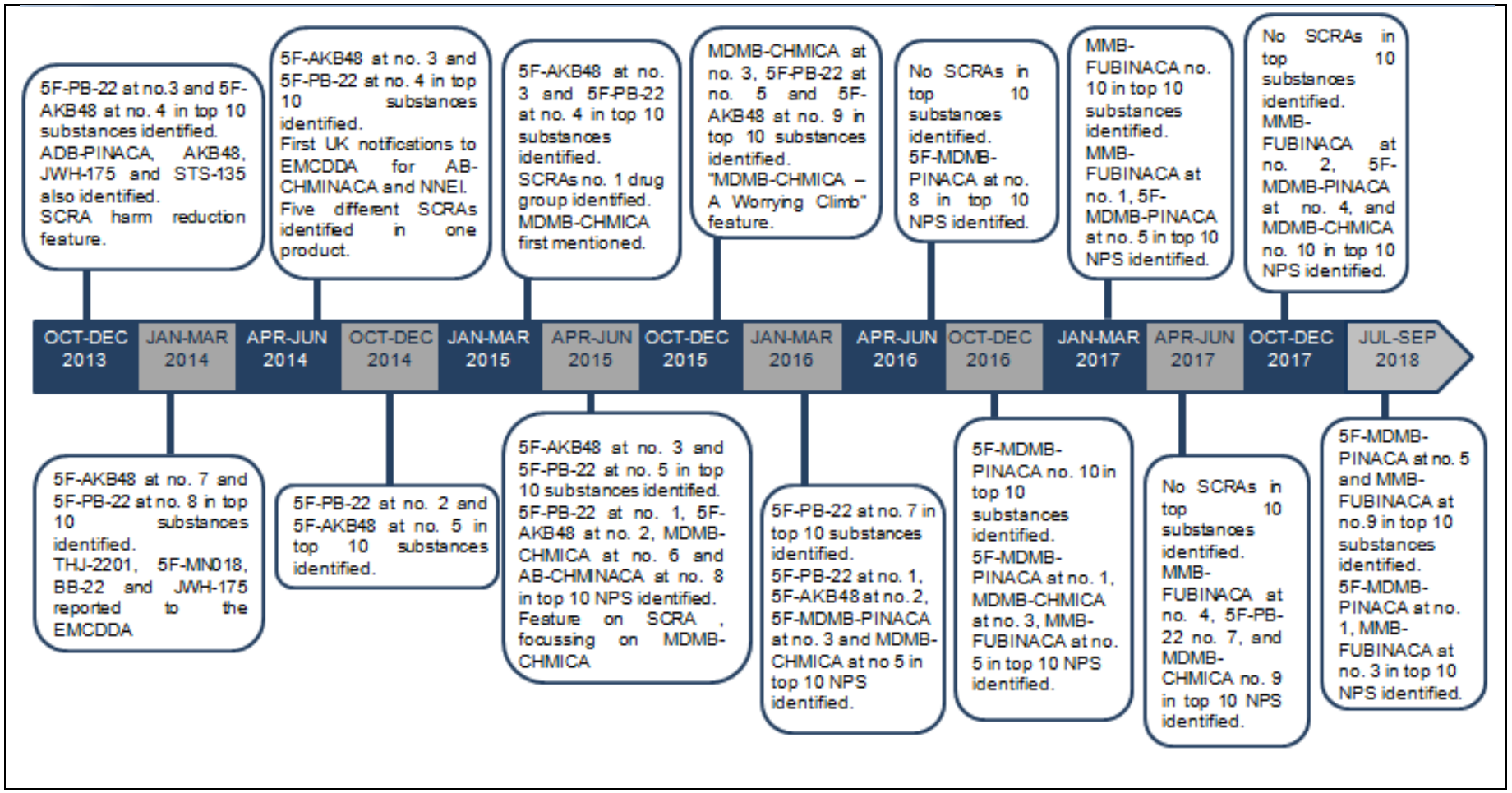


Figure 6 – Timeline of synthetic cannabinoid receptor agonist appearances in the WEDINOS PHILTRE bulletins (53-66). The shifting trends from the likes of 5F-PB-22 and 5F-AKB48 in late 2013 to MDMB-CHMICA (2014), and 5F-MDMB-PINACA and MMB-FUBINACA (both 2016) are shown.

1.5.1. Chemistry and Nomenclature

SCRAs can be classed into a variety of structural groups, varying in number by publication. A recent publication by Presley *et al.* groups them - quite specifically - into the following 18 classes (31):

- Naphthoylindoles *e.g.* JWH-018
- Halogenated naphthoylindoles *e.g*. AM2201
- Classical dibenzopyrans *e.g.* HU-210
- Cyclohexylphenyls *e.g*. CP47,497
- Benzoylindoles *e.g.* RCS-4
- Phenylacetylindoles *e.g*. JHW-250
- Tetramethylcyclopropanoylindoles *e.g.* XLR-11
- Adamantoylindoles *e.g.* AM1248
- Indolecarboxamides *e.g*. MDMB-CHMICA, APICA
- Indazolecarboxamides *e.g*. AKB48, AB-FUBINACA
- Quinolinylindolecarboxylates *e.g*. PB-22, BB-22
- Naphthoylindazoles *e.g*. THJ-2201
- Naphthoylindolecarboxylates *e.g.* NM2201
- Naphthylindazolecarboxylates *e.g.* 5F-SDB-005
- Quinolinylindazolecarboxylates *e.g.* 5F-NPB-22
- Pyrazolecarboxamides *e.g*. AB-CHFUPYCA
- Naphthoylbenzimidazoles *e.g*. FUBIMINA
- 'Others' *e.g.* methanandamide

A paper by Castaneto *et al*. groups these more broadly into 13 classes, while the ACMD condensed these further still into 7 classes (14, 33). It should be noted, though, that at the time of publication certain SCRAs representing an evolutionary step may not yet have been identified. The groups most relevant to this work are the indolecarboxamides, indazolecarboxamides, quinolinylindolecarboxylates and quinolinylindazolecarboxylates.

The classifications are based on distinguishing functional groups within the chemical structure, *i.e.* the same components of the molecules referred to in the ACMD's 3rd report to the Home Office, which led to the 2016 amendment to MDA (see section 1.5). The EMCDDA built an interactive online tool within their Perspectives on Drugs series which designates these groups the 'ring', 'link', 'core'

and ‘tail’, although ‘head’ - as features in the Cayman Chemicals Synthetic Cannabinoids Flipbook - may be a better term for ‘ring’ as some compounds do not feature a ring in this position (26, 67). The practical application of this process to MDMB-CHMICA is illustrated in Figure 7.

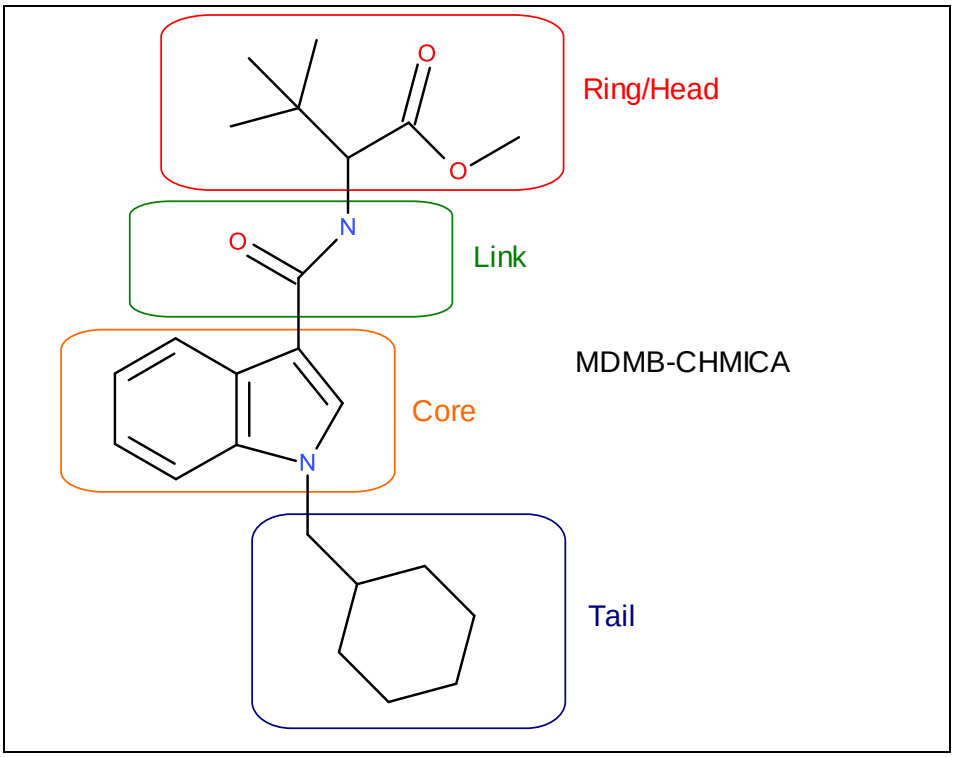


Figure 7 – Substructures of synthetic cannabinoid receptor agonists as designated by the European Monitoring Centre for Drugs and Drug Addiction Perspectives on Drugs and Cayman Chemical Synthetic Cannabinoids Flipbook (26, 67). MDMB-CHMICA is given as an example.

The process of naming SCRAs is inconsistent and confusing: many go by multiple names, different compounds can go by very similar names, and some names are practically unpronounceable. The system most commonly adopted for newer SCRAs is based on abbreviating the IUPAC systematic name for the compound and rearranging the abbreviations into the order head-tail-core-link. Although it is unclear where this system derived from, and its application is not universal, it does provide structural information on the compound to which it refers. Two examples are given using the IUPAC names for MDMB-CHMICA and AB-FUBINACA, shown below, and rearranging the abbreviated capital letters into the head-tail-core-link sequence. The coloured letters correspond to the colouring of the functional groups in Figure 7.

Methyl (S)-2-(1-(CycloHexylMethyl)-1H-Indole-3-CarboxAmido) -3,3-DiMethylButanoate

MDMB-CHMICA

N-[(1S)-1-(AminocarBonyl)-2-methylpropyl]-1-[(4-FlUorophenyl)methyl]-1H-INdAzole-3-CarboxAmide

AB-FUBINACA

While the process is relatively straightforward for deriving the name of MDMB-CHMICA, with the exception of the split in the head component name, it is not entirely comprehensive for AB-FUBINACA. Firstly, the first and third letters of the tail component, and the first, second and fourth letters of the core group are used as abbreviations, which is not entirely intuitive. A ‘b’ also appears in ‘FUBINACA’ which does not appear in the IUPAC name. It is not clear whether this arose as a requirement to make the name pronounceable or whether this refers to the benzene ring the fluorine atom is attached to. These are, however, idiosyncrasies that are consistent throughout the process and appear in other compounds, allowing the process to be learned and understood.

It appears that this naming process was brought into use after some SCRAs already had widely known names. As a result of this some drugs are known by several aliases. For example, the compound designated the name APINACA by the above process was originally called AKB48, seemingly after a Japanese girl band. When this compound went on to be halogenated at the 5’ position on the pentyl chain, both names were simply prefixed by ‘5F’, rather than completely renaming them. This is not necessarily the case with all compounds though, as 5F-APICA is also known as STS-135 (named after the US space shuttle programme).

As well as potential confusion surrounding SCRA naming, the structural components can cause issues in the interpretation of mass spectra originating from SCRAs. Certain functional groups are observed frequently in different compounds, for example the adamantyl group appears in the head position for AKB48, APICA and AM1248; the quinolinyl group appears in the head position for PB-22 and BB-22; and the indole group appears in the core position for APICA, MDMB-CHMICA and AM2201. Fragmentation within SCRAs in the mass spectrometer tends to occur in analogous positions, so if the formula weight of

different compounds is the same or similar (in the absence of suitable resolution) and they contain the same functional group, the same or similar ion transitions can result. In these circumstances, resolution must be provided by the chromatographic system or by way of High Resolution Mass Spectrometry (HRMS).

1.5.2. Pharmacology

Generally, the pharmacology of SCRAs is poorly understood: there is a lack of clinical studies, on mostly ethical grounds, and much conjecture is based on the pharmacology of Δ9-THC and other phytocannabinoids. Reports from receptor binding and animal studies for specific compounds are emerging, but the number of SCRAs means the full picture of their pharmacology remains incomplete.

SCRAs are mostly smoked in rolled-up joints either with cannabis, tobacco or alone, or inhaled *via* e-cigarette vapourisers, however they are reported to have been snorted and ingested orally as well (1, 3, 9, 13, 25, 37, 68-70). Onset of action is not well documented in published literature, but reports from user forums indicate psychoactive and physiological effects are felt almost immediately after inhalation (9, 69, 70). Effects can last from around 45 min. to several days, although it is unclear the role active metabolites or co-administered substances play in the duration of action (9, 23, 69, 71, 72).

Once administered, SCRAs distribute rapidly into brain and fat tissues due to their lipophilicity (73, 74). This results in blood concentrations of parent compounds being relatively low, even shortly after administration.

SCRAs, by definition, then act on the CB_1 and/or CB_2 receptors. Four papers investigating the pharmacology of, among others, APICA (75); AM2201, PB-22, 5F-PB-22, APICA and STS-135 (76); AB-FUBINACA, AB-PINACA and 5F-AB-PINACA (8); and MMB2201, 5F-MDMB-PINACA, MDMB-CHMICA and MDMB-CHMINACA (77) found that all were significantly more potent at both CB_1 and CB_2 receptors than Δ9-THC. With the exception of APICA and STS-135, all relevant compounds were more potent at CB_1 than CB_2. When looking at the effect of terminal fluorination on a SCRA potency between PB-22/5F-PB-22 and APICA/STS-135, a 2-3-times increase at both CB_1 and CB_2 receptors was observed in fluorinated analogues (76). Similarly, indazole-substituted forms of indole-core SCRAs (AB-FUBINACA/AB-FUBICA, AB-PINACA/AB-PICA and 5F-

AB-PINACA/5F-AB-PICA) showed an increase in CB_1 and CB_2 potency (8). Interestingly, when the indole core was substituted for indazole in MDMB-CHMICA, to form MDMB-CHMINACA, a decrease of potency was observed at the CB_2 receptor only, with no change at CB_1 (77). The potencies, in effective concentration (EC), and CB_1 selectivities of selected SCRAs with Δ9-THC for reference are given in Table 2.

Table 2 – Potencies at CB_1 and CB_2 receptors and CB_1 selectivities of selected Synthetic Cannabinoid Receptor Agonists with Δ9-tetrahydrocannabinol for reference

Compound	CB_1 potency (EC_{50}, nM)	CB_2 potency (EC_{50}, nM)	CB_1 selectivity*	Reference
Δ9-THC	250	1157	4.6	(76)
AM2201	38	58	1.5	(76)
APICA	128	29	0.2	(76)
STS-135	51	13	0.3	(76)
PB-22	5.1	37	7.3	(76)
5F-PB-22	2.8	11	3.9	(76)
AB-PINACA	1.2	2.5	2.1	(8)
5F-AB-PINACA	0.48	2.6	5.4	(8)
5F-MDMB-PINACA	0.59	7.5	12.7	(77)
MDMB-CHMICA	10	71	7.1	(77)
MDMB-CHMINACA	10	128	12.8	(77)
AB-FUBINACA	1.8	3.2	1.8	(8)
MMB2201	2.4	4.6	1.9	(77)

* Ratio of CB_2 potency to CB_1 potency.

Doses of 0.3 – 3.0 mg/kg of AB-FUBINACA and AB-PINACA lead to significant hypothermic responses in male rats, with AB-FUBINACA producing a more substantial (>2 °C decrease) and enduring (*ca.* 4 h) effect than AB-PINACA (>1.5 °C decrease, lasting *ca*. 2 h) (8). For AM2201, PB-22 and 5F-PB-22, significant hypothermic responses (>1.5 °C decrease) resulted from doses of 3 mg/kg, while no such response was observed for APICA and STS-135 until a dose of 10 mg/kg was administered (76).

Doses ranging from 0.1 – 3.0 mg/kg of AB-FUBINACA, AB-PINACA, AM2201, PB-22, 5F-PB-22, APICA and STS-135 elicited significant decreases in heart rate in rats (8, 76).

Canazza *et al.* investigated the pharmacodynamics of AKB48 and 5F-AKB48 on mice (78). The study found that a 6 mg/kg dose of AKB48 induced convulsions in 30%; hyperreflexia in 25%; myoclonias in 45%; and spontaneous and induced aggressiveness in 50% and 70% respectively of treated animals (78). 5F-AKB48 administered in 3 and 6 mg/kg doses induced convulsions in 30% and 90%, hyperreflexia in 30 and 75%; and myoclonias in 90 and 100% of treated animals respectively. A 6 mg/kg dose of 5F-AKB48 induced both spontaneous and stimulated aggressive behavior in 100% of treated animals (78). Effects of these SCRAs on core and surface body temperatures were also studied: the administration of either AKB48 or its fluorinated analogue led to reductions in body temperature, with “prolonged and significant” effects observed from AKB48 at 6 mg/kg and 5F-AKB48 at 3 and 6 mg/kg (78). Additionally, it was observed that both drugs provided analgesia during pain-inducement experiments, with even low doses (0.01 mg/kg) being effective for 5F-AKB48 in tail-pinch tests. Doses of 6 mg/kg provided increased pain threshold during equivalent thermal stimulus tests (78).

Research conducted by De Luca *et al.* studied the effects of 5F-AKB48, 5F-PB-22, BB-22 and STS-135 on rat and mouse brain and found that all were full and potent agonists exhibiting high affinity at the CB_1 receptors (79). Their results indicated that all 4 SCRAs activate G-protein receptors coupled with CB_1 and stimulate dopamine transmission. This latter activity, the authors suggested, could equate to potential abuse, through the dopamine-associated reward pathway (79).

After administration, SCRAs are sequestered in fat (due to high lipophilicity) and metabolised rapidly (73, 74). From the limited information available, there appears to be great variation in the half-lives ($t_{1/2}$) of SCRAs. Castaneto *et al.* reported the $t_{1/2}$ of CP55,940 to be 8 H, WIN55,212-2 to be 7.2 min, and STS-135 to be 3.1 min. in dog, guinea pigs and *in vitro* (human hepatocytes and human liver microsomes (HLM)) respectively (74). The metabolism of SCRAs has been more widely studied, and processes such as oxidative defluorination; mono-, di- and tri-hydroxylation; carboxylation; hydrolysis; carboxylic acid and ketone formation and glucuronidation have been identified, controlled by a variety of hepatic enzymes (31, 68, 74, 80-86). Resulting metabolites are numerous, differ in abundance, and are often shared between similar compounds leading to complications in interpretation (84, 87, 88).

Generally speaking, due to high potency and low active dose, SCRAs tend to be present at lower concentrations than many other, more 'traditional' drugs of abuse in biological matrices. Parents and metabolites can be detected in blood, serum or plasma at concentrations ranging from fractions of a nanogramme to tens of nanogrammes per millilitre (71, 72, 89-93). Concentrations in urine tend to be quantified less frequently than in whole blood or blood products. This is likely because urinary concentrations suffer more from inter-individual difference and cannot be correlated to physiological effects. Where concentrations in urine are reported, these tend to be higher than blood for metabolites, with parent compounds not detected as often (94, 95).

Hasegawa *et al.* reported the concentrations of MAB-CHMINACA in various body tissues and found urine (not-detected) < femoral blood < skeletal muscle < stomach contents ≈ heart blood < pericardial fluid < spleen ≈ adipose tissue < brain < lung < heart muscle < pancreas < kidney << liver (89).

The potency of metabolites varies, with at least some retaining significant activity (85, 96-98). Gamage *et al.* reported a decrease in affinity and an increase in selectivity for CB_2 over CB_1 in hydroxypentyl metabolites of AB-PINACA, 5F-AB-PINACA, MDMB-PINACA, 5F-MDMB-PINACA, 5F-CUMYL-PINACA, AMB-PINACA, 5F-MMB-PINACA, AKB48, and 5F-AKB48 (97). Hutchison *et al.* also found that full agonist activity remained in the 4- and 5-hydroxy pentyl metabolites of AB-PINACA (96).

1.5.3. Prevalence and Risk of Harm

The nature and scale of drug use in the general population is difficult to quantify. For this reason, prevalence studies often focus on defined sub-populations such as school-age individuals, individuals in prison, or individuals seeking or receiving treatment for substance misuse.

The Scottish Schools Adolescent Lifestyle and Substance Use Survey (SALSUS) receives questionnaire answers from secondary school pupils in local authority and independent schools on a variety of smoking and drug use habits (99). The 2015 report identified cannabis as the most commonly used drug by 15 year-old Scots, with 10% having used this drug in the last month and 17% having used it in their lifetime (99). In comparison, only 2% of 15 year olds had used "legal highs" in the last month and 5% in their lifetime, although no further information is available

on the specific type of substance used (99). It is important to consider the terminology used in the questionnaire and what respondents would think of as included in the term “legal highs”. A question relating specifically to SCRA use was included in the 2013 survey, when 2% of respondents reported having used an SCRA in their lifetime (100). (In this instance the term used was “synthetic cannabis” and the most common product names were given as examples.)

Vulnerable groups were targeted for survey completion in another study reported to the Scottish Government by MacLeod *et al* in 2016. This cohort was comprised of vulnerable young people (defined as children who are ‘looked after’ by a social work system, or accommodated, care leavers, young homeless, and/or those not in education, employment or training), people in contact with mental health services, people affected by homelessness, people who inject drugs, and men who have sex with men (101). Of 424 respondents to the survey, 252 (59%) said they had ever used NPS, 185 (74%) within the last month. SCRAs were the most commonly used NPS, with 104 respondents (41%) reporting use (101).

Cannabis was found to be the most commonly used drug in the Crime Survey England and Wales (CSEW) in 2017 – 2018, with 7.2% of individuals aged 16 – 59 having used it within the last year. This increased to 16.7% for younger adults (16 – 24 years old) (102). The same report stated that last year NPS use was 0.4% and 1.2% for 16 – 59 year olds and 16 – 24 year olds respectively (102). Of those who had used NPS in the last year, 33% of both age ranges said they had used a “herbal smoking mixture”, indicating an SCRA was the substance used (102). Questions relating specifically to ‘Spice’ and other SCRAs have not been included in the study since 2011 – 2012, when prevalence of these drugs was reported at 0.1% (100).

Conducted in Northern Ireland, The Young Persons’ Behaviour and Attitudes Survey (YPBAS) asked about SCRAs in the 2015 campaign, with 0.7% of respondents reporting having ever used a compound of this type (100).

The use of SCRAs appears to be much more significant in prisons. A 2015 report on substance misuse by Her Majesty’s Inspectorate of Prisons (HMIP) found that 6% of prisoners had used SCRAs in the 2 months prior to being incarcerated. This number rose to 10% when prisoners were asked whether they had used SCRAs since being in prison (103). In a study by User Voice, 33% of prisoners questioned

had used ‘Spice’ in the last month, and of them almost 46% reported ‘almost daily’ use (104).

In terms of drugs seized *en route* to prisons, SCRAs were detected in 39% of seizures made across 10 establishments in North West England in 2017 (105). Within these, 5F-AKB48 and 5F-PB-22 were the most commonly identified compounds, present in 29% and 24% of all seizures respectively (105).

The results of the 2012 Global Drug Survey determined that 16.8% of almost 15000 respondents had used SCRAs in their lifetime with 40.6% of these individuals having used them in the last month (51). Twenty-three respondents had sought emergency medical treatment after SCRA use, reporting symptoms such as panic and anxiety, paranoia, breathing difficulties, visual and auditory hallucinations, and extreme agitation (51). A follow up study was conducted and compared the relative risk of harm of SCRAs and cannabis, taking into account the number of days of use of both compounds (106). It was calculated that individuals who had used SCRAs in the last year had, on average, a 30-fold increased chance of seeking emergency medical treatment than the corresponding cannabis user group (106). Symptoms associated with SCRA use were similar to those reported in the 2012 survey, with cannabis users tending to report more physical than psychological symptoms and the converse true for SCRAs (106).

NEPTUNE is an initiative funded by the charity Health Foundation which aims to provide clinical guidance for the treatment of harms associated with the use of club drugs and NPS (107). A report was produced through the NEPTUNE network relating to the harms associated with SCRAs and best practice for managing these in a clinical setting (108). This highlights the variation in SCRA compounds and consequently the symptoms that may result from their use. Symptoms of acute toxicity are categorised into neurological, cognitive and psychiatric, cardiovascular, renal, and other effects including both hyper- and hypo-glycaemia and serotonin syndrome (108). As no antidote is available for SCRA toxicity, supportive and symptom-based care is recommended. Administration of benzodiazepines and antipsychotic medication such as quetiapine has been effective in treating symptoms of acute SCRA toxicity (23, 108, 109).

Tolerance to SCRAs has been reported both on practical (110, 111) and pharmacological (112) levels. Rubino *et al.* identified a decrease in cannabinoid receptor activated G-proteins in the brains of rats treated chronically with the

SCRA CP55,940, indicating desensitization and tolerance to the drug (112). In real-world terms, this is expressed as escalating use relating to amount of drug used and frequency of consumption (110, 111).

Symptoms of withdrawal on discontinuing use of SCRAs have been reported (98, 109, 110, 113). In a cohort of 47 individuals using a medical detoxification service in New Zealand, 41 reported withdrawal symptoms (113). These included mood swings (73%), anxiety (71%), and nausea and loss of appetite (12%) (113). More chronic issues such as disturbed sleep, problems in personal relationships and employment were also reported in this study (113). Treatment of withdrawal symptoms is similar to that for acute toxicity, with benzodiazepines and quetiapine having been used with some success (109).

In Scotland in 2018, there was 1 incidence where SCRAs appeared on the death certificate as implicated in, or potentially causing the death of an individual (114). By comparison, the numbers for methadone, etizolam and cocaine are 564, 551 and 278 respectively (114). By this measure SCRAs cause relatively little harm, however harmful effects on the individual's general health and quality of life, family and dependents and society on the whole should be considered along with this. Social harms are more challenging to measure and little information is available on SCRAs in this respect.

1.5.4. Analysis in Biological Matrices

As discussed previously, SCRAs and their metabolites are present in biological matrices in low, often sub-nanogramme per mililitre, concentrations, and many have similar structures. For these reasons it is important that the instrumentation used to detect and quantify these drugs is sensitive and specific.

The most widely-used analytical instruments in forensic toxicology are Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS). GC-MS tends to be used for compounds which are of relatively low molecular mass, basic in chemical nature, and are thermally labile. SCRAs are generally neutral-to-acidic in chemical nature and are of higher molecular mass than compounds associated with GC-MS analysis. LC-MS analysis, and specifically LC-MS/MS analysis, has the potential to provide sensitive and specific detection and quantitation for SCRA compounds in biological matrices.

LC-MS/MS (Figure 8) involves a mixture of compounds, for example an extracted blood sample, being separated by an analytical column before being drawn into a series of charged channels which filter specific components to be quantified by a detector.

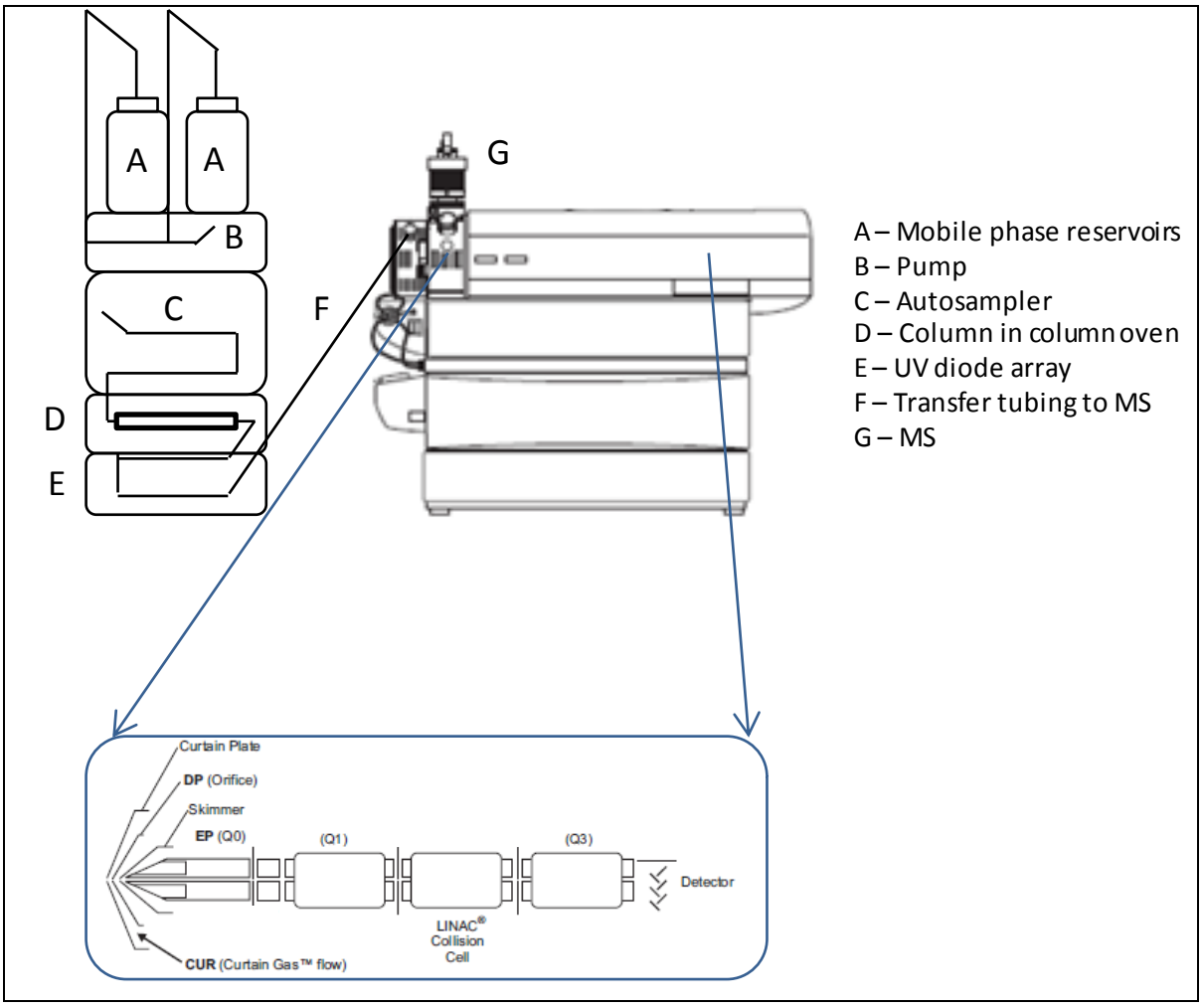


Figure 8 – Schematic representation of a Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS) system

An aliquot of the extract is injected into a mixture of aqueous and solvent liquids (the mobile phase; MP) which carries it, at high pressure, into the analytical column. The analytical column is packed with sorbent particles (the stationary phase; SP) with chemical qualities selected specifically for the application. For SCRAs, for example, the column packing might be C18 chains. The analytes of interest interact with the SP to differing degrees, such that different analytes spend different lengths of time in the analytical column. The time spent retained on the analytical column (the retention time, t_R) is characteristic of the compound, and can be specific for each compound in a certain chromatographic system. Once

elution from the column has taken place, the – now separated – components enter the mass spectrometer (MS).

The first stage of mass spectrometric analysis is desolvation. The eluted component has come straight from the LC system and is consequently still enclosed within droplets of MP. In order to remove the MP, heat and nebulizer gas (nitrogen) are applied to the droplets. Once the component has been isolated from the MP, it is ionized in the ion source of the MS through application of an ionspray voltage to the capillary connecting the LC and the MS. This converts the neutral particles eluting from the LC into charged ions, the path of which can then be dictated through the use of applied voltages. The declustering potential (DP) is applied to the orifice plate, which the ions pass through to enter the MS. This prevents ions from forming adducts with other compounds, such as sodium. The entrance potential (EP) is applied to Q_0 and is set at a voltage to guide the ions of interest through the aperture of the quadrupoles into the collision cell. The collision cell entrance potential (CEP) focuses the ions of interest into the collision cell and is mass-dependant. Once in the collision cell, the ions are fragmented by collision with nitrogen (the collision gas) and the application of collision energy (CE). The higher the CE, the more the precursor ion is fragmented and this can be optimised to give the best sensitivity and selectivity. For example, a CE can be selected that provides a high response for a fragment characteristic for one compound, and avoids the production of fragments common between other compounds included in the method. The amount of time the instrument spends applying the CE to each selected ion transition is termed the dwell time (DT). The route out of the collision cell is controlled by the collision cell exit potential (CXP). This focuses and accelerates the fragments of interest out of the collision cell, through Q_3, and towards the detector. In summary, precursor ions are selected and filtered in Q_0, fragmented into selected product ions in the collision cell, and product ions are filtered to the detector through Q_3. The voltages at each section of the MS are automatically optimised by the instrument when compound optimisation is conducted.

In this work, an AB Sciex 3200 QTrap MS system was used, which employed the QTrap apparatus as the collision cell.

LC-MS/MS has been widely used for the analysis of SCRAs in biological matrices. Huppertz et al. (115) and Kneisel and Auwärter (30) developed methods for the

detection of SCRAs in blood with limits of detection (LODs) ranging from 0.1 – 0.5 ng/mL and 0.1 – 2.0 ng/mL respectively. Method of analysis in whole blood were developed by Knittel *et al.* (116), Ambroziak and Adamowicz (117), Hess *et al.* (118) and Kacinko *et al.* (119), with LODs ranging from 0.025 – 0.1 ng/mL, 0.01 – 0.48 ng/mL, 0.01 – 8.2 ng/mL and 0.006 – 0.016 ng/mL respectively. Knittel *et al.* (116), Borg, Tverdovsky and Stripp (120), Jang *et al.* (121), Gaunitz *et al.* (122) and Staeheli *et al.* (123) developed method of analysis in urine. LODs in these methods were 0.5 ng/mL, 0.01 – 0.5 ng/mL, 0.01 – 1 ng/mL, 0.025 – 0.5 ng/mL and 0.05 – 2.5 ng/mL respectively.

Analytical methods have been also been reported for alternative matrices such as hair (124-126) and oral fluid (127).

While LC-MS/MS can provide the necessary sensitivity and selectivity required for detection and quantitation of SCRAs, consideration must also be made into the most appropriate extraction techniques.

Many researchers have employed liquid-liquid extraction (LLE), potentially due to the varied structures of SCRAs and the relatively non-specific nature of the extraction technique.

LLE manipulates the solubilities of compounds in polar and non-polar solvents to concentrate analytes of interest and enable their separation from impurities such as proteins. Generally, the polar solvent used is an aqueous buffer at a pH that controls the degree of ionisation of acidic or basic analytes, while the non-polar solvent is one in which the analytes of interest have good solubility. The theory is that, upon mixing and centrifugation, the analytes of interest migrate into the non-polar solvent which is separated and retained, while the unwanted impurities remain in the aqueous solvent and can be discarded. This is true for SCRA metabolites, which tend to be acidic in nature. Parent SCRAs, however, are neutral molecules. During LLE of these, the manipulation of the pH – and therefore degree of ionisation – of the aqueous phase rather than molecules themselves means the neutral compounds are preferentially compartmentalised into the non-polar solvent. The non-polar solvent containing the analytes of interest can then be directly injected into the analytical instrumentation, diluted prior to injection, or evaporated and reconstituted into a solvent system more suitable for instrumental analysis.

Kneisel and Auwärter (30), Huppertz *et al.* (115), Knittel *et al.* (116), and Hess *et al.* (118) have all applied LLE with a basic buffer (pH 9.3 or 10 carbonate buffer) and n-hexane:ethyl acetate (99:1) solvent system to whole blood or serum. Knittel *et al.* (116) also employed LLE to urine samples, using a phosphate buffer of pH 6.8 and 50:2 mixture of chlorobutane:hydrochloric acid. Staeheli *et al.* conducted LLE on urine using sodium acetate buffer (1M, pH5) and ACN with ammonium acetate (123). Kacinko *et al.* (119) applied to whole blood, an LLE method with the same solvent system as previously described for serum, but using saturated solutions of sodium bicarbonate and sodium chloride as the aqueous phase. The limits of detection in these methods ranged from 0.01 – 2.0 ng/mL indicating sufficient efficiency of extraction by LLE protocols using a variety of solvent systems.

Protein precipitation (PP) is another broad extraction technique. This is similar to LLE but no aqueous component is used: non-polar solvent is added to the sample and these are then mixed and centrifuged. The analytes of interest are in the solvent layer while proteins and other unwanted impurities are compacted in the pellet. The solvent can then be treated as with LLE prior to injection into the instrument.

Ambroziak and Adamowicz (117) applied a PP method using acetonitrile (ACN) as the solvent to whole blood samples.

Supported Liquid Extraction (SLE) is similar to LLE in that polar and non-polar solvents are used to extract the compounds of interest. In SLE, though, a porous material is contained within a plastic cartridge to provide a high-surface area support on which extraction can occur. Disadvantages associated with this extraction type are the financial and environmental costs of the SLE cartridges, and the requirement for vacuum manifolds.

Scheidweiler, Jarvis and Huestis (95) employed SLE in their method for the detection of SCRAs in urine.

Solid Phase Extraction (SPE) is used less frequently with SCRAs, as it is quite specific for the compounds it separates from the biological matrix. In SPE, the – usually buffered – sample is passed through a column containing a porous polymer with certain physicochemical properties among a series of aqueous and non-polar solvents. The pH and ionisation of the analytes of interest are

manipulated by the solvents so they are retained on the polymer, until an elution solvent rinses them into a collection vial. As well as the disadvantages associated with SLE, SPE is relatively time consuming. Jang *et al*. (121) and Gaunitz *et al.* (122) did, however, apply this extraction technique to urine samples.

As described previously, SCRAs can form glucuronidated metabolites which are eliminated in the urine. For this reason it is necessary to conduct hydrolysis prior to the extraction of urine samples, if glucuronidated reference standards are not being used. Many researchers employ the hydrolyzing activity of the β-glucuronidase enzyme in SCRA urinalysis. Knittel *et al.* (116) used 20 µL of β-glucuronidase from *Escherichia coli* (1250 units) per 2 mL of urine, and incubated the samples at 55 °C for 20 min prior to extraction. Jang *et al.* (121) used 40 µL of β-glucuronidase from *Helix pomatia* (*ca.* 10000 units) per 100 µL urine, and incubated at 60 °C for 1 H before extracting. Staeheli *et al.* also used β-glucuronidase from *Helix pomatia*, employing 25 µL (2500 units) per 250 µL urine, and incubating at 60 °C for 1 H prior to extraction (123). Borg, Tverdovsky and Stripp (120) and Scheidweiler, Jarvis and Huestis. (95) both used β-glucuronidase from abalone, 50 µL (1250 units) and 40 µL (625 units) respectively. The former incubated at 56 °C for 45 min., and the latter 55 °C for 1 H prior to extracting the samples.

2. Aims and Objectives

The overall aim of this project was to evaluate the scale and nature of the use of SCRAs in the Scottish population. In order to do so, the following objectives were set:

1. To identify the most commonly encountered drugs and their most abundant metabolites and to develop and validate analytical methods for the detection and quantitation of these compounds in whole blood and urine.
2. To apply these methods to blood and/or urine samples collected from a variety of different Scottish sub-populations, namely:
    a. Individuals undergoing treatment in an Emergency Department (ED) for suspected Novel Psychoactive Substances intoxication;
    b. Individuals undergoing admission to and liberation from Scottish Prison Service (SPS) facilities;
    c. Individuals receiving treatment from the National Health Service (NHS) Forensic Directorate (FD) in the Greater Glasgow and Clyde (GGC) region, for psychiatric illness with substance misuse co-morbidities;
    d. Individuals under the jurisdiction of the Glasgow Drug Court (GDC), who have agreed to comply with drug abstinence and treatment in place of a custodial sentence for offending behaviour; and
    e. Deceased individuals undergoing post-mortem (PM) examination in East, West and North Fiscal Regions of Scotland, where SCRA use is either suggested by case circumstances or specifically requested by the pathologist.
3. To collect demographic information, where possible, to determine the sub-populations where SCRA use may be more prevalent.

It was hypothesised that SCRA use would be generally low, particularly when compared to prevalence of traditional drugs of abuse. SCRA use was expected in SPS, FD and GDC cohorts due to their tendency to go undetected in current Mandatory Drug Tests and the requirement for observed drug abstinence in these participants. Higher rates of SCRA detection were expected in ED and PM cohorts as the samples underwent analysis because of the suspicion of their use by these participants.

3. Development and Validation of a Method for the Detection and Quantitation of MDMB-CHMICA in Blood

3.1. Introduction

On the 25th April 2015 an alert was issued by the EMCDDA European Union Early Warning System warning of 2 deaths and 3 non-fatal intoxications in Germany resulting from ingestion of MDMB-CHMICA (128). These events took place between September 2014 and January 2015 and in all cases the products ingested were found to contain MDMB-CHMICA, although this drug was only analytically confirmed in biological samples from one case. The drug products consumed in these cases were listed as AK47 Loaded, Manga Hot, Cloud 9 Second Generation Mad Hatters Incense, and Black Diamond. Causes of death in the fatal cases were recorded as suffocation after aspiration of gastric contents under ethanol intoxication and probable methadone intoxication. Symptoms of suspected MDMB-CHMICA toxicity in the non-fatal intoxications included tremor, unresponsiveness, cramping seizures, 'permanent' vomiting, severe motor impairment, and slurred speech.

On the 30th of June the same year a similar alert was issued by the Welsh Emerging Drugs and Identification of Novel Substances (WEDINOS) Project, containing details of an adverse reaction in North Wales following ingestion of what was – erroneously – referred to as 'MMB-CHMINACA aka MDMD-CHMICA' (129). A young male had inhaled 2 or 3 times from a rolled cigarette confirmed to contain MDMB-CHMICA and had been hospitalised overnight suffering from dizziness, shortness of breath, nausea, chest pains, irregular heart beat and convulsions. In addition to the German cases detailed within the EMCDDA EU EWS alert, further reports of deaths and adverse reactions were given within the WEDINOS alert. These included 4 deaths and 6 non-fatal intoxications in Sweden and 7 non-fatal intoxications in Austria. The specific product suspected of causing the adverse event in Wales was not provided, but products confirmed by WEDINOS to contain MDMB-CHMICA were given as Sweet Leaf Obliteration, SKYHIGH and Vertex Pirate Edition.

The following month, Issue 2 of the Police Scotland Drug Trend Monitoring Bulletin reprinted the WEDINOS alert alongside details of a similar adverse reaction in an individual following ingestion of Sweet Leaf Obliteration in Glasgow (130).

Due to the significant concern surrounding this drug, it was important to develop and validate a method to accurately identify and quantify this drug in whole blood.

3.2. Aims and Objectives

The aim of this work was to develop and validate a LC-MS/MS method suitable for the accurate identification and quantitation of MDMB-CHMICA in whole blood.

The objectives were two-fold:

- To implement a simple extraction technique coupled to a targeted LC-MS/MS method;
- To validate this to ensure its fitness-for-purpose as an accurate quantitative method.

3.3. Materials

MDMB-CHMICA (crystalline solid, >98% purity) was purchased from Chiron (Trondheim, Norway) and JWH-200-d_5 (100 µg/mL solution in ACN) was purchased from LGC Standards (Teddington, UK). Phosphate buffer (pH 6, 0.1M) was prepared in-house from disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate dehydrate from Fisher Scientific (Loughborough, UK) and deionised water produced from a Purite (Thame, UK) deionised water system. *Tertiary* methyl butyl ether (*t*BME), formic acid, sodium chloride and ammonium acetate were purchased from Sigma Aldrich (Gillingham, UK). Methanol (MeOH) and acetonitrile (ACN), both HPLC grade, and formic acid were obtained from VWR (Lutterworth, Leicestershire, UK). Blood products were purchased from the Scottish National Blood Transfusion Service (SNBTS) based at Gartnavel Hospital (Glasgow, UK).

3.3.1. Solutions

3.3.1.1. 2M Ammonium acetate

15.4 g of ammonium acetate was weighed accurately into a 100 mL volumetric flask and made up to volume with deionised H_2O. The flask was inverted several times before transferring the contents to a reagent bottle and storing at RT for up to 6 months.

3.3.1.2. MeOH with 0.1% Formic Acid and 2mM Ammonium Acetate

1 mL of concentrated formic acid and 1 mL of 2M ammonium acetate were added to 1 L of MeOH. This was inverted several times and sonicated at room temperature for 15 min. This was stored at RT for up to 3 months.

3.3.1.3. H_2O with 0.1% Formic Acid and 2mM Ammonium Acetate

1 mL of concentrated formic acid and 1 mL of 2M ammonium acetate were added to 1 L of deionised H_2O. This was inverted several times and sonicated at RT for 15 min. This was stored at RT for up to 3 months.

3.3.1.4. 50:50 MeOH:H_2O 0.1% Formic Acid and 2mM Ammonium Acetate (Infusion Solution)

1 mL of concentrated formic acid and 1 mL of 2M ammonium acetate were added to a mixture of 500 mL of deionised H_2O and 500 mL of MeOH. This was inverted several times and stored at RT for up to 3 months.

3.3.1.5. 100 µg/mL MDMB-CHMICA solution

500 µL of a 1 mg/mL MDMB-CHMICA solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer (≤-20 °C) for up to 12 months.

3.3.1.6. 10 µg/mL MDMB-CHMICA solution

500 µL of a 100 µg/mL MDMB-CHMICA solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 6 months.

3.3.1.7. 1 µg/mL MDMB-CHMICA solution

500 µL of a 10 µg/mL MDMB-CHMICA solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 6 months.

3.3.1.8. 100 ng/mL MDMB-CHMICA solution

500 µL of a 1 µg/mL MDMB-CHMICA solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 6 months.

3.3.1.9. 1 µg/mL MDMB-CHMICA in 50:50 MeOH:H_2O 0.1% Formic Acid and 2mM Ammonium Acetate

3 µL of a 1 mg/mL MDMB-CHMICA solution were added to 3 mL of infusion solution. The remainder of this solution was discarded after use.

3.3.1.10. 10 µg/mL JWH-200-d_5 solution

500 µL of a 100 µg/mL JWH-200-d_5 solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 12 months.

3.3.1.11. 1 µg/mL JWH-200-d_5 solution

500 µL of a 10 µg/mL JWH-200-d_5 solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 6 months.

3.3.1.12. 100 ng/mL JWH-200-d_5 solution

500 µL of a 1 µg/mL JWH-200-d_5 solution were added to ACN in a 5 mL volumetric flask and made up to volume with ACN. This was inverted several times, transferred to a reagent bottle and stored in the freezer for up to 6 months.

3.3.1.13. 1 µg/mL JWH-200-d_5 in 50:50 MeOH:H_2O 0.1% Formic Acid and 2mM Ammonium Acetate

300 µL of a 10 µg/mL JWH-200-d_5 solution were added to 2.7 mL of infusion solution. The remainder of this solution was discarded after use.

3.3.1.14. 0.1M pH6.0 phosphate buffer

1.7 g of disodium hydrogen orthophosphate anhydrous were weighed out and added to a 1 L beaker. 12.14 g of sodium dihydrogen orthophosphate monohydrate were weighed and added to the same 1 L beaker. *Ca.* 800 mL deionised H_2O were added to the beaker and the pH of the resulting solution was adjusted to pH6.0 with 0.1 M dibasic sodium phosphate (to increase pH) or 0.1 M monobasic sodium phosphate (to decrease pH). The solution was then transferred to a 1 L volumetric flask and made to volume with deionised H_2O. This was inverted several times and transferred to a reagent bottle. This was stored at RT for up to 3 months.

3.3.1.15. 1% Saline Solution

9.5 g of sodium chloride were transferred to a 1 L volumetric flask and made to volume with deionised H_2O. This was inverted several times, transferred to a reagent bottle and stored at RT for up to 6 months.

3.3.1.16. Blank Blood

Expired packed red cells (whole blood with the plasma portion removed) were mixed 1:1 with 1% saline solution in a beaker. This was transferred to a reagent bottle, capped and inverted several times, then stored in the fridge (2 – 8 °C) for up to 6 months.

3.4. Method Development and Optimisation

3.4.1. Liquid Chromatography – Mass Spectrometry

The instrument used for this method was an Agilent 1260 Infinity HPLC system coupled to an AB Sciex 3200 Qtrap MS. Chromatographic separation was undertaken using a Phenomenex Gemini C18 column (150 mm x 2.0 mm, 5 µm) fitted with a guard cartridge of the same packing material and held at 40 °C. Analyte detection was made using positive ESI and MRM.

3.4.1.1. Optimisation of Analyte Precursor and Product Ions

Determination of analyte ion transitions was made by infusing a solution of the analyte (at 1 µg/mL in infusion solution) directly in to the ion source of the MS using the in-built syringe driver on the instrument. The Compound Optimisation function of the Analyst Software (version 1.6.3) was employed to manipulate instrumental parameters for optimum analyte response. This process involves ramping the declustering potential (DP), entrance potential (EP), cell entrance potential (CEP) and cell exit potential (CXP) within the MS and recording the responses obtained for the precursor ion and 8 most abundant product ions. The 4 most abundant product ions and their abundances were then recorded by the software and automatically incorporated into a method. These were then scrutinised with reference to the scientific literature and the structural formulae of the analytes to ensure they originated from the analytes and were not present due to contamination.

This process was repeated for JWH-200-d_5 which was used as an I.S.

3.4.1.2. Investigation Into Analyte Retention

Isocratic mobile phase (MP) compositions of 0.1% formic acid and 2mM ammonium acetate in aqueous solutions of 50, 60, 70 and 80% MeOH were tested for best analyte retention, at a flow rate of 300 µL/min with an injection volume of 20 µL. Retention times for analyte and I.S. were noted and considered acceptable for use in the final method if they were between 2 – 10 min.

3.4.1.3. Final Method

The instrumental parameters found to be most conducive to analyte sensitivity and specificity are given in Table 3. These comprise the final method and are discussed in more detail in section 3.5.

Table 3 – Final instrumental parameters of MDMB-CHMICA method. Ion transitions for the analyte and Internal Standard, the Liquid Chromatography Mobile Phase programme, and Mass Spectrometer voltages are shown.

Parameter	Values					
MDMB-CHMICA Ion Transitions	385.1 → 240.2 (QT), 144.1 (QL1), 116.1 (QL2)					
JWH-250-d_5 Ion Transition	390.1 → 155.1					
Liquid Chromatography Mobile Phase Programme	Isocratic at 20:80 H_2O:MeOH with 0.1% formic acid and 2mM ammonium acetate					
Mass Spectrometer Voltages	Ion Transition	Declustering Potential (V)	Entrance Potential (V)	Cell Entrance Potential (V)	Collision Energy (eV)	Cell Exit Potential (V)
	385.1 → 240.2	36	4	32	23	4
	385.1 → 144.1	36	4	32	47	4
	385.1 → 116.1	36	4	32	87	4
	390.1 → 155.1	61	9.5	20	29	4

3.4.2. Extraction

A relatively broad Liquid-Liquid Extraction (LLE) procedure was employed as previously developed (131): an aliquot of 100 µL blood was buffered with 2 mL 0.1M pH6.0 phosphate buffer with I.S. present at 25 ng/mL. During each batch, matrix-matched calibrators prepared at 1, 5, 10, 25, 50 and 100 ng/mL were extracted alongside a ‘blank’ MDMB-CHMICA-free standard and a ‘spike’ at 42 ng/mL prepared from a stock distinct from the calibrator stock solution. This

concentration was chosen as a spike due to it being within the mid-range of calibrators but not being equal to any calibrator. Calibrators and spike were prepared according to Table 4 and a 100 µL aliquot of each was added to the buffered blood. ACN (100 µL) was added to the blank and samples as well, to ensure equal volumes of solvent between standards and samples.

Analyte extraction was induced through the addition of 2 mL *t*BME. All standards, samples and blank were vortex mixed vigorously before and after addition of this then centrifuged at 3000 rpm for 10 min. The solvent layer was then removed for evaporation under nitrogen at RT. Samples were then reconstituted in 500 µL of an aqueous solution of 50% MeOH before injection into the final LC-MS method (see Table 3).

Table 4 – Preparation details for the calibrators and QC used in the analytical method for the detection and quantitation of MDMB-CHMICA in blood

Standard	Concentration (ng/mL)	Volume of Working Standard/Spike Solution (µL) (1 µg/mL or *100 ng/mL)	Volume of ACN (µL)
CAL 1	1	10*	990
CAL 2	5	5	995
CAL 3	10	10	990
CAL 4	25	25	975
CAL 5	50	50	950
CAL 6	100	100	900
QC	42	42	958

3.4.3. Method Validation

The method was validated for linearity, selectivity, sensitivity (LLOQ and LOD), inter- and intra-day precision and accuracy, process efficiency and matrix effects.

3.4.3.1. Linearity

Linearity was assessed with 1/χ weighting over the calibration range 1 – 100 ng/mL, in triplicate. The linear model was determined and a correlation co-efficient using this model was deemed acceptable at ≥0.99, ensuring a minimum of 4 of 6 calibration points were within 80 – 120% accuracy. Linearity was assessed in every batch which included samples to ensure the aforementioned criteria were met.

3.4.3.2. Selectivity

Selectivity was monitored by analysing MDMB-CHMICA-free samples and ensuring any peak observed in the resultant chromatogram not originating from MDMB-CHMICA was baseline resolved from this analyte.

3.4.3.3. Sensitivity

The LOD was designated as the lowest standard at which the signal-to-noise ratio (SNR) of the ion transition with the lowest response was 3, with the LLOQ being the lowest standard at which the SNR of the same ion transition was 10. To assess this, unextracted calibrators of decreasing concentration were analysed and the SNR of the peak with the lowest response was assessed visually. The LLOQ was employed as the lowest calibrator.

3.4.3.4. Accuracy

Accuracy was calculated inter- and intra-day at 10 and 42 ng/mL using the calculation given in Equation 1, where $\bar{\chi}$ denotes the mean concentration of replicate measurements and χ denotes the expected concentration, *i.e.* 10 or 42 ng/mL. For intra-day (n=4) measurements, replicates were injected within 1 batch, and for inter-day (n=2) measurements, batches of triplicate standards were run on separate days.

Equation 1

$$Accuracy\ (\%)\ = \left(\frac{\bar{\chi}}{\chi}\right) \times 100$$

Accuracy was deemed acceptable when the above equation yielded a result between 80 – 120%.

3.4.3.5. Precision

Precision was calculated inter- and intra-day at the same concentrations as accuracy by using the calculation for %CV. This is given by Equation 2, where σ denotes the standard deviation of the measurements as calculated using Equation 3, and n denotes the number of measurements made.

Equation 2

$$\%CV = \left(\frac{\sigma}{\bar{\chi}}\right) \times 100$$

Equation 3

$$\sigma = \sqrt{\frac{\sum(\chi - \bar{\chi})^2}{n}}$$

Precision was deemed acceptable when %CV values were ≤15.

3.4.3.6. Matrix Effects and Process Efficiency

Matrix effects (ME) were calculated at 50 ng/mL and process efficiency (PE) was calculated at 5 and 50 ng/mL extracted in triplicate from 6 sources of blank blood according to the Matuszewski method (132), shown in Equation 4 and Equation 5.

Equation 4

$$Matrix\ Effects = \left(\frac{B}{C}\right) \times 100$$

Equation 5

$$Process\ Efficiency = \left(\frac{A}{C}\right) \times 100$$

Where:

A= peak area of an extracted standard

B= peak area of a double blank extracted standard reconstituted in the unextracted standard

C= peak area of an unextracted standard

3.5. Results and Discussion

3.5.1. Liquid-Chromatography – Mass Spectrometry

3.5.1.1. Optimisation of Analyte Precursor and Product Ions

From the analyte infusion work described in Section 3.4.1 the ion transitions and MS parameters in Table 5 provided the optimum response. These transitions relate to the fragmentation of MDMB-CHMICA and JWH-200-d_5 as shown in Figure 9. Transition 385.109 → 240.2 gave the highest response and was therefore used as the quantitation transition (QT). Transitions 385.1 → 144.1 and 385.1 → 116.1 also gave sufficiently high instrument responses and were used as qualifier transitions 1 and 2 (QL1 and QL2) respectively.

Table 5 – Ion transitions and Mass Spectrometer parameters for MDMB-CHMICA and internal standard JWH-200-d_5 as determined for the analytical method by instrumental optimisation

Analyte	Ion Transition	Dwell Time (ms)	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
MDMB-CHMICA	385.1 → 240.2	150	36	4	32	23	4
	385.1 → 144.1	150	36	4	32	47	4
	385.1 → 116.1	150	36	4	32	87	4
JWH-200-d_5	390.1 → 155.1	150	61	9.5	20	29	4

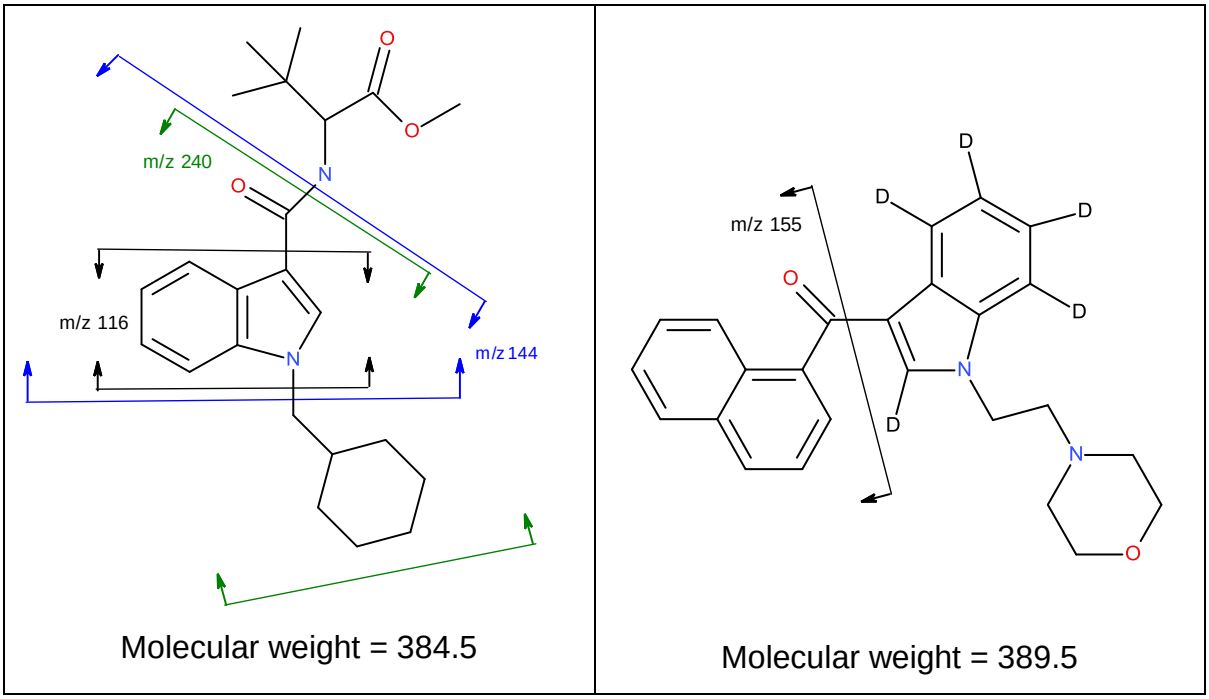


Figure 9 – Fragmentation in the Mass Spectrometer of MDMB-CHMICA (left) and JWH-200-d_5 (right). For MDMB-CHMICA this takes place between the carboxamide link and the indole moiety (either between the carboxide and the indole, or between the amide and the indole), and between the indole moiety and the cyclohexylmethyl group.

3.5.1.2. Investigation Into Analyte Retention

From experiments into MP composition and retention time, 80:20 MeOH:H_2O with 0.1 % formic acid and 2mM ammonium acetate was found to be most appropriate. This gave a retention time of *ca*. 2 min for JWH-200-d_5 and *ca*. 5.5 min for MDMB-CHMICA. Increased retention for the I.S. on the column would have been desirable, however even a slight increase in the aqueous content of the MP caused a detrimental widening of MDMB-CHMICA peak shape, with very strong retention of MDMB-CHMICA resulting from lower solvent contents. These effects

can be observed in Figure 10 with example XICs of MDMB-CHMICA shown at 60%, 70% and 80% MP B (bottom, middle and top traces respectively; note the difference in χ-axis scale on 60% B trace).

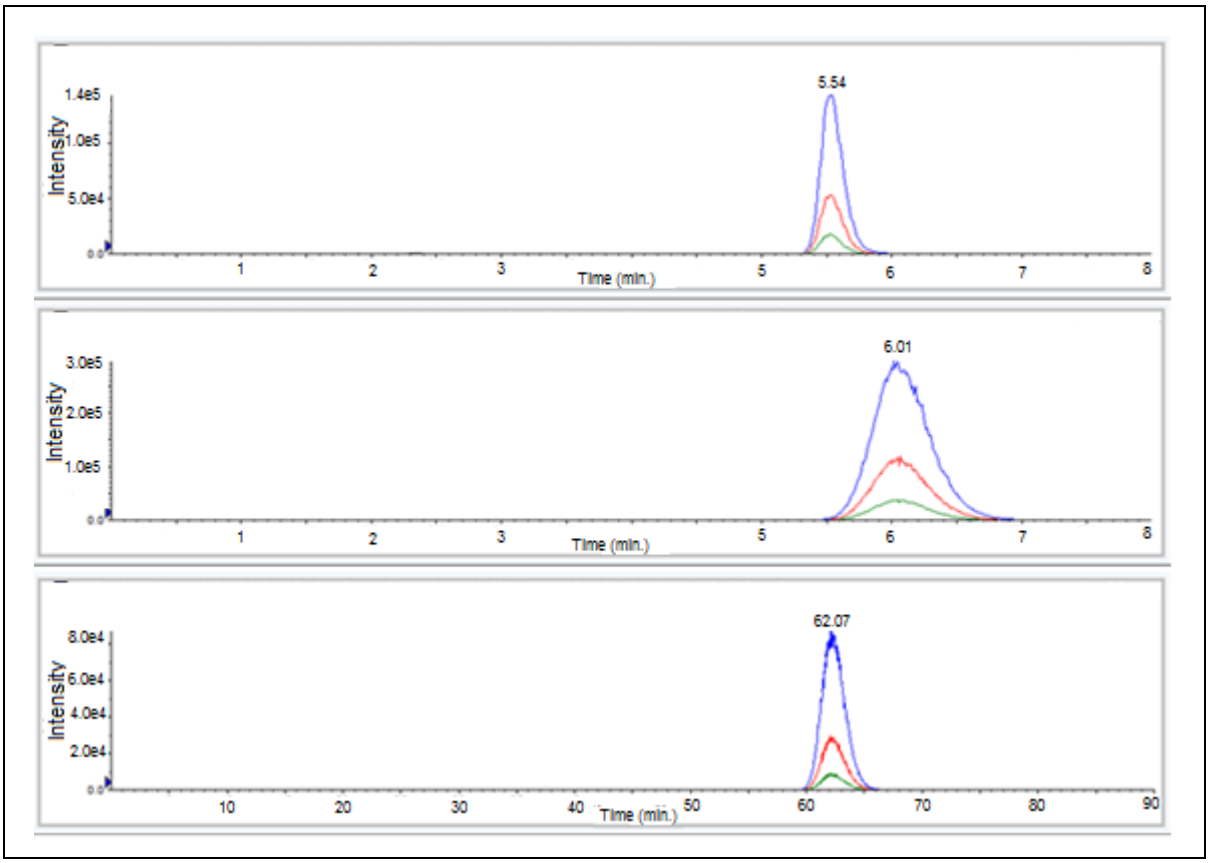


Figure 10 – Retention times of MDMB-CHMICA at 60% (bottom), 70% (middle) and 80% (top) methanolic mobile phase (isocratic). Note the difference in χ-axis scale on 60% methanolic mobile phase trace. Blue, red and green lines indicate QT, QL1 and QL2 ion transitions respectively and intensity is given in counts per second.

3.5.1.3. Extraction

The extraction procedure detailed in Section 3.4.2 provided good results in terms of peak area, peak shape and baseline noise and allowed method validation to go ahead.

3.5.2. Method Validation

3.5.2.1. Linearity

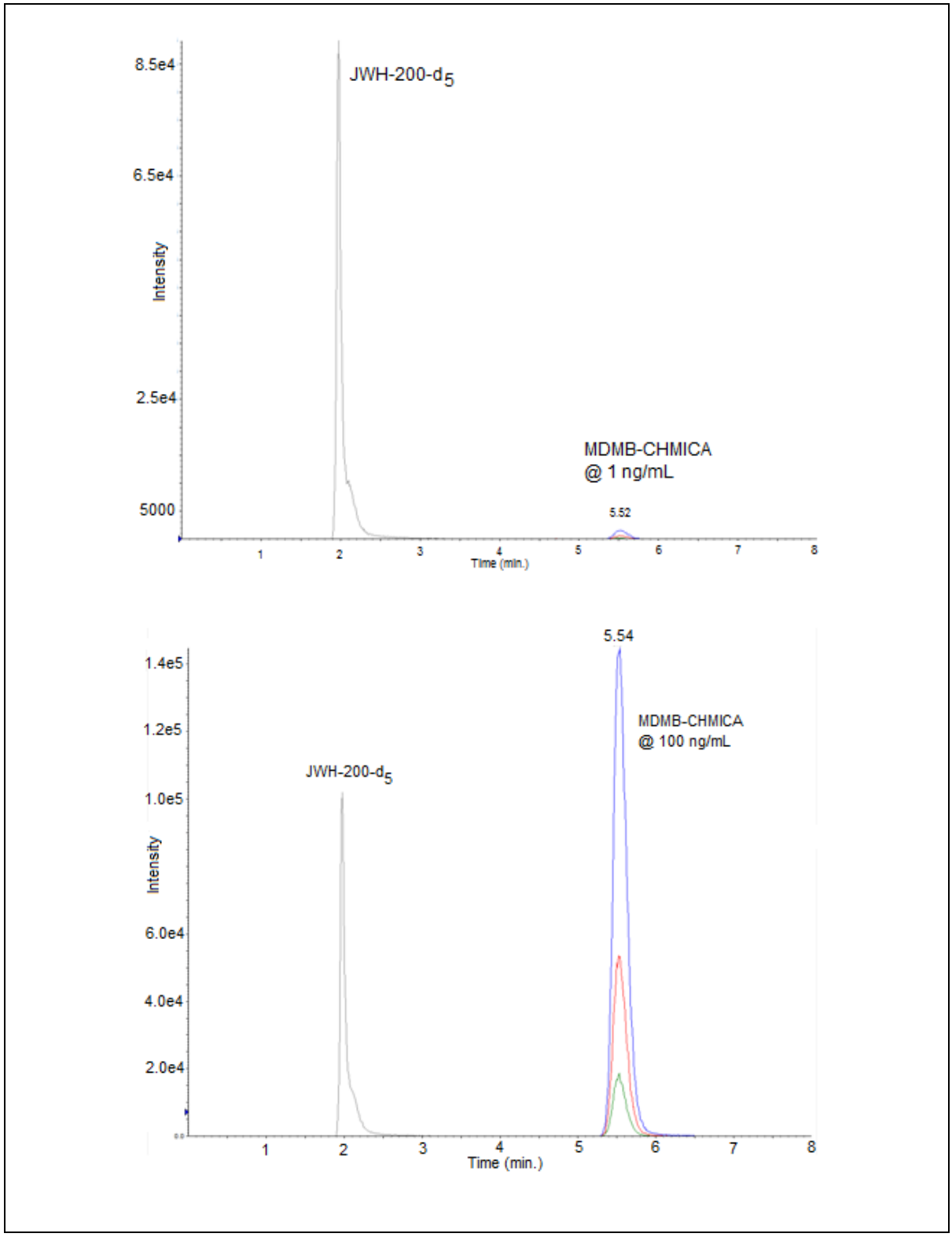


Figure 11 – Example extracted ion chromatograms of extracted MDMB-CHMICA calibrators at 1 (top) and 100 (bottom) ng/mL run isocratically at 80% methanolic mobile phase. QT, QL1 and QL2 ion transitions are shown in blue, red and green respectively and intensity is given in counts per second.

Linearity was established between 1 – 100 ng/mL using 1/χ-weighting yielding a correlation co-efficient of ≥0.99 with a minimum of 4 points in all replicates. Example XICs of extracted calibrators at 1 and 100 ng/mL are given in Figure 11 and an example calibration is shown in Figure 12. It was believed that concentrations of MDMB-CHMICA found in case samples would be towards the bottom end of the calibration range, where absolute error would be higher, so 1/χ-weighting was chosen to normalise the error over the range, taking the domination of the calibration curve away from the higher concentrations, where absolute error would be lower.

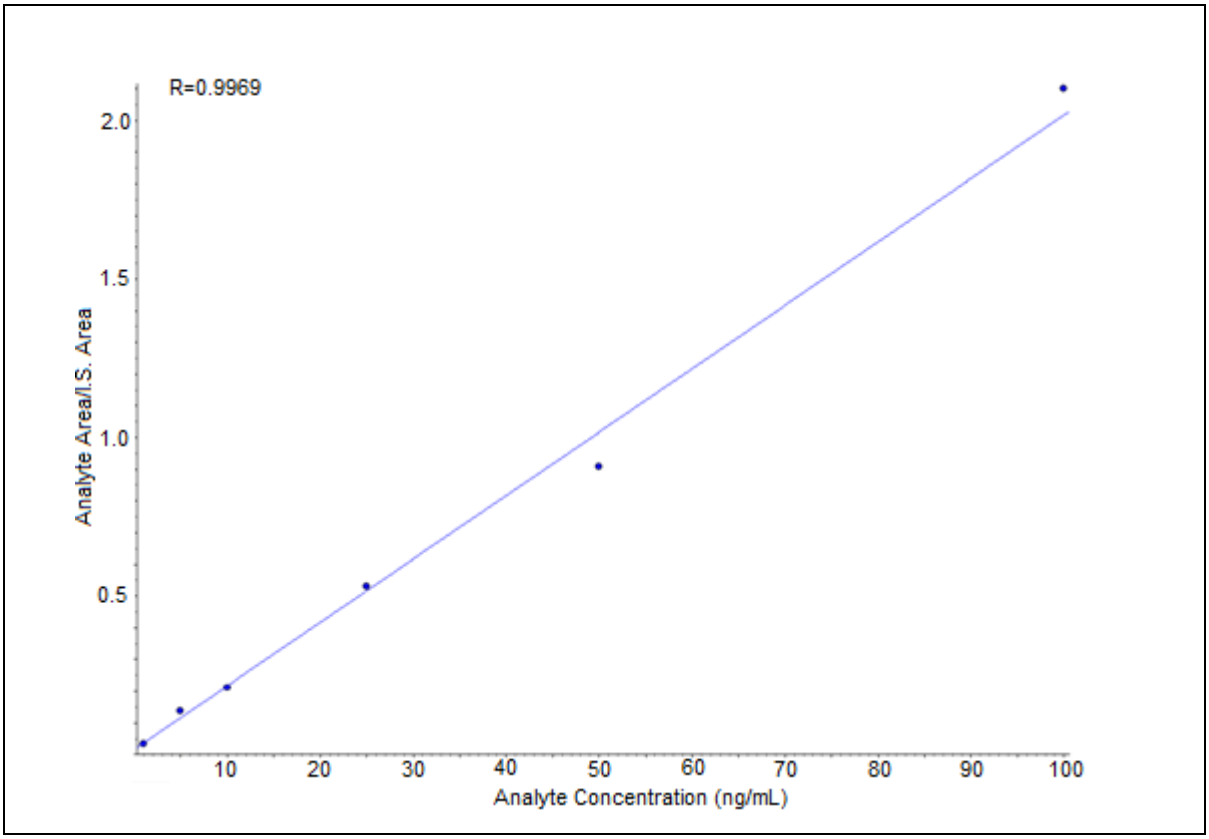


Figure 12 – Example calibration of MDMB-CHMICA showing linearity, with 1/χ weighting, between 1 – 100 ng/mL with R=0.9969.

During analysis, it was observed that the 100 ng/mL calibrator would be removed on occasion to improve the linearity and accuracy of the calibrators. The calibration range was maintained as described for the duration of method validation for consistency, but taking the concentrations found in case samples into account, it was decided that the range should ideally contain lower

concentration calibrators and that a top calibrator of 50 ng/mL would be more appropriate. This was implemented in future methods.

3.5.2.2. Selectivity

Selectivity was established by observing the lack of a peak in MDMB-CHMICA ion transition channels in an MDMB-CHMICA-free standard, as exemplified in Figure 13. A 'blank' (MDMB-CHMICA-free) standard was included in every batch of samples analysed to ensure this remained the case.

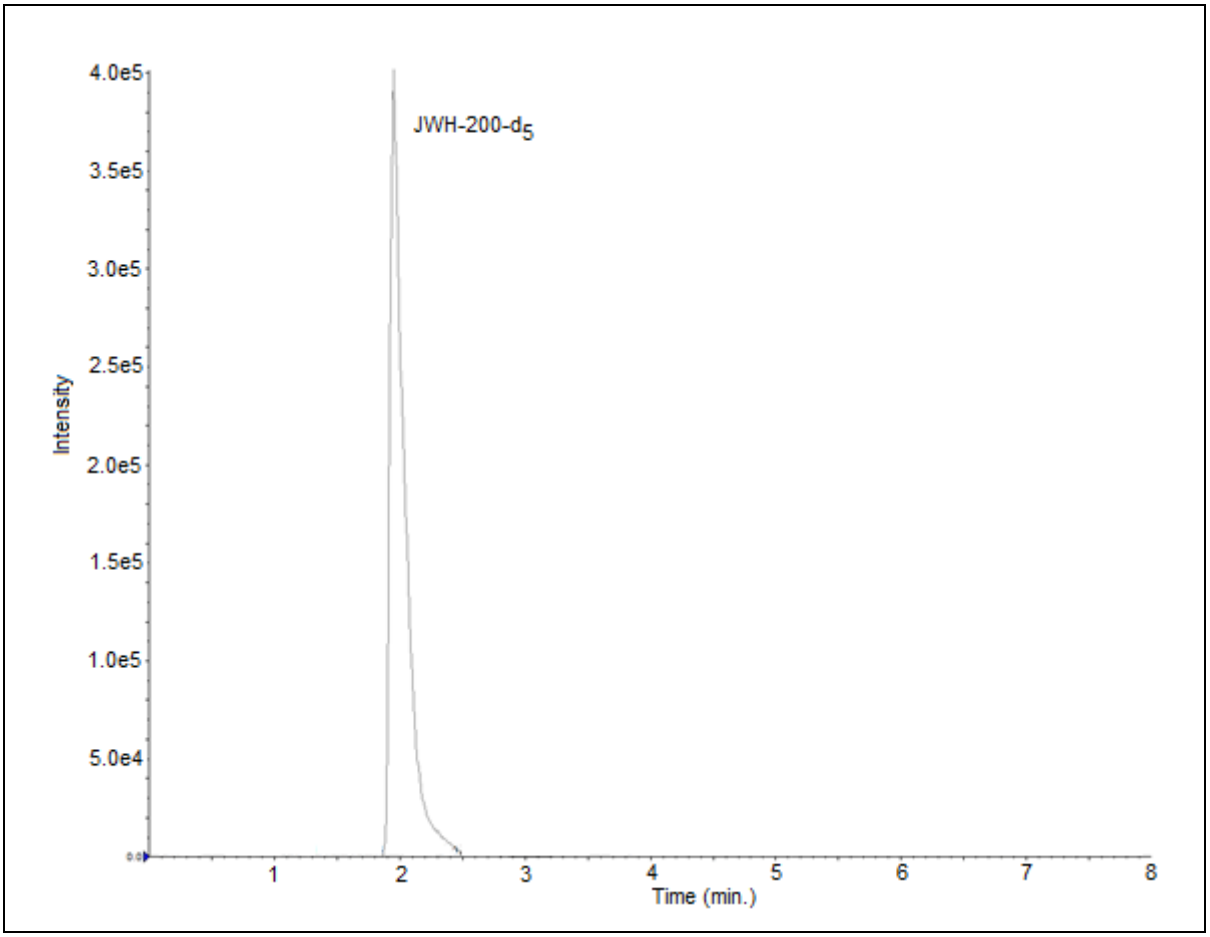


Figure 13 – Example of method selectivity exhibited by a lack of response for MDMB-CHMICA ion transitions in an analyte-free standard. Intensity is given in counts per second.

3.5.2.3. Sensitivity

The LOD and LLOQ of the assay were determined to be 0.5 and 1 ng/mL respectively. Example XICs of standards at these concentrations are given in Figure 14 and Figure 15 for 0.5 and 1 ng/mL respectively. The SNR of the lowest responding ion transition, in this case qualifier ion 2, was used to determine LOD and LLOQ in order to maintain specificity for MDMB-CHMICA. It is noted that the

use of diluted packed red cells rather than whole blood would affect the sensitivity of the extraction as components such as plasma are absent and SCRAs may bind preferentially to this. As a result, the sensitivity of the method may be artificially improved by the use of diluted packed red cells. This is current standard practice in FMS and no whole blood was available at the time of the research.

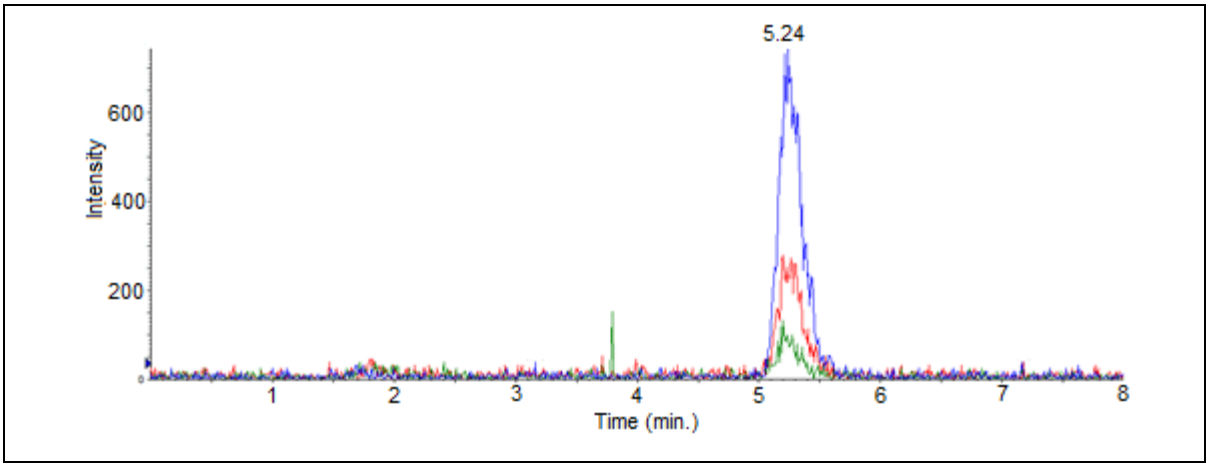


Figure 14 – Example extracted ion chromatogram from an unextracted standard at 0.5 ng/mL MDMB-CHMICA, the Limit of Detection (a signal-to-noise ratio of ≥3). The QT, QL1 and QL2 ions are shown in blue, red and green respectively and intensity is given in counts per second.

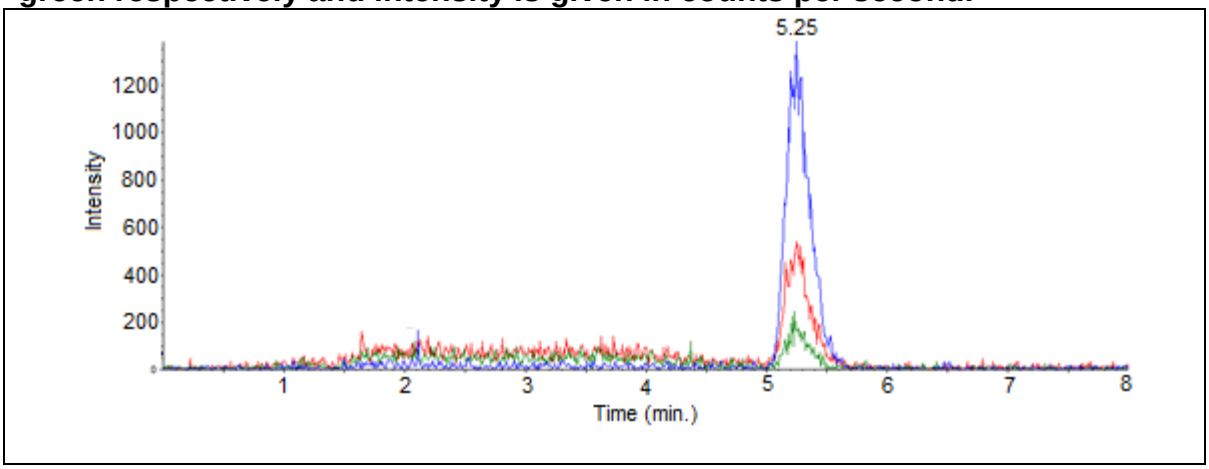


Figure 15 – Example extracted ion chromatogram from an unextracted standard at 1 ng/mL MDMB-CHMICA, the Lower Limit of Quantitation (a signal-to-noise ratio of ≥10). The QT, QL1 and QL2 ions are shown in blue, red and green respectively and intensity is given in counts per second.

3.5.2.4. Accuracy and Precision

Mean inter- and intra-day accuracy was found to be 102% and 96% respectively at 10 ng/mL, with the equivalents being 104% and 108% respectively at 42 ng/mL. Inter- and intra-day precision was calculated to be 8.33% and 4.68% respectively at 10 ng/mL, and 5.31% and 3.65% respectively at 42 ng/mL. The data used to calculate these values can be found in Table 6 and Table 7.

Table 6 – Data used to calculate inter-day accuracy and precision at 10 and 42 ng/mL (n=4) for MDMB-CHMICA. Accuracy and precision were both within acceptable limits of 100 ± 20% and ≤15% respectively.

Expected conc. (ng/mL)	Calculated conc. (ng/mL)	$\bar{\chi}$ (ng/mL)	Accuracy (%)	σ (ng/mL)	%CV
10	11.16	10.17	101.68	0.85	8.33
	10.01				
	8.88				
	10.62				
42	43.19	43.72	104.09	2.32	5.31
	42.06				
	41.97				
	47.65				

Table 7 – Data used to calculate mean intra-day accuracy and precision at 10 and 42 ng/mL (n=2) for MDMB-CHMICA. Accuracy and precision were both within acceptable limits of 100 ± 20% and ≤15% respectively.

Expected conc. (ng/mL)	Day	Calculated conc. (ng/mL)	$\bar{\chi}$ (ng/mL)	Accuracy (%)	σ (ng/mL)	% CV	Mean Accuracy (%)	Mean %CV
10	1	8.88	9.16	91.63	0.45	4.92	95.8	4.68
		8.81						
		9.80						
	2	9.62	10.00	99.97	0.44	4.44		
		10.62						
		9.75						
42	1	41.97	42.04	100.1	1.94	4.60	108.3	3.65
		44.45						
		39.71						
	2	47.65	48.94	116.52	1.32	2.70		
		50.76						
		48.41						

An accuracy of 100 ± 20% was deemed acceptable, and all the values obtained fell within this range. Similarly, all precision was less than 9%, within the deemed acceptable criterion of ≤15%.

3.5.2.5. Matrix Effects and Process Efficiency

Process efficiency was calculated to be 58 and 90% at 5 and 50 ng/mL respectively. The data used to calculate these values are given in Table 8, where unextracted standards are denoted by 'UE'.

Table 8 – Data used to calculate process efficiency at 5 and 50 ng/mL for MDMB-CHMICA. Process efficiency at 50 ng/mL was within the desirable range, but the value for 5 ng/mL was sub-optimal.

Standard	Peak Area	Mean Peak Area	Process Efficiency %	Mean Process Efficiency % (%CV)
UE 5 ng/mL A	81036	80142.5	101.11	100
UE 5 ng/mL B	79249		98.89	
5 ng/mL A	44322	46166	55.30	57.6 (2.8)
5 ng/mL B	47208		58.91	
5 ng/mL C	46968		58.61	
UE 50 ng/mL A	558639	566927.5	98.54	100
UE 50 ng/mL B	575216		101.46	
50 ng/mL A	533157	511992	94.04	90.3 (3.2)
50 ng/mL B	492801		86.92	
50 ng/mL C	510018		89.96	

The sub-optimal process efficiency exhibited at 5 ng/mL is of concern as SCRAs tend to be present in low concentrations, and the process needs to be efficient to ensure low concentrations can be detected. Given the novelty of this compound, it was unknown at this stage in method development just how low concentrations of MDMB-CHMICA in blood would be, *i.e.* frequently <1 ng/mL. The LOD as calculated on unextracted standards, was therefore higher than desired when you take the process efficiency into account. Development and optimisation of a more comprehensive SCRAs method, including MDMB-CHMICA, was conducted at a later stage and addressed this.

During the measurement of matrix effects, slight ion enhancement was detected, with the area of an extracted peak being 116% of its unextracted equivalent in the most significant example. The mean degree of ion enhancement was calculated to be 109%, with the median being 106%. The data used to calculate these values are presented in Table 9.

Table 9 – Data used to calculate matrix effects at 50 ng/mL for MDMB-CHMICA. These were satisfactory at ≤17%.

Standard	Peak Area	Mean Peak Area	% Matrix Effect	Mean (%) (%CV)	Median (%)
UE 50 ng/mL A	558639	566927.5	100	100	100
UE 50 ng/mL B	575216				
50 ng/mL 1A	601401	593384.5	104.7	108.6 (5.6)	105.9
50 ng/mL 1B	585368				
50 ng/mL 2A	571627	572893	101.1		
50 ng/mL 2B	574159				
50 ng/mL 3A	600903	600327.5	105.9		
50 ng/mL 3B	599752				
50 ng/mL 4A	657195	651192.5	114.9		
50 ng/mL 4B	645190				
50 ng/mL 5A	661079	661079	116.6		

The injection of standard 50 ng/mL 5B failed, hence only the result for 50 ng/mL 5A is shown.

Given the low process efficiency at 5 ng/mL, measuring matrix effects at this concentration would have provided more context as to what might be contributing towards this low value. Matrix effects for MDMB-CHMICA were further investigated during development, optimisation and validation of the more comprehensive method in Section 4.

Overall, matrix effects as calculated at 50 ng/mL do not pose a significant threat to accurate quantitation of MDMB-CHMICA at similar concentrations.

The comments made about the use of packed red cells in place of whole blood in Section 3.5.2.3 are valid as a limitation here too. The presence of plasma may affect the process efficiency and matrix effects of the extraction, and the ability of the extraction protocol to efficiently extract plasma-bound SCRAs has not been assessed.

While no formal interference testing was conducted in the scope of this validation, it became apparent that the SCRA BB-22 exhibited the same ion transitions during MS analysis and could not be resolved from MDMB-CHMICA using the chromatographic method detailed in Section 3.4.1.3. It was therefore essential that the ratios of QT and QL ions were calculated to ensure designation as the correct

analyte. The ratios of extracted peak areas for QT to QL1 ion transitions, QT to QL2 ion transitions and QL1 to QL2 ion transitions were noted for 6 calibrators from 5 batches (n=30 calibrators) for MDMB-CHMICA. These were compared to the ratios for the same ion transitions for calibrators of BB-22 extracted and analysed in replicate at 0.5 ng/mL (n=6), 1 ng/mL (n=4) and 50 ng/mL (n=4). Ratios were calculated over different concentrations to ensure that they were not concentration dependant. The results from these experiments are shown, by concentration, in Table 10.

Table 10 – Comparison of ion ratios of MDMB-CHMICA and BB-22 to determine whether these compounds can be distinguished. Distinction can be made using the QT/QL2 ratio.

Conc. (ng/mL)	QT/QL1				QT/QL2				QL1/QL2			
	MDMB-CHMICA		BB-22		MDMB-CHMICA		BB-22		MDMB-CHMICA		BB-22	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
0.5	N/A	N/A	3.01	3.0 - 3.1	N/A	N/A	10.94	10.8 - 11.0	N/A	N/A	3.63	3.6 - 3.7
1	2.42	2.1 - 2.6	3.04	3.0 - 3.1	8.27	6.2 - 10.7	10.96	10.8 - 11.2	3.39	2.9 - 4.1	3.60	3.6 - 3.7
5	2.73	2.7 - 2.9	N/A	N/A	8.40	7.9 - 9.3	N/A	N/A	3.07	3.0 - 3.2	N/A	N/A
10	2.70	2.6 - 2.9	N/A	N/A	8.05	7.8 - 8.6	N/A	N/A	2.99	2.9 - 3.1	N/A	N/A
25	2.72	2.6 - 2.8	N/A	N/A	8.06	7.9 - 8.2	N/A	N/A	2.97	2.9 - 3.1	N/A	N/A
50	2.68	2.7 - 2.7	3.01	3.0 - 3.0	8.19	7.9 - 8.3	10.94	10.9 - 11.1	3.05	3.0 - 3.1	3.63	3.6 - 3.7
100	2.72	2.7 - 2.7	N/A	N/A	8.24	8.1 - 8.3	N/A	N/A	3.03	3.0 - 3.1	N/A	N/A
Mean	**2.66**	**2.1 - 2.9**	**3.02**	**3.0 - 3.1**	**8.20**	**6.2 - 10.7**	**10.95**	**10.8 -11.2**	**3.08**	**2.9 - 4.1**	**3.62**	**3.6 - 3.7**

It is acknowledged that the data set included is limited and, while no overlap exists for QT/QL1 and QT/QL2 ratios in the data presented, the ranges are very close and would likely vary further in a larger sample. Similarly, it is not known how the ratios would be affected by the presence of both BB-22 and MDMB-CHMICA.

The distinction between MDMB-CHMICA and BB-22 was noted as a limitation of the method, and was taken into account during further method development and optimisation.

3.6. Conclusion

The work conducted and presented in this section resulted in a validated LC-MS/MS method which was shown to be able to accurately and precisely quantify the SCRA MDMB-CHMICA between 1 – 100 ng/mL in whole blood. Whilst no chromatographic distinction can be made between this analyte and the structurally similar SCRA BB-22, the ratio of the QT/QL2 ion transitions allows the identity of the analyte to be known, albeit with relatively limited certainty.

A further limitation of this work is the use of diluted packed red cells rather than whole blood, including plasma. While this would have an effect on validated parameters such as matrix effects and sensitivity, it is current standard practice in FMS and no drug-free whole blood was available at the time of the research. Whole blood was used in the validation of matrix effects, process efficiency and recovery for the optimised method discussed in Chapter 4.

4. Development and Validation of a Method for the Detection and Quantitation of Synthetic Cannabinoid Receptor Agonists in Blood and Urine

4.1. Aims and Objectives

The aim of this project was to develop and validate a method for the detection and quantitation of the most common SCRA compounds available in the UK and apply it to real forensic case samples.

In order to do so, the following 4 objectives were set:

- To identify the SCRA compounds most likely to be encountered in the UK population;
- To develop extraction protocols for these compounds applied to blood and urine;
- To develop an LC-MS/MS method to detect and quantitate these compounds; and
- To validate the resultant methods to ensure fitness-for-purpose as accurate quantitative methods.

4.2. Materials

Standards of 5F-AB-PINACA, 5F-ADB-FUBINACA, 5F-AKB48, 5F-AKB48 N4OH pentyl metabolite, 5F-MDMB-PINACA, 5F-MDMB-PINACA *O*-desmethyl acid metabolite, 5F-NPB-22, 5F-PB-22, AB-CHMINACA, AB-FUBINACA, AB-FUBINACA valine metabolite, AB-PINACA, AB-PINACA N4OH pentyl metabolite, AKB48, AKB48 N5OH pentyl metabolite, AM2201, AM2201 N4OH pentyl metabolite, AM2201 N5OH indole metabolite, APICA, APICA N4OH pentyl metabolite, BB-22, FUB-PB-22, MAB-CHMINACA, MAM2201 N4OH pentyl metabolite, MDMB-CHMICA, MDMB-CHMICA *O*-desmethyl acid metabolite, MDMB-CHMINACA, MMB2201, MMB-CHMICA, MMB-FUBINACA, PB-22, STS-135, STS-135 N4OH pentyl, AKB48-d_{11}, AM2201-d_5, were purchased from Chiron (Trondheim, Norway). AB-CHMINACA metabolite 1A, AB-CHMINACA metabolite 2, AB-FUBINACA metabolite 2B, AKB48 N-pentanoic acid metabolite, BB-22 3-carboxyindole metabolite, CUMYL-PeGACLONE, MAB-CHMINACA metabolite 1, PB-22 N5OH pentyl metabolite, PB-22 N-pentanoic acid metabolite, PB-22 N-pentanoic acid 3-carboxyindole metabolite, AB-FUBINACA-d_4, and PB-22-d_9 were purchased from LGC Standards (Teddington, UK). AKB48 N5 hydroxy pentyl

metabolite-d_4, β-glucuronidase from *Helix pomatia*, *tertiary* methyl butyl ether (*t*BME), potassium hydroxide, sodium acetate trihydrate and ammonium acetate were purchased from Sigma Aldrich (Gillingham, UK). Phosphate buffer (pH 6, 0.1M) was prepared in-house from disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate dehydrate from Fisher Scientific (Loughborough, UK), who also provided acetic acid. Deionised water was produced from a Purite (Thame, UK) deionised water system. MeOH (HPLC grade) and ACN (LC-MS gradient grade), cyclohexane, ethyl acetate, ammonium carbonate, sodium hydroxide and formic acid were obtained from VWR (Lutterworth, Leicestershire, UK). Packed red blood cells were purchased from the Scottish National Blood Transfusion Service (SNBTS) based at Gartnavel Hospital (Glasgow, UK). Blank urine was obtained from healthy adult volunteers. Drug-free whole blood was purchased from Biological Specialty Corporation, Pennsylvania, U.S..

4.3. Method

4.3.1. Solutions

4.3.1.1. Stock Standards of Analytes and I.S.

Individual stocks of 10 µg/mL in ACN were prepared by taking 50 µL of a 1 mg/mL or 500 µL of a 100 µg/mL solution as received from supplier and making up to 5 mL in a volumetric flask. These were inverted several times, transferred to glass vials fitted with screw caps and stored in the freezer for 1 year.

If the analyte was received in solid form, stocks of 1 mg/mL were produced in ACN or MeOH as solubility dictated by taking a minimum of 2 mg (weighed accurately) and dissolving in the equivalent volume of solvent. These solutions were mixed thoroughly, ensuring complete dissolution of solid and placing in an ultrasonic bath if dissolution wasn't immediate. Solutions were stored in the freezer for 1 year. Prior to each use, the solutions were checked to ensure no precipitation had occurred (and sonicated again prior to use in case of precipitation).

4.3.1.2. Infusion Solutions of Analytes and I.S.

Infusion solutions of all analytes individually were prepared at a starting concentration of 1 µg/mL by diluting the stock solutions with an aqueous solution of 50% MeOH containing 0.1% formic acid and 2mM ammonium acetate. Further dilutions, or solutions at higher concentrations, were prepared if it was required to

attain the necessary instrumental response. As it was a certain magnitude of instrumental response that was the goal, the concentration did not need to be accurate.

4.3.1.3. Mixed Working Solutions of Analytes

One solution containing the parent analytes and another containing the metabolites in ACN were prepared at 5 µg/mL. This was achieved by adding 25 µL of solutions at 1 mg/mL or 250 µL of solutions at 100 µg/mL to a 5 mL volumetric flask and making up to the line with ACN. The flask was inverted several times, the contents transferred to a glass vial fitted with a lid and stored in the freezer for 1 year.

These were then further diluted, by taking 1 mL of each solution into a 10 mL volumetric flask and making up to 10 mL with ACN, to form one solution at 500 ng/mL containing all the analytes. This solution was stored in the freezer for 6 months.

A further solution containing all the analytes at 50 ng/mL was prepared by taking 500 µL of the 500 ng/mL solution into a 5 mL volumetric flask and making up to the line with ACN. The flask was inverted several times, the contents transferred to a glass vial fitted with a lid and stored in the freezer for 6 months.

4.3.1.4. Mixed Working Solutions of I.S.

A mixed solution at 1 µg/mL was prepared by taking 500 µL of each individual 10 µg/mL stock solutions into a 5 mL volumetric flask and making up to the line with ACN. The flask was inverted several times, the contents transferred to a glass vial fitted with a lid and stored in the freezer for 1 year.

Latterly, a second mixed solution at 500 ng/mL was prepared by taking 500 µL of each individual 10 µg/mL stock into a 10 mL volumetric flask and making up to the line with ACN. The flask was inverted several times, the contents transferred to a glass vial fitted with a lid and stored in the freezer for 1 year.

4.3.1.5. pH4.5 0.04M Sodium Acetate Buffer

Sodium acetate trihydrate (5.86 g) was weighed out and dissolved in a small amount of deionized H_2O in a beaker. This was then transferred to a 1 L volumetric flask and made up to the line with deionized H_2O. To this, glacial acetic

acid was added drop-wise until the pH reached 4.5. The flask was inverted several times, the buffer transferred to a glass bottle and stored at RT for 1 month.

4.3.1.6. pH6.0 0.1M Phosphate Buffer

See Section 3.3.1.14.

4.3.1.7. 0.1M pH7.4 Phosphate Buffer

Potassium dihydrogen orthophosphate (13.6g) was added to *ca.* 800 mL deionized H_2O and adjusted to pH7.4 with 10M potassium hydroxide. This was transferred to a 1 L volumetric flask and made up to the line with deionized H_2O. This was then inverted several times, transferred into a glass bottle and stored in the fridge for 1 month.

4.3.1.8. 10M Potassium Hydroxide

56g of potassium hydroxide was added to 100 mL deionized H_2O, transferred to a glass bottle, inverted several times and stored at RT for 6 months.

4.3.1.9. 0.01M pH9.3 Carbonate Buffer

0.96g of ammonium carbonate was added to *ca.* 800 mL deionized H_2O and adjusted to pH9.3 with 1M potassium hydroxide. This was then transferred to a 1 L volumetric flask, made up to the mark with deionized H_2O and inverted several times. The resulting solution was transferred to a glass bottle and stored at RT for 6 months.

4.3.1.10. 1M NaOH

40g sodium hydroxide was weighed out and added to a 1 L volumetric flask. The volume was made up to the mark with deionised H_2O and the flask was inverted several times. The solution was then transferred to a glass bottle and stored at RT for 6 months.

4.3.1.11. Cyclohexane with 1% Ethyl Acetate

Ethyl acetate (1 mL) was added to a 100 mL volumetric flask approximately half full of cyclohexane. The volume was made up to the mark with cyclohexane and the flask was inverted several times. The solution was then transferred to a glass bottle and stored at RT for 6 months.

4.3.1.12. Acetonitrile:Deionised H_2O (95:5) with 0.1% Formic Acid and 2mM Ammonium Acetate

Concentrated formic acid (1 mL) and 2M ammonium acetate (1 mL, for preparation details see Section 3.3.1.1) were added to 50 mL of deionised H_2O. ACN (950 mL) was added to this and the solution was sonicated at RT for 15 min. to remove dissolved gases. This solution was stored at RT for 3 months.

For aqueous (MP A) and methanolic (MP B) MP preparation details see Sections 3.3.1.3 and 3.3.1.2 respectively.

4.3.2. Selection of Analytes

The parent compounds most relevant to the Scottish population were selected through a search of the published literature; by identifying the compounds detected most frequently by the WEDINOS service; and according to alerts issued by the EMCDDA and other agencies. When undertaking the literature search, peer-reviewed journal articles discussing cases of analytically confirmed SCRA use in members of the public in Western European countries were deemed of highest value. The availability and cost of certified reference materials for the SCRA compounds were also taken into account.

Once a list of parent compounds had been devised, information was sought on the major metabolites of these compounds through a second search of the scientific literature. The findings from this search were cross-referenced with suppliers' product listings, and one or two of the metabolites most likely to be encountered in urine were chosen for inclusion.

Internal standards (I.S.) were selected by studying the chemical structures of the parent compounds and selecting a deuterated form of a structurally similar SCRA. Availability and cost of deuterated certified reference materials were the limiting factors with regards to selection of I.S.

Due to constant flux in the SCRA market, compounds were added and, less frequently removed, from the method to maintain its fitness for purpose. In addition, further information was garnered from the analysis of residue from packets of SCRA products to justify and guide the analyte panel.

4.3.3. Liquid Chromatography – Mass Spectrometry

4.3.3.1. Infusion of compounds

Compounds were infused directly into the ion source of the mass spectrometer using a syringe pump at a rate of 5 – 10 µL/min (controlled by the instrument), employing the compound optimization infusion feature of the Analyst© software. Infusion solutions were prepared at a starting concentration of 1 µg/mL in an aqueous solution of 50% MeOH with 2mM ammonium acetate and 0.1% formic acid, mimicking the anticipated MP starting conditions. A response of approximately $1x10^6$ counts was considered desirable and infusion solution concentrations were amended if the compound elicited a response significantly higher or lower than this value.

All compounds were infused in positive mode with unit resolution, targeting the molecular ion. Initially, the individual compound methods were built with the 4 ion transitions which provided the highest responses. These were then reviewed, ensuring aspects such as peak shape were acceptable, and two ion transitions were selected based on sensitivity (response) and selectivity (number of other compounds employing that transition). In some cases a third ion transition was retained in the method, to be monitored in case the method sensitivity allowed a more selective ion transition (albeit giving a lower response) to be employed.

During the infusion process, the m/z values of the fragments were checked against the chemical structure to ensure the drug compound could be confirmed as their source.

4.3.3.2. Mobile Phase Experiments

SCRA parent compounds have a strong retention on reverse phase (RP) HPLC columns due to their non-polar nature, so as a starting point a MP composed of 2mM ammonium acetate and 0.1% formic acid in deionised water (MP A) and 2mM ammonium acetate and 0.1% formic acid in methanol (MP B) at an isocratic ratio of 10:90 was employed. An unextracted standard of each individual compound at a concentration of 100 ng/mL in 50% methanol was run down this isocratic system and their retention time noted. A mixed standard containing all compounds at this concentration was also run in order to assess peak shape and spread of elution throughout the run time. This was repeated at ratios of 20:80 and

30:70 where elution of the compounds was too quick; a retention time between 2.5 – 20 min. was considered desirable.

When the approximate extent of retention for each compound was known, experiments into a gradient elution system were conducted. The MP gradient systems are shown in Figure 48 - Figure 65 in Appendix 9.1 and were devised to investigate analyte separation.

For gradient systems A – J, MPs A and B were deionised water and methanol respectively with 2mM ammonium acetate and 0.1% formic acid. Systems K – N employed deionised water and ACN as MP A and C respectively, again with 2mM ammonium acetate and 0.1% formic acid. MP C for gradient systems O and P was a mixture of ACN and methanol (90:10) with 2mM ammonium acetate and 0.1% formic acid; whilst for gradient systems Q and R, MP C was a mixture of ACN and deionised water (95:5) with 2mM ammonium acetate and 0.1% formic acid.

4.3.4. Extraction of Analytes

A simple, non-specific LLE protocol was used as the starting point for extraction of analytes from both blood and urine. This involved the addition of 1 mL pH6.0 1M phosphate buffer, I.S. (50 µL of a 500 ng/mL solution to give a final concentration of 50 ng/mL) and analyte working solution (100 µL for calibrators and QCs only) to 500 µL matrix. For urine samples 50 µL β-glucuronidase and I.S. were added (plus 100 µL for calibrators and QCs only). Details of how the working solution of calibrators and QCs were prepared are provided in Table 11. Calibrator and QC working standards were different solutions, either prepared on different days, or by different individuals.

Table 11 – Preparation details of calibrator and QC solutions for the extraction, detection and quantitation of selected Synthetic Cannabinoid Receptor Agonists in blood and urine

Final Concentration of Calibrator or QC (ng/mL)	Volume of 50 ng/mL working solution (µL)	Volume of 500 ng/mL working solution (µL)	Volume of ACN (µL)
CAL 0.05	5	0	995
CAL 0.10	10	0	990
CAL 0.20	20	0	980
CAL 0.50	0	5	995
CAL 1.00	0	10	990
CAL 2.00	0	20	980
CAL 5.00	0	50	950
CAL 10.00	0	100	900
CAL 25.00	0	250	750
CAL 50.00	0	500	500
QC 0.10	10	0	990
QC 0.20	20	0	980
QC 0.42	42	0	958
QC 2.50	0	25	975
QC 15.00	0	150	850
QC 42.00	0	420	580

Extraction of analytes was induced by the addition of *t*BME (2 mL) to the prepared sample. The tubes were vortex mixed for *ca*. 30 sec. and centrifuged at 3000 rpm for 10 min. The solvent layer was then transferred to clean and labeled vials and evaporated under a stream of nitrogen at 40 °C. The analytes were reconstituted in 200 µL of a solution prepared to the MP starting conditions (*e.g.* MeOH:deionised H_2O (50:50)).

4.3.4.1. Optimisation of the Extraction Method for Application to Blood

To gain the optimal recovery and minimal matrix effects for extraction, variations of extraction conditions were assessed. These are detailed in Table 12.

Table 12 – Experimental conditions for the optimisation of the extraction of selected Synthetic Cannabinoid Receptor Agonists from blood

Experiment	Mix Time (min.)	Buffer Type	Vol. Buffer (mL)	Extraction Solvent	Vol. Extraction Solvent (mL)
1	5	pH 6 0.1M phosphate	1	*t*BME	2
2	2	pH 6 0.1M phosphate	1	*t*BME	2
3	10	pH 6 0.1M phosphate	1	*t*BME	2
4	5	pH 6 0.1M phosphate	0.5	*t*BME	2
5	5	pH 9.3 0.01M carbonate	0.5	*t*BME	2
6	5	pH 9.3 0.01M carbonate	1	*t*BME	2
7	5	pH 6 0.1M phosphate	1	*t*BME	1
8	5	pH 6 0.1M phosphate	1	Cyclohexane (1% ethyl acetate)	1
9	5	pH 6 0.1M phosphate	1	Cyclohexane (1% ethyl acetate)	2
10	2	pH 6 0.1M phosphate	0.5	*t*BME	1

Mixing was conducted on a flatbed mixer, and 4 mL plastic test tubes were used. Reconstitution was in ACN:deionised H_2O (95:5):deionised H_2O (30:70) with 2mM ammonium acetate and 0.1% formic acid. All variables of the experiments not detailed in Table 12 remained consistent with the method detailed in Section 4.3.4.

Duplicate standards of 5 ng/mL extracted as per section 4.3.4 were compared with the corresponding standard extracted as per the varied technique in terms of recovery, matrix effects and process efficiency (Equation 6 – Equation 8).

Equation 6

$$Recovery = \left(\frac{A}{B}\right) \times 100$$

Equation 7

$$Matrix\ Effects = \left(\frac{B}{C}\right) \times 100$$

Equation 8

$$Process\ Efficiency = \left(\frac{A}{C}\right) \times 100$$

Where:

A= peak area of an extracted standard

B= peak area of a double blank extracted standard reconstituted in the unextracted standard

C= peak area of an unextracted standard

Recoveries and extent and type of ME were compared to determine the optimal process, with highest recovery and lowest ME being preferable.

4.3.4.2. Optimisation of the Extraction Method for Application to Urine

Optimisation of the extraction of SCRAs from urine was also undertaken, varying conditions as detailed in Table 13.

Table 13 – Experimental conditions for the optimisation of the extraction of selected Synthetic Cannabinoid Receptor Agonists from urine

Experiment	Mix Time (min.)	Buffer Type	Vol. Buffer (mL)	Extraction Solvent	Vol. Extraction Solvent (mL)
1	5	pH 6 0.1M phosphate	1	tBME	2
2	2	pH 6 0.1M phosphate	1	tBME	2
3	2	pH 6 0.1M phosphate	0.5	tBME	1
4	5	pH4.5 0.04M sodium acetate	1	tBME	2
5	5	pH4.5 0.04M sodium acetate	0.5	tBME	2
6	5	pH4.5 0.04M sodium acetate	0.5	tBME	1
7	5	pH 9.3 0.01M carbonate	1	tBME	2
8	5	pH 9.3 0.01M carbonate	0.5	tBME	2
9	2	pH 9.3 0.01M carbonate	0.5	tBME	1
10	2	pH 6 0.0.04M sodium acetate	0.5	tBME	1
11	2	1M NaOH	0.5	tBME	2
12	5	pH 7.4 0.1M phosphate	0.5	tBME	2
13	5	None	N/A	MeOH	2
14	5	None	N/A	MeOH	3
15	5	None	N/A	ACN	2

As with the blood experiments, 4 mL plastic test tubes and the flatbed mixer were used, as was reconstitution performed in the MP starting conditions. Comparison of the conditions was made by way of the results of Equation 6 – Equation 8 with the same desirable criteria as with blood.

Examination of the optimal hydrolysis conditions for extraction of analytes from urine was also performed by comparing the process efficiency of extractions conducted according to Table 14 with unextracted standards of the same concentration incubated at room temperature for 1 H.

Table 14 – Experimental conditions for the optimisation of the hydrolysis of selected Synthetic Cannabinoid Receptor Agonists in urine

Experiment	Buffer	β-glucuronidase	Incubation Conditions
1	0.5 mL, pH4.5 0.1M sodium acetate	50 µL from *Helix pomatia*	1 H at RT
2	0.5 mL, pH4.5 0.1M sodium acetate	50 µL from *Helix pomatia*	1 H at 60 °C
3	None	50 µL from *Helix pomatia*	1 H at RT
4	None	50 µL from *Helix pomatia*	1 H at 60 °C
5	None	None	1 H at RT
6	None	None	1 H at 60 °C

4.3.5. Method Validation – Blood

The Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines for method validation were used as a basis for the parameters validated and the acceptance criteria as detailed below.

4.3.5.1. Linearity

Linearity was assessed over 10 extracted calibrations using the calibration ranges given in Table 15 and using 1/χ weighting. The calibration was deemed acceptable if the correlation co-efficient was ≥0.99 with at least 4 calibration points within 100% ± 20 accuracy.

Table 15 – Concentrations of calibrators included in assessment of linearity for selected Synthetic Cannabinoid Receptor Agonists

Calibrator	Concentration of Parent Compound (ng/mL)	Concentration of Metabolite (ng/mL)
1	0.10	0.20
2	0.20	0.50
3	0.50	1.00
4	1.00	5.00
5	5.00	10.00
6	10.00	25.00
7	25.00	50.00

4.3.5.2. Selectivity

Selectivity was assessed by running drug-free standards and observing a response ≤25% of the peak area of the lowest calibrator in analyte channels.

4.3.5.3. Sensitivity

The LOD was designated as the lowest standard at which the SNR of the ion transition giving the lowest response was ≥4. The LLOQ was set as 0.10 or 0.20 ng/mL for parent compounds and metabolites respectively ensuring that this was ≥ the LOD for the compound.

In order to determine the SNR a series of extracted standards were injected at 0.01, 0.02, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00 and 5.00 ng/mL. The height of the compound peak was measured as the signal, and the maximum height of the baseline in the area immediately adjacent to the compound retention time was measured as the noise. The signal height was divided by the noise to calculate the SNR.

4.3.5.4. Accuracy

Accuracy was assessed at 0.1, 2.5, and 15 ng/mL (parent compounds) or 0.2, 2.5 and 42 (metabolites) over 5 replicates using the calculation given in Equation 1. Inter- and intra-assay accuracy were both calculated and deemed acceptable if within ± 20%.

4.3.5.5. Precision

Precision was assessed at 0.1, 2.5, and 15 ng/mL (parent compounds) or 0.2, 2.5 and 42 (metabolites) over 5 replicates using the calculation given in Equation 2. Inter- and intra-assay precision were both calculated and deemed acceptable if the %CV was < 15.

4.3.5.6. Recovery, Matrix Effects and Process Efficiency

Recovery, matrix effects and process efficiency were all calculated for each analyte at 2.5 and 15 ng/mL, in duplicate, and according to Equation 6 – Equation 8. Recovery of ≥ 50% and matrix effects not exceeding ± 30% were preferred. Ten sources of blank whole blood were used (*i.e.* not prepared as per Section 3.3.1.16) for these experiments.

4.3.5.7. Interference Testing

An unextracted solution containing the most commonly encountered prescription and abused drugs was prepared to give a concentration of 1 mg/L. This was injected on the instrument using the optimised method in triplicate and the

resulting XICs interrogated for peaks at or around the retention times of SCRA or I.S. peaks.

The drugs included in the solution used are given in Table 16 .

Table 16 – Panel of prescription and abused drugs included in the interference tests

7-Aminoflunitrazepam	Amitriptyline
Amphetamine	Benzoylecgonine
Chlordiazepoxide	Chlorpheniramine
Chlorpromazine	Citalopram
Cocaine	Codeine
Cyclizine	Desmethyldiazepam
Diazepam	Dihydrocodeine
Diltiazem	Diphenhydramine
Etizolam	Lignocaine
Lorazepam	MDA*
MDEA	MDMA
Methadone	Methamphetamine
Mirtazapine	6-Monoacetylmorphine
Morphine	Nitrazepam
Oxazepam	Phenazepam
Phencyclidine	Temazepam
Tetrahydrocannabinol	Tetrahydrocannabinol acid metabolite
Tramadol	Zolpidem

* Methylenedioxymethamphetamine, to distinguish from Misuse of Drugs Act 1971

4.3.5.8. Autosampler Stability

Five pooled extracted standards (1 mL extract) prepared at 2.5 and 15 ng/mL were injected 30 times each, in groups of 3, so the final injection of a concentration was approximately 46 H after the first injection. The peak areas and PAR were plotted over time to show the stability over the time period as compensated by the IS.

4.3.6. Method Validation – Urine

As compounds present in urine are no longer active pharmacodynamically, the concentrations of these compounds were not deemed as important as their presence or absence. The exception to this is the requirement to determine the concentration of a compound to corroborate or refute results of mandatory drug testing (MDT) in the prison setting (England and Wales), where a cut-off concentration is used to distinguish between active and passive inhalation. For this reason, the method was only validated quantitatively for the most commonly encountered analytes, with qualitative validation being deemed sufficient for the remaining analytes. The compounds for which quantitative validation was

undertaken were 5F-MDMB-PINACA *O*-desmethyl acid metabolite, MDMB-CHMICA *O*-desmethyl acid metabolite and AB-FUBINACA valine metabolite.

4.3.6.1. Linearity

This was assessed for 5F-MDMB-PINACA *O*-desmethyl acid metabolite, MDMB-CHMICA *O*-desmethyl acid metabolite and AB-FUBINACA valine metabolite as per 4.3.5.1.

4.3.6.2. Selectivity

This was assessed for all compounds as per 0.

4.3.6.3. Sensitivity

This was assessed for all compounds as per 4.3.5.3.

4.3.6.4. Accuracy

This was assessed for 5F-MDMB-PINACA *O*-desmethyl acid metabolite, MDMB-CHMICA *O*-desmethyl acid metabolite and AB-FUBINACA valine metabolite as per 4.3.5.4.

4.3.6.5. Precision

This was assessed for 5F-MDMB-PINACA *O*-desmethyl acid metabolite, MDMB-CHMICA *O*-desmethyl acid metabolite and AB-FUBINACA valine metabolite as per 4.3.5.5.

4.3.6.6. Recovery, Matrix Effects and Process Efficiency

This was assessed for all compounds as per 4.3.5.6, using blank urine in place of blood.

4.3.6.7. Interference Testing

This was assessed as per 4.3.5.7.

4.3.6.8. Autosampler Stability

This was assessed for all compounds as per 4.3.5.8.

4.3.7. Validation – Intermediate Methods

Due to the timing of this research, samples were received prior to full method optimisation and validation. In order to begin testing these samples, two intermediate methods were developed distinctly from the more comprehensive

method undergoing optimisation and these were validated for use with specific projects further described. These intermediate methods are denoted method 1.1 and 1.2 and were assessed on selectivity and sensitivity as per sections 0 and 4.3.5.3 respectively. Full details of these methods are provided in Table 24 and Table 25.

4.3.8. Comparison of Prison 'A' and 'B' Samples

During MDT in prisons, 2 samples of urine are collected from prisoners: one sample (the 'A' sample) is tested by the original laboratory, while the other (the 'B' sample) is stored for potential testing if the original result is challenged. Forensic Medicine and Science provides testing of 'B' samples as an additional service. A report is issued stating the compounds which have been detected above the cut-off value (5 ng/mL) and the concentrations at which these were detected. A comment is then made stating whether the 'B' results are consistent with those reported by the original laboratory. The decision as to whether the results are consistent was made based on the finding of the same compounds at concentrations similar to the original findings, taking into account time since original analysis, storage during sample transfer and possible analyte instability. No statistical analysis is made to calculate the similarity because these factors are unknown. The accuracy was calculated for this work, however, using Equation 1, where $\bar{\chi}$ denotes the 'B' sample result and χ denotes the original 'A' sample result.

The results of the 'B' sample analysis were compared to those of the original testing laboratory to ensure accuracy, and to assess stability of these compounds in urine.

In order to do this, the 'A' and 'B' results were plotted against each other using the 'A' and 'B' sample results as the y- axis and x-axis values respectively. The correlation co-efficient was then calculated using linear regression. An assessment of agreement and nature and extent of any random and/or systematic error was considered, along with potential analyte stability issues.

The School of Medicine, Veterinary and Life Sciences Research Ethics Committee were contacted regarding this work, and provided a Letter of Comfort that a Research Ethics Application was not required for this work. This is included within Appendix B, in Section 9.2.

4.3.9. Analysis of Drug Packets

Throughout 2016 packets of suspected SCRA products were analysed. Photographs of these packets are given in Figure 16 and show the front and back of products Afghan Black Ultra (formula 2A), Blueberry Haze Ultra (formula 4A), Kuber Khaini, Lunar Diamond, Pandora's Box Unleashed and Tribal Warrior Ultimate. With the exception of Lunar Diamond, all the packets were empty of material.

For analysis, rinse solutions of the packets were made with 2 mL MeOH. Approximately 5 mg of Lunar Diamond material was weighed out and 2 mL of MeOH was added to this. From these rinses, a 1 in 10 dilution was made by adding 100 µL of rinse to 900 µL of MeOH and mixing. LC-MS analysis was conducted on 1 in 100 dilutions of these solutions, adding 10 µL of diluted rinse to 990 µL of reconstitution solution (ACN:H_2O 30:70).

These solutions were injected in duplicate, with an unextracted standard at 50 ng/mL and analysed using method 1.2.

Figure 16 – Photographs of packets suspected of containing Synthetic Cannabinoid Receptor Agonists front (L) and back (R). These were analysed to determine their contents.

Figure 16 – Photographs of packets suspected of containing Synthetic Cannabinoid Receptor Agonists front (L) and back (R). These were analysed to determine their contents.

4.4. Results and Discussion

Due to the fluid nature of the drugs market and chronology of the projects conducted within this research, 3 distinct methods were developed through the process detailed in Section 4.3.2 onwards: Methods 1.1, 1.2 and 2.1.These varied by drugs included and MP gradient (details given in Table 24 and Table 25). This ensured the panels of drugs included in the analysis for the projects were kept up to date with likely available compounds.

4.4.1. Selection of Analytes

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

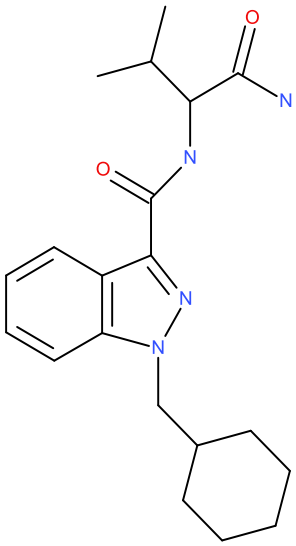
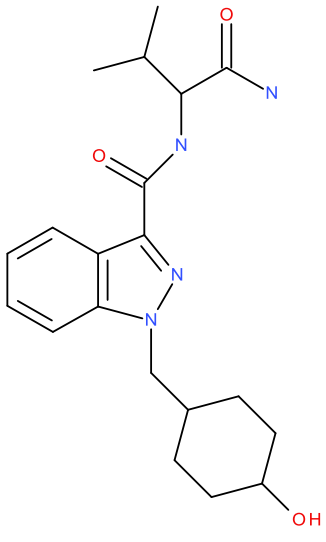
Compound Name	Aliases	Structure	Formula (MW)
AB-CHMINACA	N/A		$C_{20}H_{28}N_4O_2$ (356.5)
AB-CHMINACA metabolite 1A	N/A		$C_{20}H_{28}N_4O_3$ (372.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

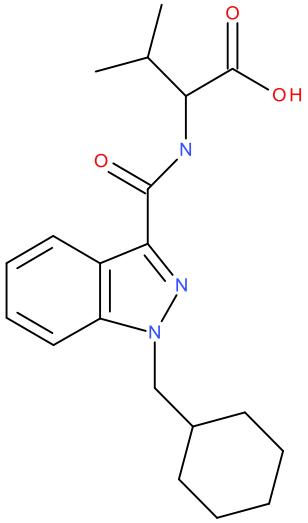
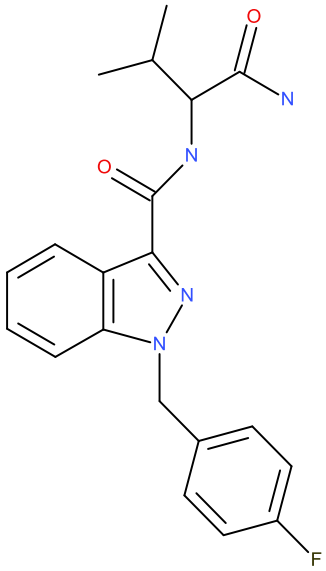
Compound Name	Aliases	Structure	Formula (MW)
AB-CHMINACA metabolite 2	N/A		$C_{20}H_{27}N_3O_3$ (357.4)
AB-FUBINACA	N/A		$C_{20}H_{21}FN_4O_2$ (368.4)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

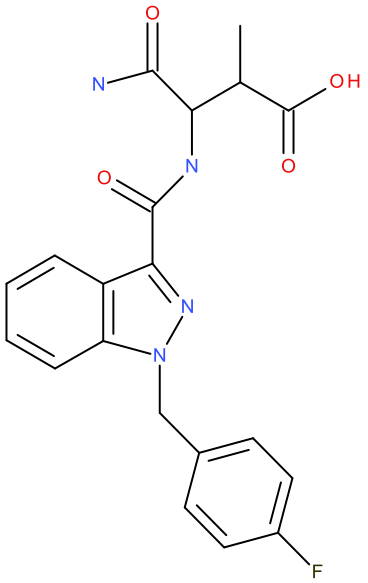
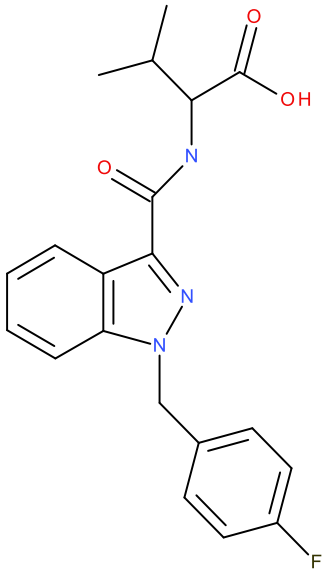
Compound Name	Aliases	Structure	Formula (MW)
AB-FUBINACA metabolite 2B	N/A		$C_{20}H_{19}FN_4O_4$ (398.4)
AB-FUBINACA valine metabolite	MMB-FUBINACA metabolite AB-FUBINACA metabolite 3		$C_{20}H_{20}FN_3O_3$ (369.4)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

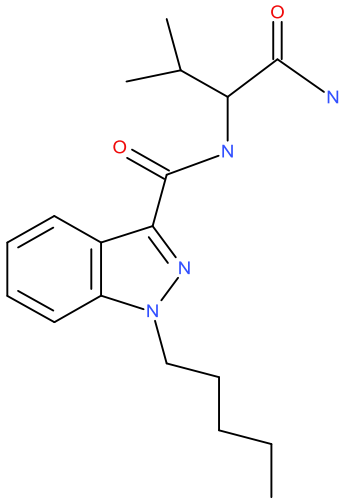
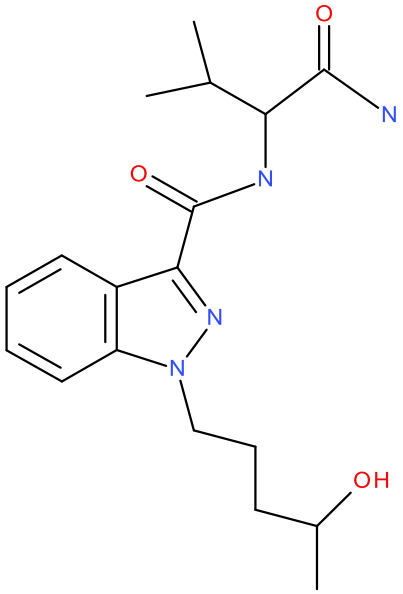
Compound Name	Aliases	Structure	Formula (MW)
AB-PINACA	N/A		$C_{18}H_{26}N_4O_2$ (330.4)
AB-PINACA N4OH pentyl	N/A		$C_{18}H_{26}N_4O_3$ (346.2)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

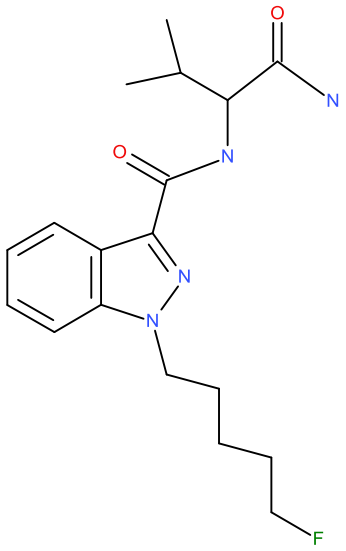
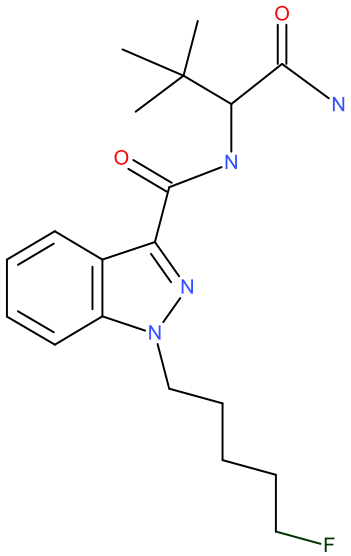
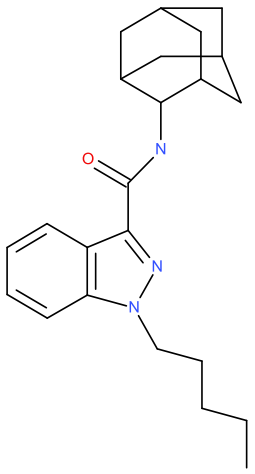
Compound Name	Aliases	Structure	Formula (MW)
5F-AB-PINACA	N/A		$C_{18}H_{25}FN_4O_2$ (348.4)
5F-ADB-PINACA	N/A		$C_{19}H_{27}FN_4O_2$ (362.4)
AKB48	Apinaca		$C_{23}H_{31}N_3O$ (365.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

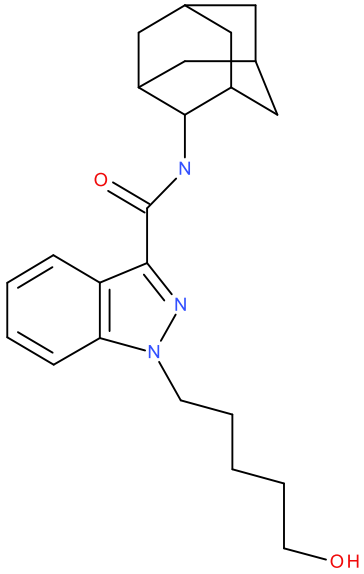
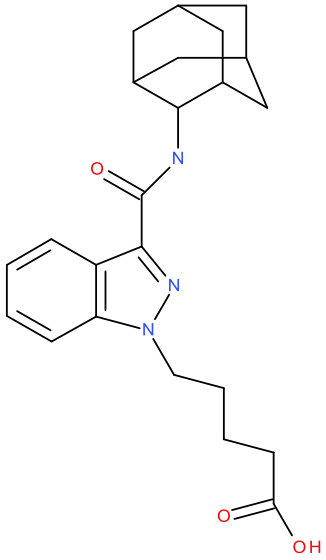
Compound Name	Aliases	Structure	Formula (MW)
AKB48 N5OH pentyl	Apinaca N5OH pentyl		$C_{23}H_{31}N_3O_2$ (381.5)
AKB48 N-pentanoic acid	Apinaca N-pentanoic acid		$C_{23}H_{29}N_3O_3$ (395.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

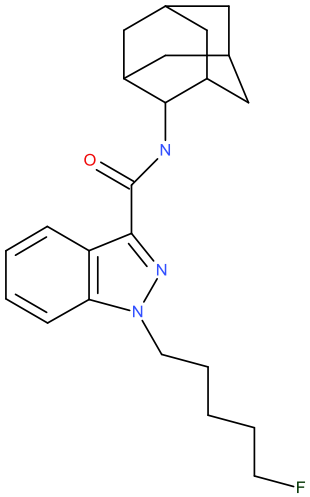
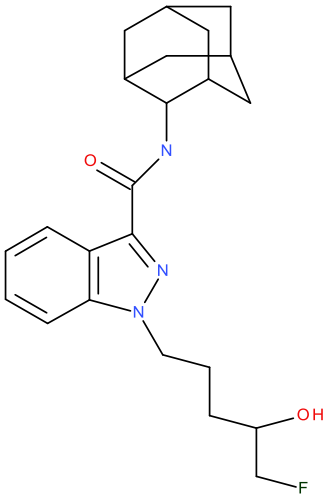
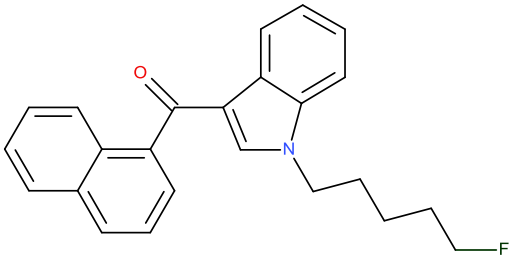
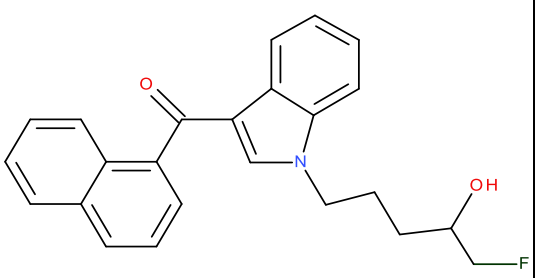
Compound Name	Aliases	Structure	Formula (MW)
5F-AKB48	5F-Apinaca		$C_{23}H_{30}FN_3O$ (383.5)
5F-AKB48 N4OH pentyl	5F-Apinaca N4OH pentyl		$C_{23}H_{30}FN_3O_2$ (399.5)
AM2201	5F-JWH-018		$C_{24}H_{22}FNO$ (359.4)
AM2201 N4OH pentyl	5F-JWH-018 N4OH pentyl		$C_{24}H_{22}FNO_2$ (375.4)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

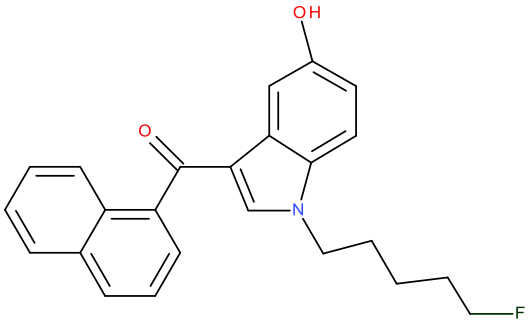
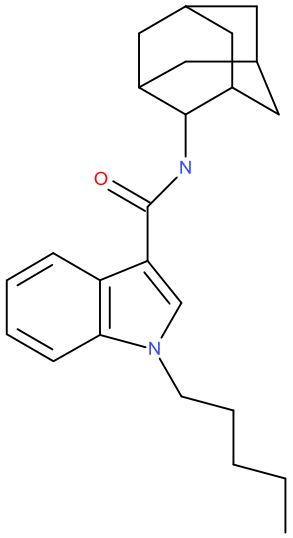
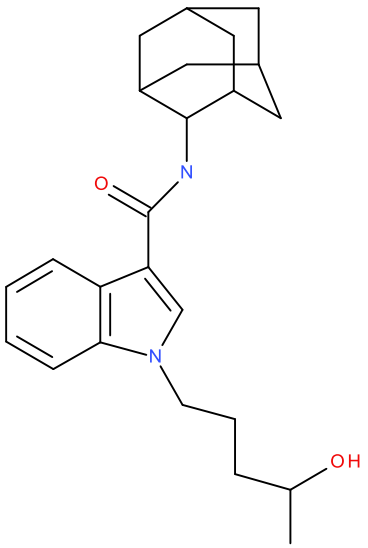
Compound Name	Aliases	Structure	Formula (MW)
AM2201 N5OH indole	5F-JWH-018 N5OH indole		$C_{24}H_{22}FNO_2$ (375.4)
APICA	2NE1 SDB-001 JWH-018 adamantyl carboxamide		$C_{24}H_{32}N_2O$ (364.5)
APICA N4OH pentyl	2NE1 N4OH pentyl SDB-001 N4OH pentyl JWH-018 adamantyl carboxamide N4OH pentyl		$C_{24}H_{32}N_2O_2$ (380.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

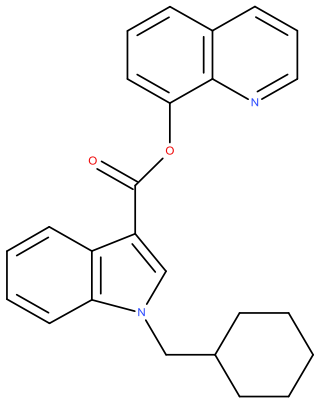
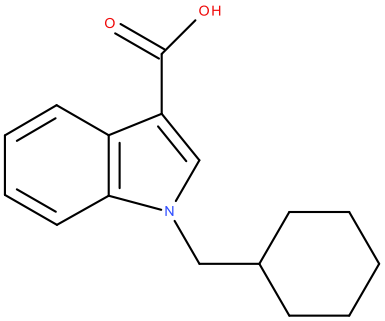
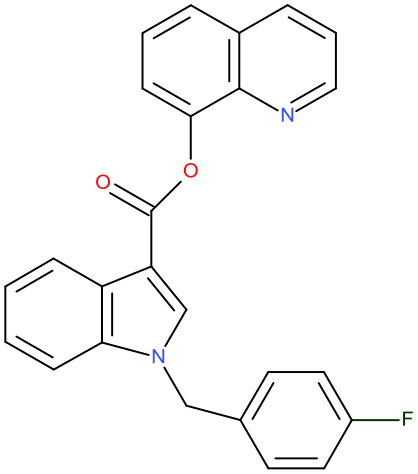
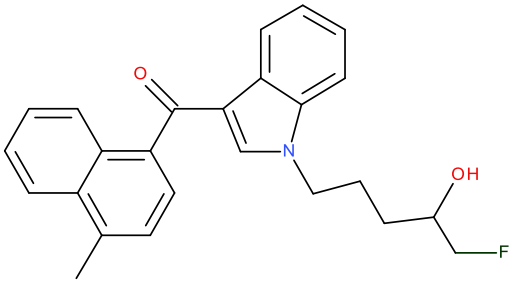
Compound Name	Aliases	Structure	Formula (MW)
BB-22	Quchic		$C_{25}H_{24}N_2O_2$ (384.5)
BB-22 3-carboxy indole	Quchic 3-carboxy indole		$C_{16}H_{19}NO_2$ (257.3)
FUB-PB-22	N/A		$C_{25}H_{17}FN_2O_2$ (396.4)
MAM-2201 N4OH pentyl	5F-JWH-122 N4OH pentyl		$C_{25}H_{24}FNO_2$ (389.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

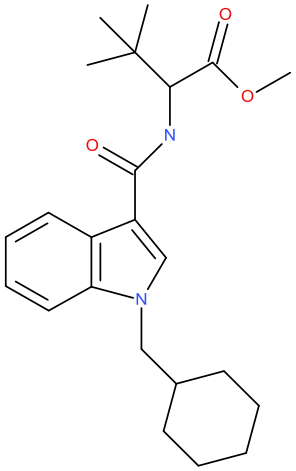
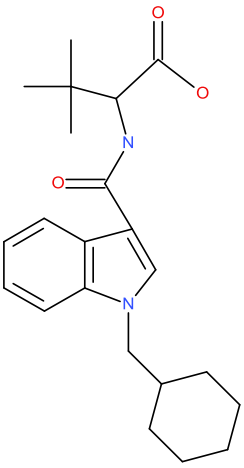
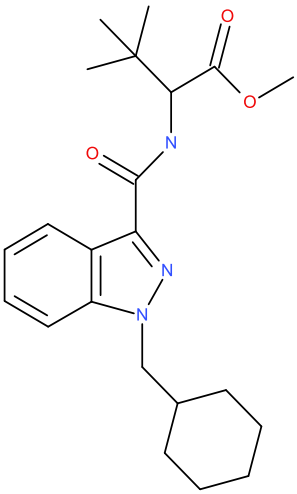
Compound Name	Aliases	Structure	Formula (MW)
MDMB-CHMICA	MMB-CHMINACA		$C_{23}H_{32}N_2O_3$ (384.5)
MDMB-CHMICA *O*-desmethyl acid metabolite	MMB-CHMINACA *O*-desmethyl acid metabolite		$C_{22}H_{30}N_2O_3$ (370.5)
MDMB-CHMINACA	N/A		$C_{22}H_{31}N_3O_3$ (385.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

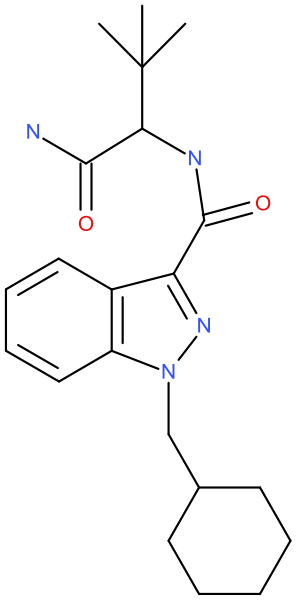
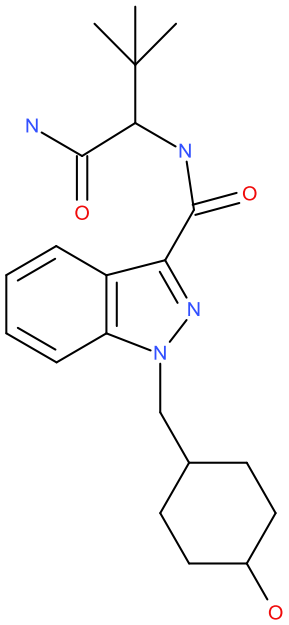
Compound Name	Aliases	Structure	Formula (MW)
MAB-CHMINACA	ADB-CHMINACA		$C_{21}H_{30}N_4O_2$ (370.5)
MAB-CHMINACA M1	ADB-CHMINACA M1		$C_{21}H_{30}N_4O_3$ (386.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

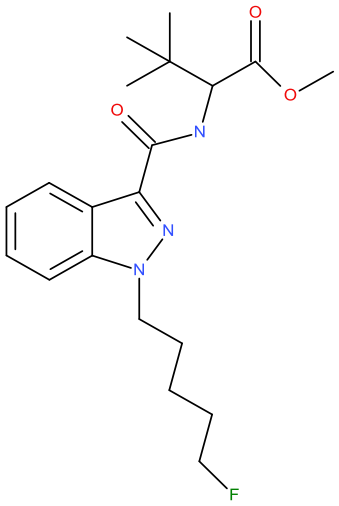
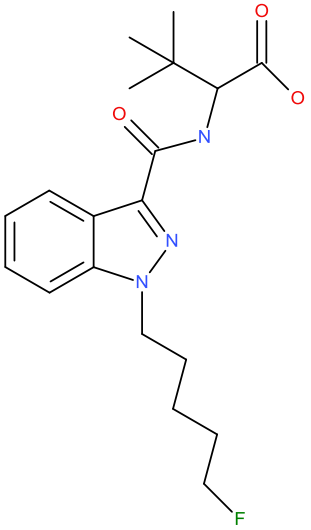
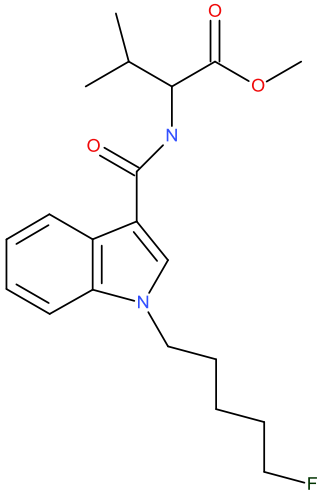
Compound Name	Aliases	Structure	Formula (MW)
5F-MDMB-PINACA	5F-ADB		$C_{20}H_{28}FN_3O_3$ (377.5)
5F-MDMB-PINACA *O*-desmethyl acid metabolite	5F-ADB *O*-desmethyl acid metabolite		$C_{19}H_{26}FN_3O_3$ (363.4)
MMB2201	AMB-PICA I-AMB		$C_{20}H_{27}FN_2O_3$ (362.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

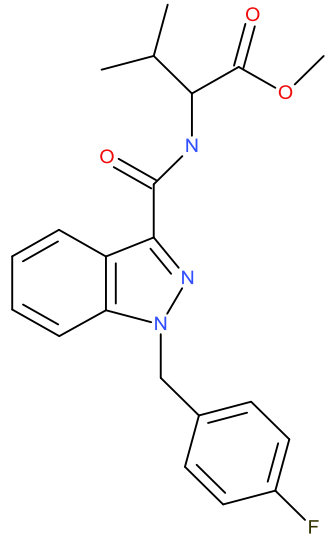
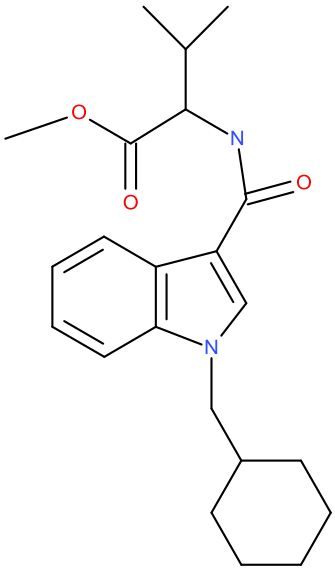
Compound Name	Aliases	Structure	Formula (MW)
MMB-FUBINACA	AMB-FUBINACA FUB-AMB		$C_{21}H_{22}FN_3O_3$ (383.4)
MMB-CHMICA	AMB-CHMICA		$C_{22}H_{30}N_2O_3$ (370.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

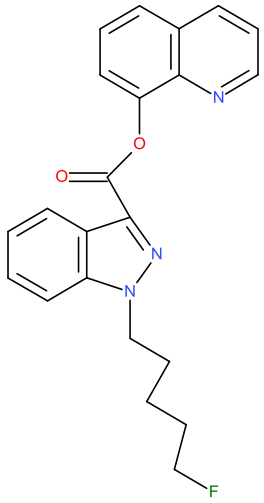
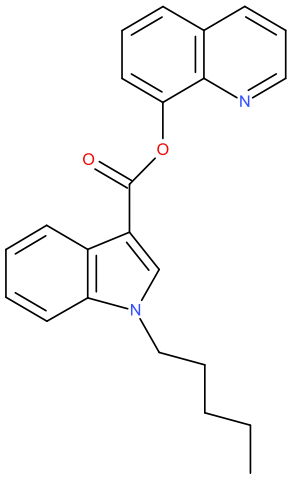
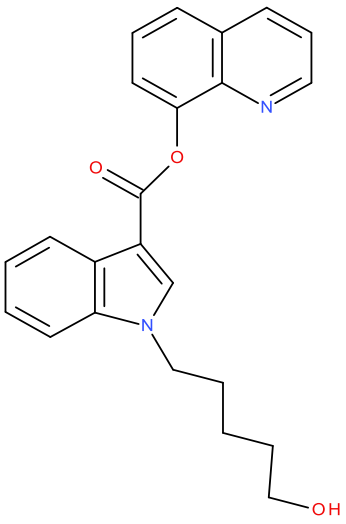
Compound Name	Aliases	Structure	Formula (MW)
5F-NPB-22	5F-PB-22 indazole analogue		$C_{22}H_{20}FN_3O_2$ (377.4)
PB-22	Qupic		$C_{23}H_{22}N_2O_2$ (358.4)
PB-22 N5OH pentyl	Qupic N5OH pentyl		$C_{23}H_{22}N_2O_3$ (374.4)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

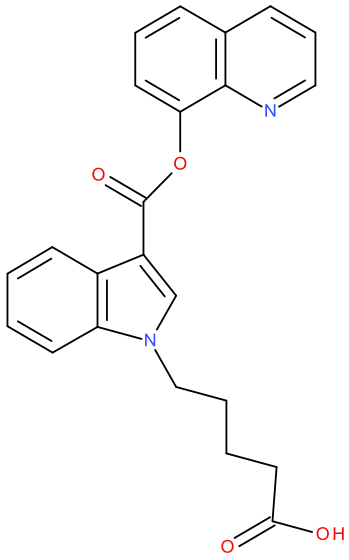
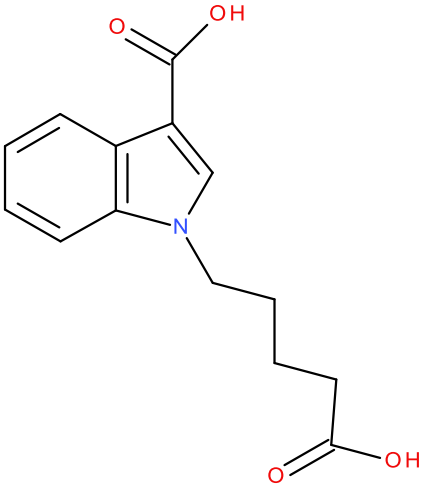
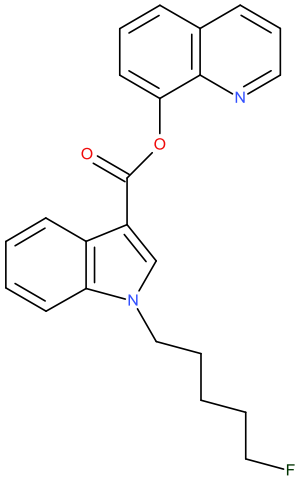
Compound Name	Aliases	Structure	Formula (MW)
PB-22 N-pentanoic acid	Qupic N-pentanoic acid		$C_{23}H_{20}N_2O_4$ (388.4)
PB-22 N-pentanoic acid 3-carboxyindole	Qupic N-pentanoic acid 3-carboxy indole		$C_{14}H_{15}NO_4$ (261.3)
5F-PB-22	5F-Qupic		$C_{23}H_{21}FN_2O_2$ (376.4)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

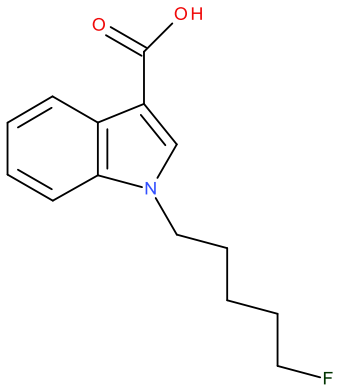
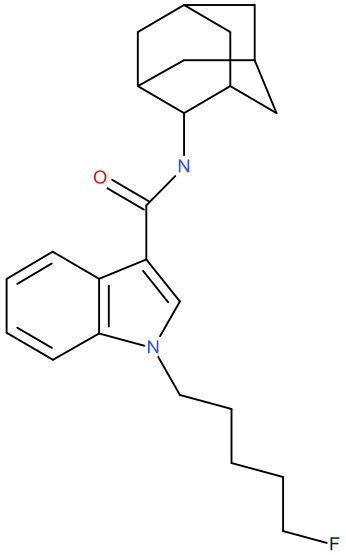
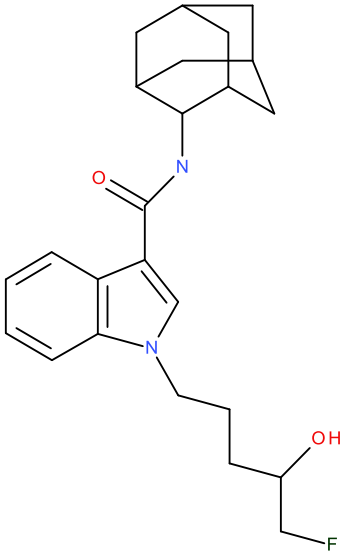
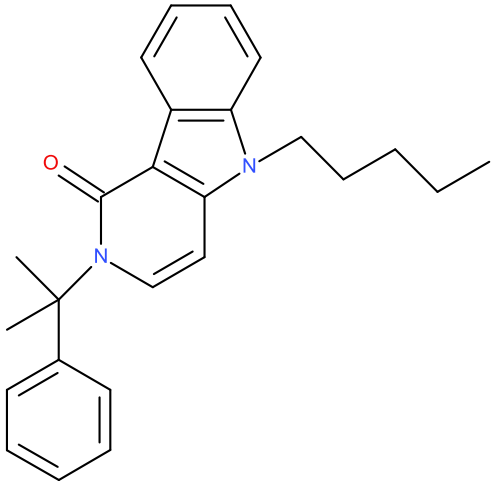
Compound Name	Aliases	Structure	Formula (MW)
5F-PB-22 3-carboxy indole	5F-Qupic 3-carboxy indole		$C_{14}H_{16}FNO_2$ (249.3)
STS-135	5F-APICA N-adamantyl-1-fluoropentyl indole-3-carboxamide		$C_{24}H_{31}FN_2O$ (382.5)
STS-135 N4OH pentyl	5F-APICA N4OH pentyl N-adamantyl-1-fluoropentyl indole-3-carboxamide N4OH pentyl		$C_{24}H_{31}FN_2O_2$ (398.5)

Table 17 – Synthetic Cannabinoid Receptor Agonists included in one or more of the methods detailed in this research. Details of chemical formula, structure, molecular weight and any known aliases are given.

Compound Name	Aliases	Structure	Formula (MW)
CUMYL-PeGACLONE	SGT-151		$C_{25}H_{28}N_2O$ (372.5)

After the conclusion of the practical aspect of the research, information was received from the retailer that the 5F-PB-22 3-carboxyindole metabolite drug standard used was actually a 5F-PB-22 ester isomer. As such the data relating to this compound was removed from the validation presented here. This compound was in the panel of a method employed for some cases discussed as a previously validated method was applied to these cases, using a drug standard from a different source. These cases will be highlighted as such. The parent compound, 5F-PB-22, and another metabolite, PB-22 N-pentanoic acid, were included in the method so the detection of 5F-PB-22 use was possible.

Figure 17 gives chemical and structural information relating to the intended and actual product received. This figure shows that the molecular weights are identical with respect to the resolution of the instrumentation used in this research. The most abundant ion transitions resulting from the fragmentation of both molecules were the same when infused on the MS.

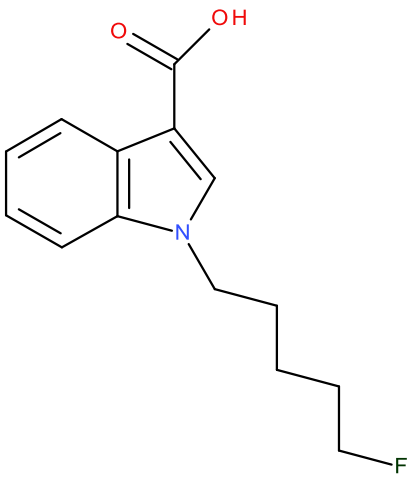
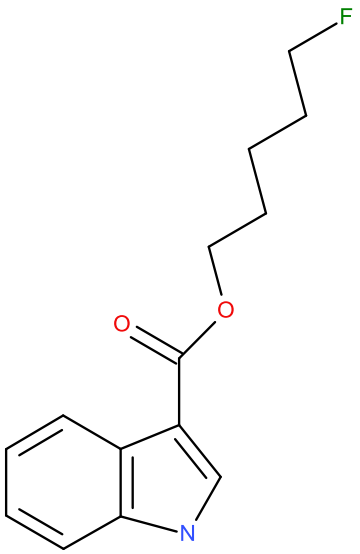
	
5F-PB-22 3-Carboxyindole $C_{14}H_{16}FNO_2$ MW=249.28	1H-Indole-carboxylic acid 5-fluoropentyl ester $C_{14}H_{16}FNO_2$ MW=249.28

Figure 17 – Structural and chemical formulae and molecular weights for the intended and actual products provided as 5F-PB-22 3-carboxyindole.

4.4.2. Liquid Chromatography – Mass Spectrometry

4.4.2.1. Infusion of Compounds

Infusion of the compounds resulted in optimised parameters for the mass spectral fragmentation, collection and detection for the analytes. These parameters are shown in Table 18.

Table 18 – Optimised tandem mass spectrometric parameters for Synthetic Cannabinoid Receptor Agonists included in one or more methods detailed in this research

Analyte	Precursor Mass	Product Mass	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
AB-CHMINACA	357.2	241.2	31	4.0	28	29	4
	357.2	145.1	31	4.0	28	55	4
AB-CHMINACA M1A	373.2	257.3	31	4.5	28	29	6
	373.2	145.1	31	4.5	28	55	4
AB-CHMINACA M2	358.1	145.1	41	4.5	26	49	4
	358.1	241.1	41	4.5	26	25	6
AB-FUBINACA	369.1	109.1	31	6.5	28	55	4
	369.1	253.0	31	6.5	28	27	6
AB-FUBINACA M2B	399.0	109.1	31	5.0	30	53	4
	399.0	253.0	31	5.0	30	27	6
AB-FUBINACA valine metabolite	370.1	109.2	41	7.5	24	49	4
	370.1	253.0	41	7.5	24	25	4
AB-PINACA	331.2	215.2	26	4.5	16	27	4
	331.2	145.1	26	4.5	16	53	4
AB-PINACA N4OH pentyl	347.2	213.1	31	9.0	26	41	4
	347.2	145.1	31	9.0	26	53	4
5F-AB-PINACA	349.2	233.2	26	4.5	28	25	4
	349.2	304.2	26	4.5	28	17	6
5F-ADB-PINACA	363.2	233.1	31	7.0	26	29	4
	363.2	145.1	31	7.0	26	59	4
AKB48	366.2	135.2	56	4.0	28	27	4
	366.2	93.1	56	4.0	28	63	4
AKB48 N5OH pentyl	382.2	135.2	51	5.0	28	29	4
	382.2	93.2	51	5.0	28	69	4
AKB48 N-pentanoic acid	396.2	135.2	46	7.0	28	29	4
	396.2	107.2	46	6.5	20	61	4
5F-AKB48	384.2	135.2	46	7.5	22	29	4
	384.2	93.2	46	7.5	22	67	4
5F-AKB48 N4OH pentyl	400.2	135.2	56	6.5	30	29	4
	400.2	93.2	56	6.5	30	73	4
AM2201	360.1	155.1	61	8.0	22	31	4
	360.1	127.2	61	8.0	22	65	4
AM2201 N4OH pentyl	376.1	155.1	61	7.0	26	33	4

Table 18 – Optimised tandem mass spectrometric parameters for Synthetic Cannabinoid Receptor Agonists included in one or more methods detailed in this research

Analyte	Precursor Mass	Product Mass	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
	376.1	127.1	61	7.0	26	67	4
AM2201 N5OH indole	376.1	155.0	66	7.5	28	37	4
	376.1	127.1	66	7.5	28	71	4
APICA	365.2	135.2	76	7.0	26	41	4
	365.2	107.2	71	8.0	26	57	4
APICA N4OH pentyl	381.2	135.2	66	7.0	28	41	4
	381.2	107.2	71	4.5	28	63	4
FUB-PB-22	397.2	109.1	36	4.5	20	51	4
	397.2	252.1	36	4.5	20	19	4
BB-22	385.2	240.3	36	4.0	32	23	4
	385.2	144.1	36	4.0	32	47	4
	385.2	116.1	36	4.0	32	87	4
BB-22 3-carboxy indole	258.1	118.1	56	5.5	14	31	4
	258.1	132.1	56	5.5	14	25	4
MAM2201 N4OH pentyl	390.2	168.9	66	8.0	18	39	4
	390.2	141.1	66	8.0	18	59	4
MDMB-CHMICA	385.1	240.2	36	4.0	32	23	4
	385.1	144.1	36	4.0	32	47	4
	385.1	116.1	36	4.0	32	87	4
MDMB-CHMICA *O*-desmethyl acid metabolite	371.2	240.2	31	6.5	28	21	6
	371.2	144.2	31	6.5	28	49	4
MDMB-CHMINACA	386.2	241.2	56	5.0	22	27	4
	386.2	326.2	56	5.0	22	19	6
MAB-CHMINACA	371.2	240.2	41	8.5	20	21	4
	371.2	144.1	41	8.5	20	51	4
MAB-CHMINACA M1	387.2	257.2	41	8.5	22	29	4
	387.2	145.2	41	8.5	22	57	4
5F-MDMB-PINACA	378.2	233.1	46	8.5	20	27	4
	378.2	145.1	46	8.5	20	57	4
5F-MDMB-PINACA *O*-desmethyl acid metabolite	364.2	233.2	41	9.0	18	25	4
	364.2	145.2	41	9.0	18	55	4
MMB2201	363.2	232.1	31	7.5	20	21	4
	363.2	144.1	31	7.5	20	53	4
MMB-FUBINACA	384.1	109.2	51	5.5	28	55	4
	384.1	253.0	51	5.5	28	25	6
MMB-CHMICA	371.2	241.2	41	8	28	29	4
	371.2	145.1	41	8	28	57	4
5F-NPB-22	378.2	233.1	46	8.5	20	27	4
	378.2	145.1	46	8.5	20	57	4
PB-22	359.1	214.1	31	4.5	26	19	4
	359.1	144.1	31	4.5	26	51	4

Table 18 – Optimised tandem mass spectrometric parameters for Synthetic Cannabinoid Receptor Agonists included in one or more methods detailed in this research

Analyte	Precursor Mass	Product Mass	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
PB-22 N5OH pentyl	375.1	230.1	31	5.0	26	21	4
	375.1	144.1	31	5.0	26	49	4
PB-22 N-pentanoic acid	389.0	244.1	26	4.5	30	19	4
	389.0	144.1	26	4.5	30	47	4
5F-PB-22	377.1	232.0	26	4.5	28	21	4
	377.1	144.1	26	4.5	28	53	4
5F-PB-22 N-pentanoic acid 3-carboxyindole	262.1	244.2	31	10.0	14	15	4
	262.1	144.1	31	10.0	14	33	4
STS-135	383.2	135.2	71	8.5	28	41	4
	383.2	107.2	76	8.0	28	59	4
STS-135 N4OH pentyl	399.1	135.1	81	8.0	20	41	4
	399.1	93.2	81	8.0	20	69	4
CUMYL-PeGACLONE	373.2	255.2	36	6.5	26	19	4
	373.2	167.2	36	6.5	26	65	4
AB-FUBINACA-d_4	373.1	109.2	26	7.0	26	53	4
PB-22-d_9	368.2	223.2	26	4.5	24	19	4
AM2201-d_5	365.2	155.1	61	8.0	26	31	4
AKB48-d_{11}	377.3	135.2	46	8.5	20	27	4
AKB48 N5OH pentyl-d_4	386.2	135.2	51	9.0	28	29	4

The compounds detailed in Table 17 and Table 18 can be grouped together based on their structures and how these fragment in the MS source.

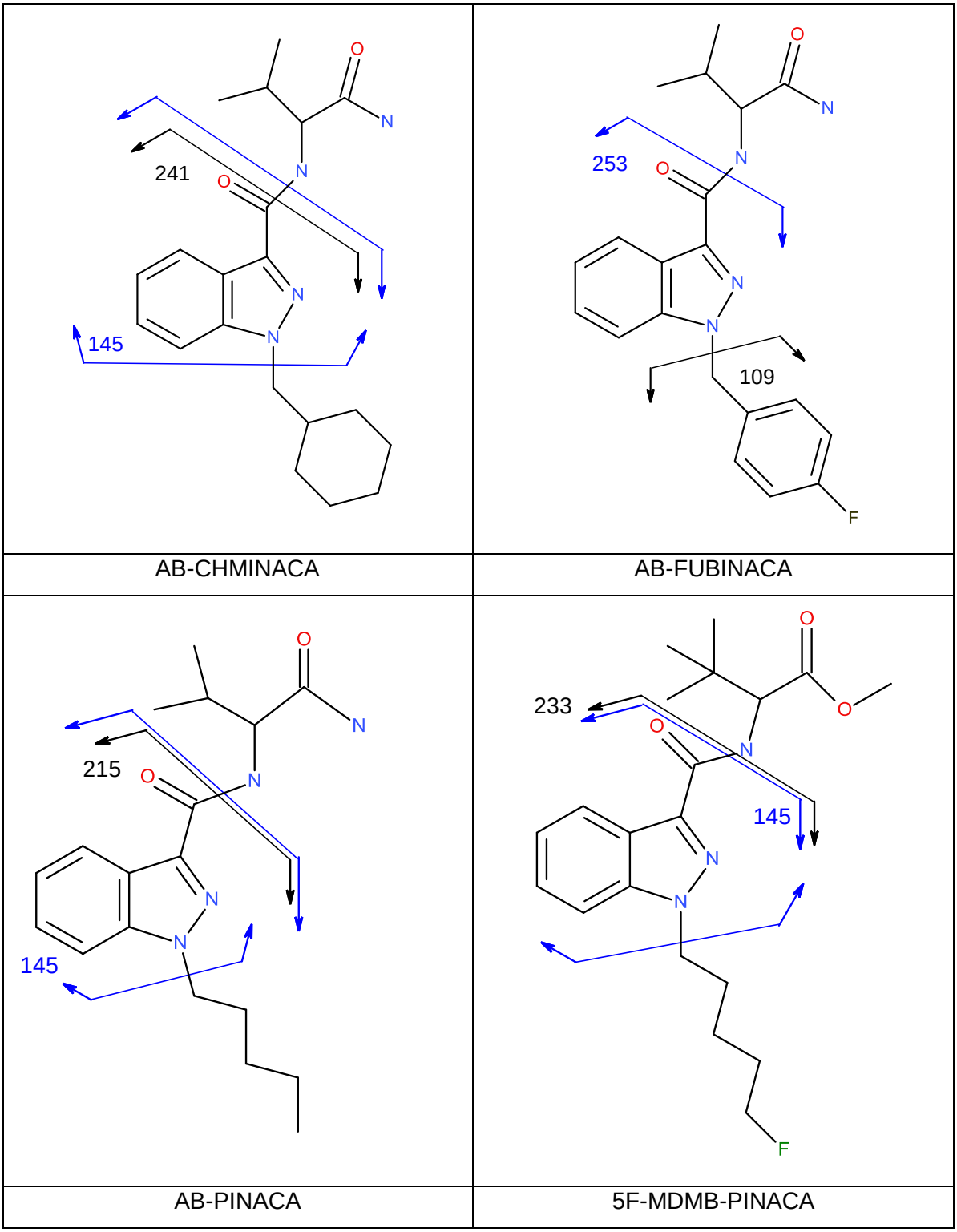


Figure 18 – Fragmentation at the carboxamide linkage, and between the indole/indazole core and tail in selected Synthetic Cannabinoid Receptor Agonists

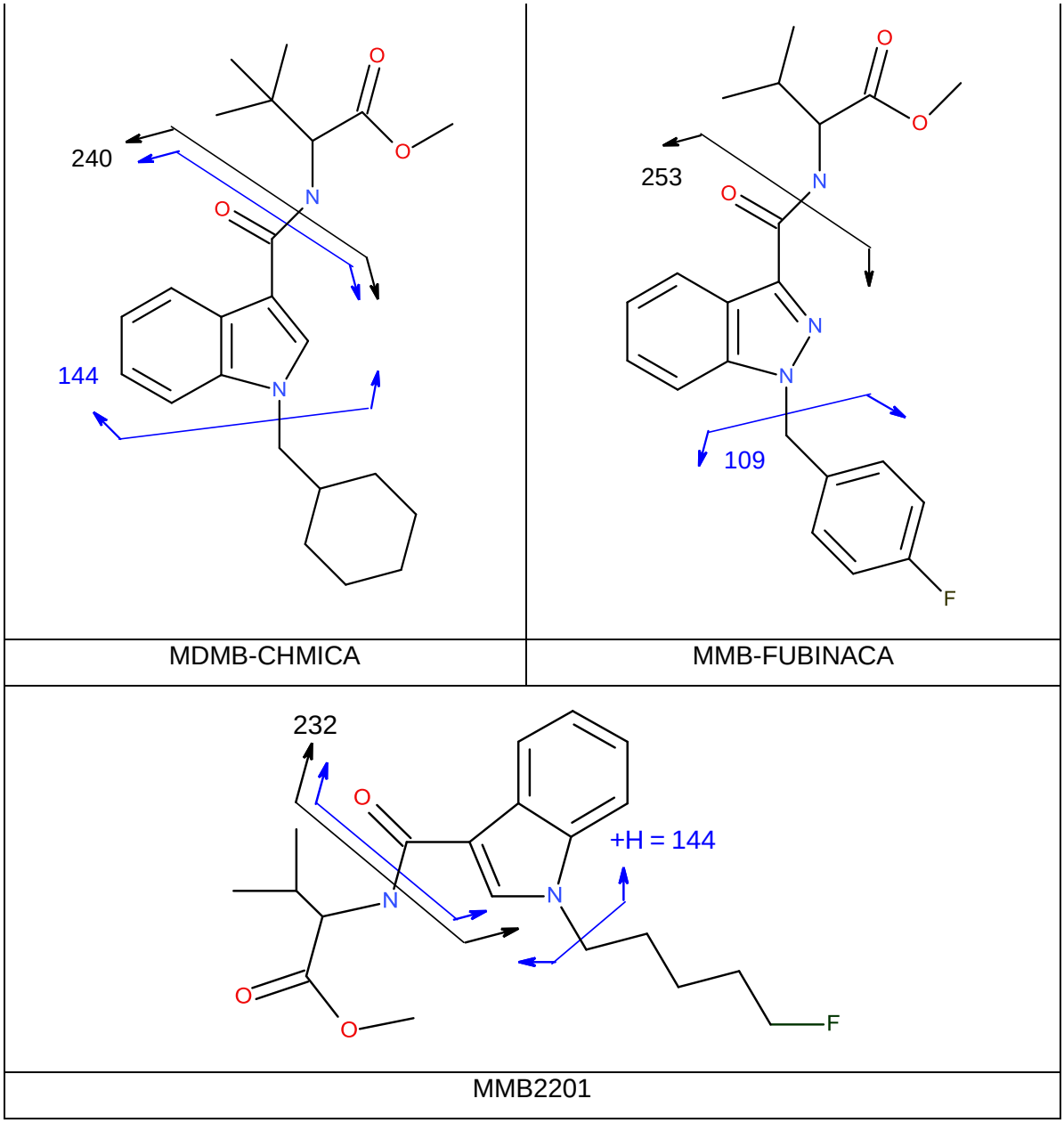
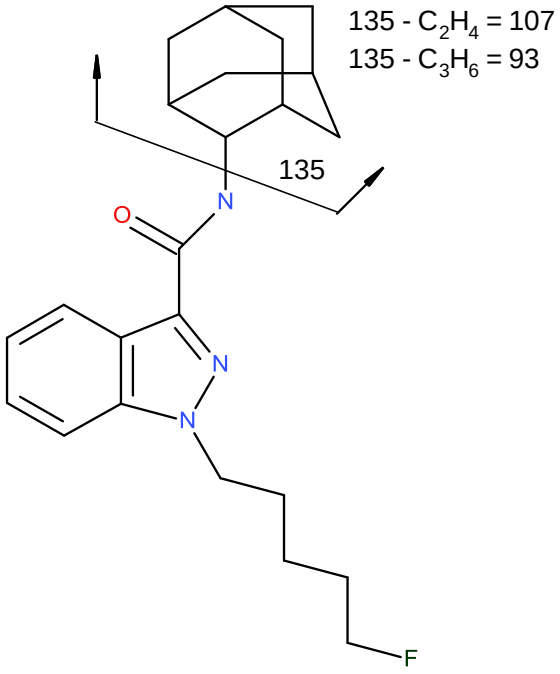


Figure 18 – Fragmentation at the carboxamide linkage, and between the indole/indazole core and tail in selected Synthetic Cannabinoid Receptor Agonists

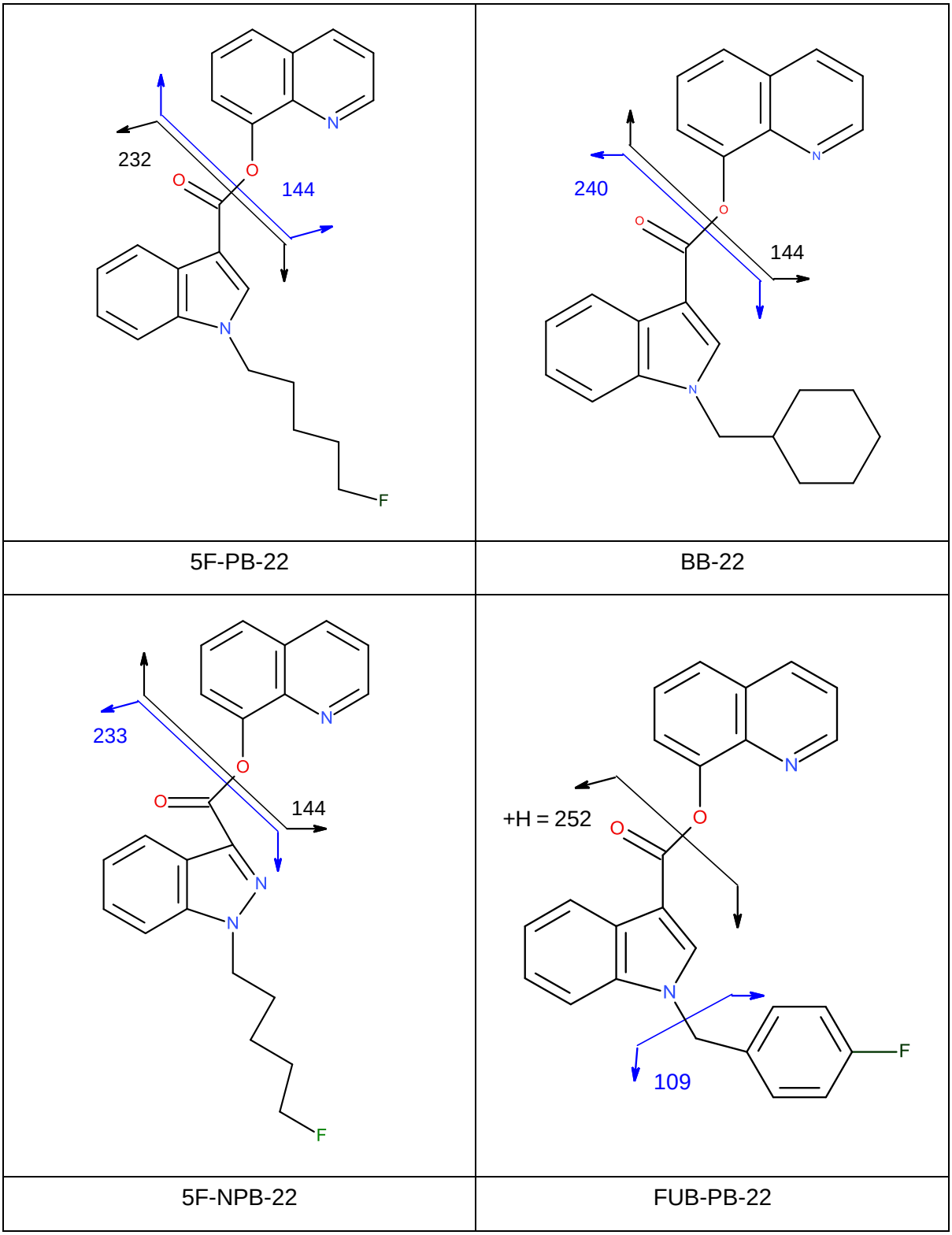
Fragmentation can take place between the nitrogen and carbon atoms in the carboxamide linkage, and between the nitrogen and carbon atoms joining the indole or indazole group to the tail group (Figure 18). This fragmentation pattern occurs in AB-CHMINACA and metabolites, AB-FUBINACA, metabolites and deuterated analogue, AB-PINACA and metabolite, 5F-AB-PINACA, 5F-ADB-PINACA, MDMB-CHMICA and metabolite, MDMB-CHMINACA, 5F-MDMB-PINACA and metabolite, MMB-FUBINACA, MMB2201, MAB-CHIMINACA and metabolite, and MMB-CHMICA.



5F-AKB48

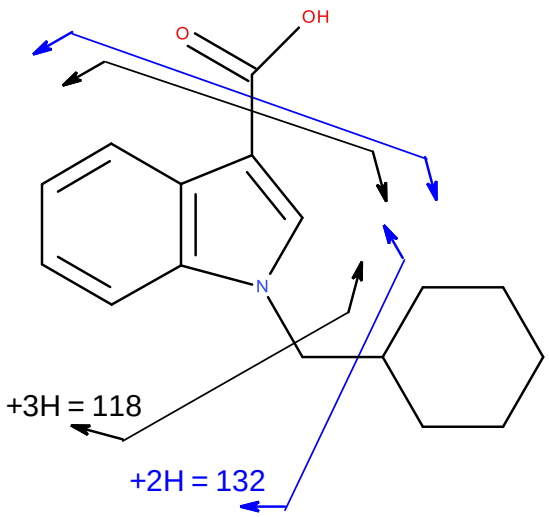
Figure 19 – Fragmentation at the adamantyl group in selected Synthetic Cannabinoid Receptor Agonists

For AKB48, metabolites and deuterated analogues, 5F-AKB48 and metabolite, APICA and metabolite, and STS-135 and metabolite the fragmentation occurs at the adamantyl group (Figure 19). Cleavage of this after the nitrogen atom results in the m/z 135 ion, and the loss of C_2H_4 or C_3H_6 from this group results in the ions at m/z 107 and 93 respectively.



5F-PB-22	BB-22
5F-NPB-22	FUB-PB-22

Figure 20 – Fragmentation at the carboxyl linkage in selected Synthetic Cannabinoid Receptor Agonists



BB-22 3-carboxy indole

Figure 20 – Fragmentation at the carboxyl linkage in selected Synthetic Cannabinoid Receptor Agonists

Compounds containing the carboxyl linkage fragment at this position, either between the two oxygen atoms, or between the carboxyl group and the indole or indazole group (Figure 20). The two component parts are the primary fragments for PB-22, metabolites and deuterated analogue, 5F-PB-22, BB-22 and 5F-NPB-22. Fragmentation also occurs between the indole core and tail for FUB-PB-22 and the BB-22 3-carboxy indole metabolite. This happens before the first carbon atom in the tail group, and includes the remainder of the molecule for FUB-PB-22. In BB-22 3-carboxy indole, fragmentation occurs both before and after the first carbon atom in the tail group, resulting in fragments comprising the indole group alone and the indole group plus the first carbon atom from the tail.

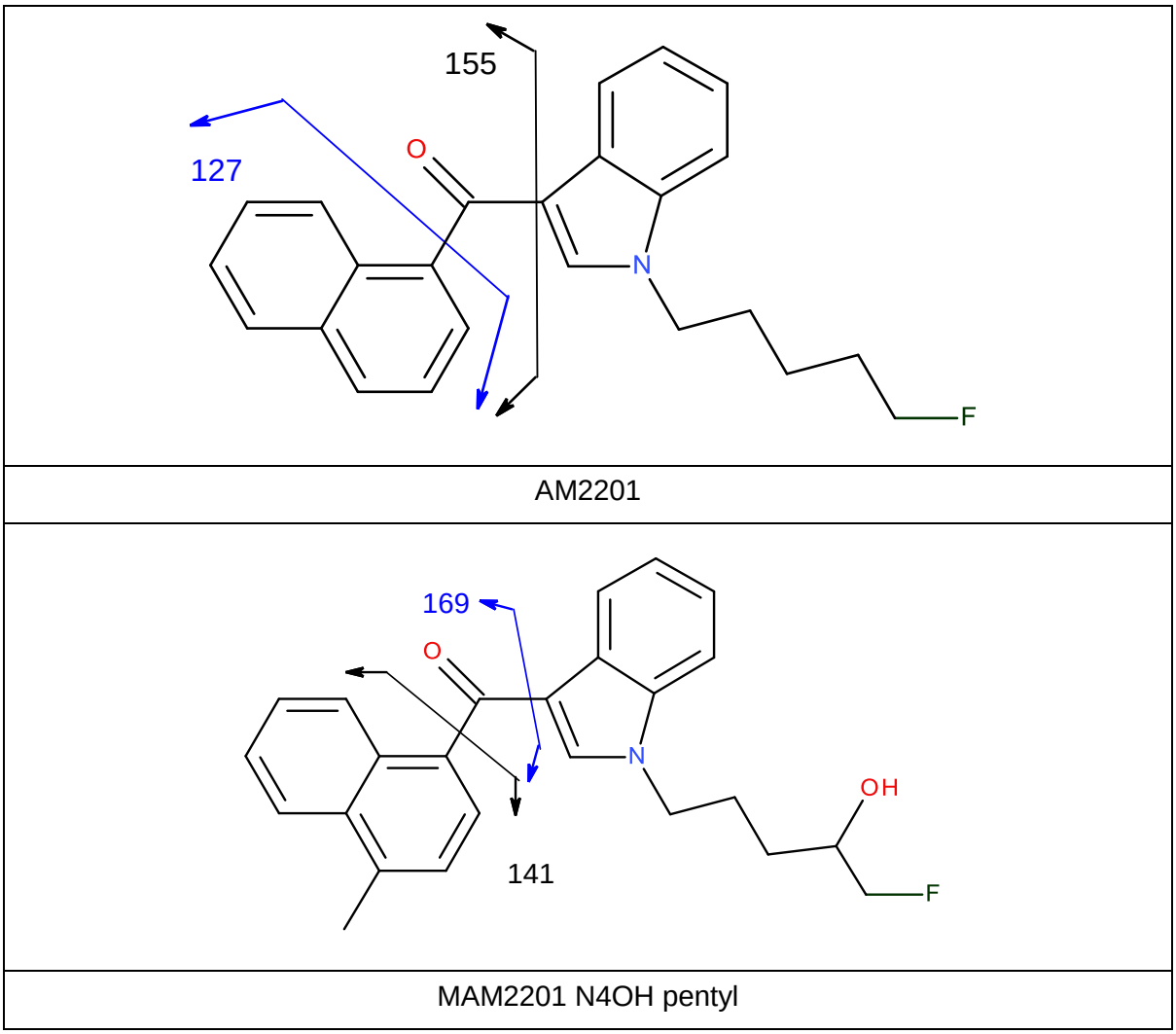
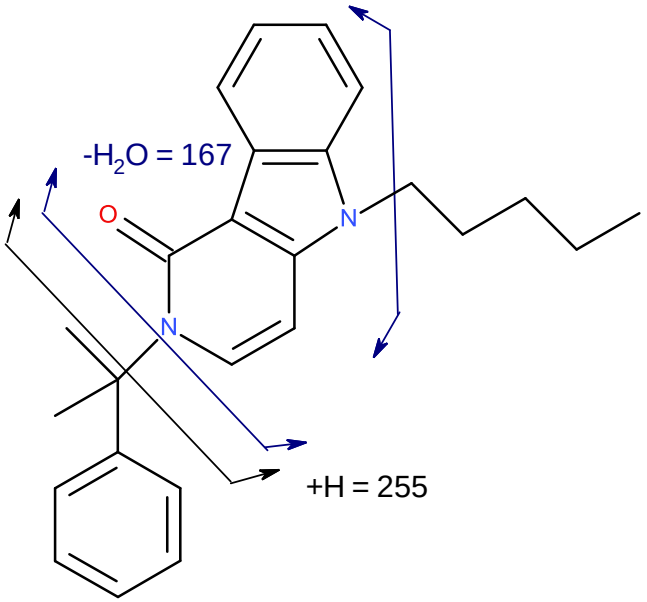


Figure 21 – Fragmentation at the methanone linkage in selected Synthetic Cannabinoid Receptor Agonists

AM2201, metabolites and deuterated analogue, and MAM2201 N4OH pentyl fragment around the methanone linkage (Figure 21). Fragmentation happens both before and after the oxygen molecule in this group, and resulting fragments include the naphthyl group and, in MAM2201 N4OH pentyl, the methyl group attached to this.



CUMYL-PeGACLONE

Figure 22 – Fragmentation in the Synthetic Cannabinoid Receptor Agonist CUMYL-PeGACLONE

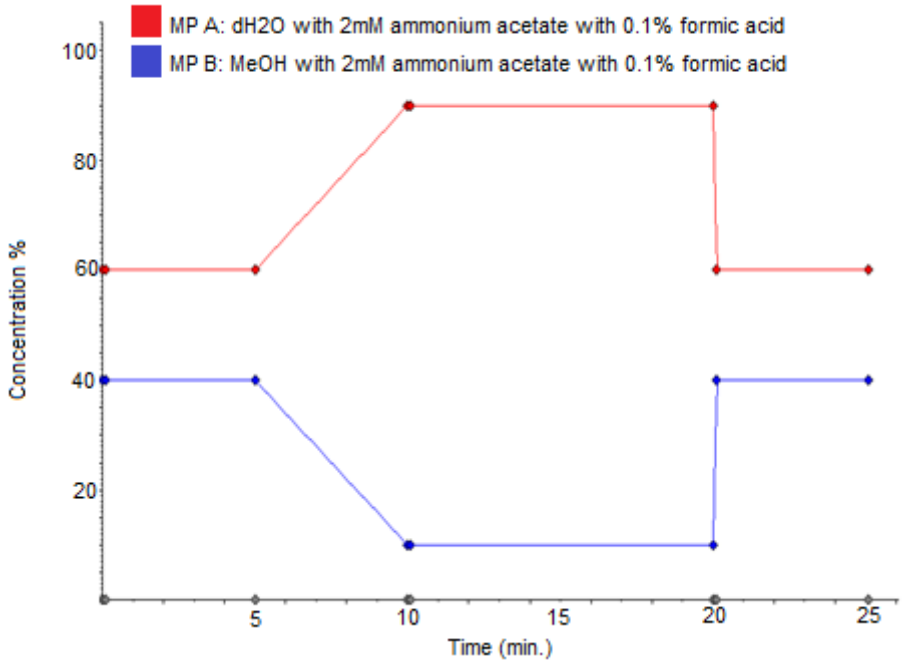
Within the CUMYL-PeGACLONE molecule, fragmentation takes place between the tricyclic group and the pentyl chain, and the tricyclic group and the benzyl group. For the production of the fragment at m/z 255, the tricyclic and pentyl chain remain intact, while for the fragment at m/z 167, the tricyclic group is fully detached, and the carbonyl group is also removed.

Because of these close structural resemblances and fragmentation patterns between compounds in the same classes of SCRAs, several compounds shared, or had very similar, ion transitions as far as the resolving power of the instrument allowed. These were: AM2201 N4OH pentyl/AM2201 N5OH indole, MDMB-CHMICA/BB-22, 5F-ADB-PINACA/MMB2201 and 5F-MDMB-PINACA/5F-NPB-22, which shared identical transitions. In addition, MDMB-CHMICA and BB-22 were similar but not identical to MDMB-CHMINACA; 5F-ADB-PINACA and MMB2201 were similar but not identical to 5F-MDMB-PINACA *O*-desmethyl acid metabolite; and 5F-MDMB-PINACA and 5F-NPB-22 were similar but not identical to 5F-PB-22. The ion transitions which were similar but not identical to each other were AKB48/APICA, 5F-AKB48/STS-135, AKB48 N5OH pentyl/APICA N4OH pentyl, 5F-AKB48 N4OH pentyl/STS-135 N4OH pentyl, AB-CHMINACA/AB-CHMINACA metabolite 2, and MMB-CHMICA/MAB-CHMINACA. For these reasons it was

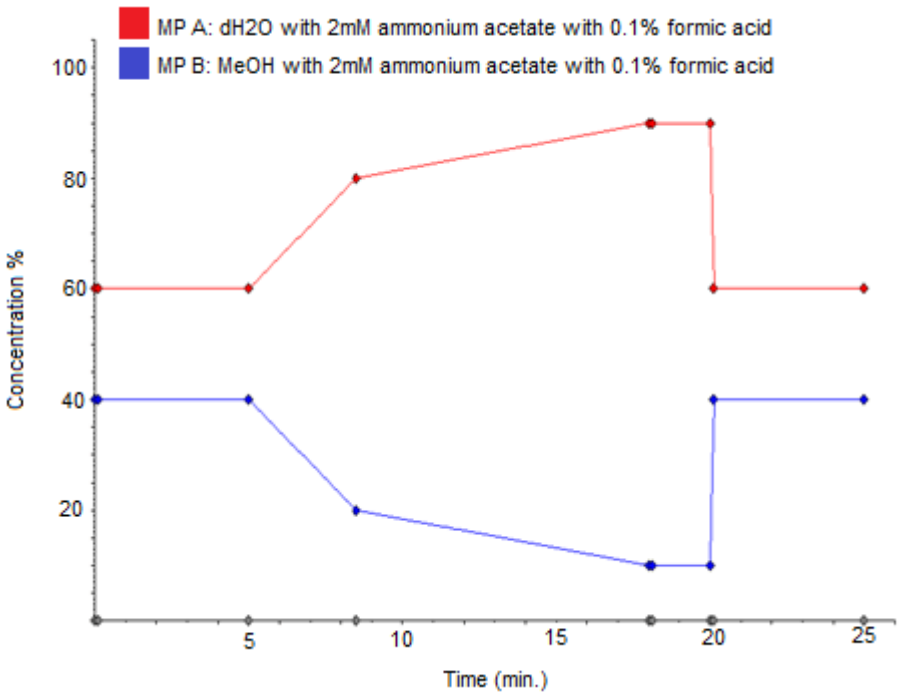
important to have a chromatographic method with sufficient resolution between these compounds with identical or similar transitions.

4.4.2.2. Mobile Phase Experiments

From the experiments conducted in 4.3.3.2 it was determined that MP gradients F, H and R gave satisfactory retention and separation of analytes on the column for methods 1.1, 1.2 and 2.1 respectively. These MP gradient programmes are represented graphically in Figure 23. An example chromatogram from each method is shown in Figure 24 .

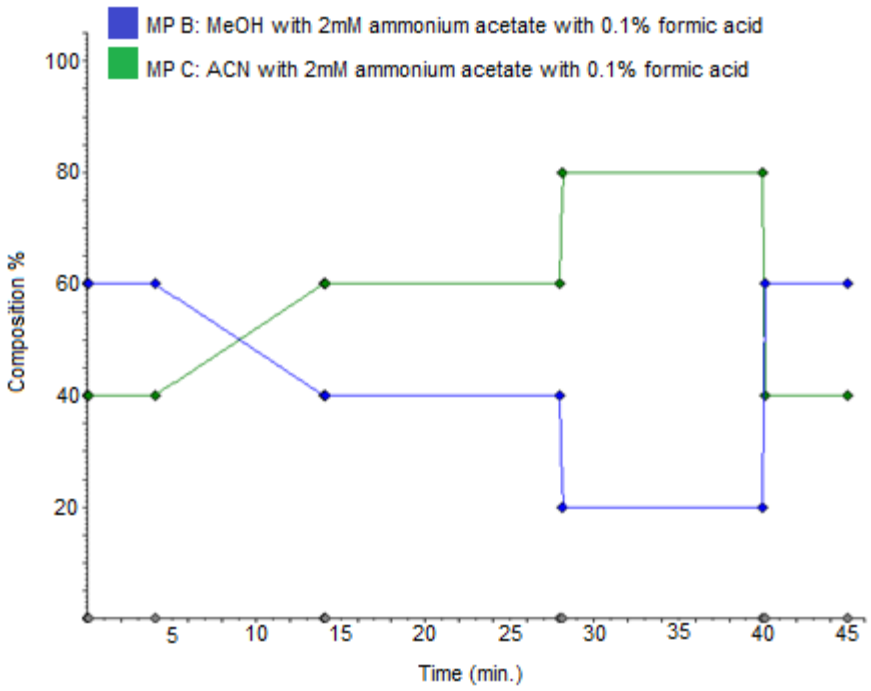


Mobile Phase programme F



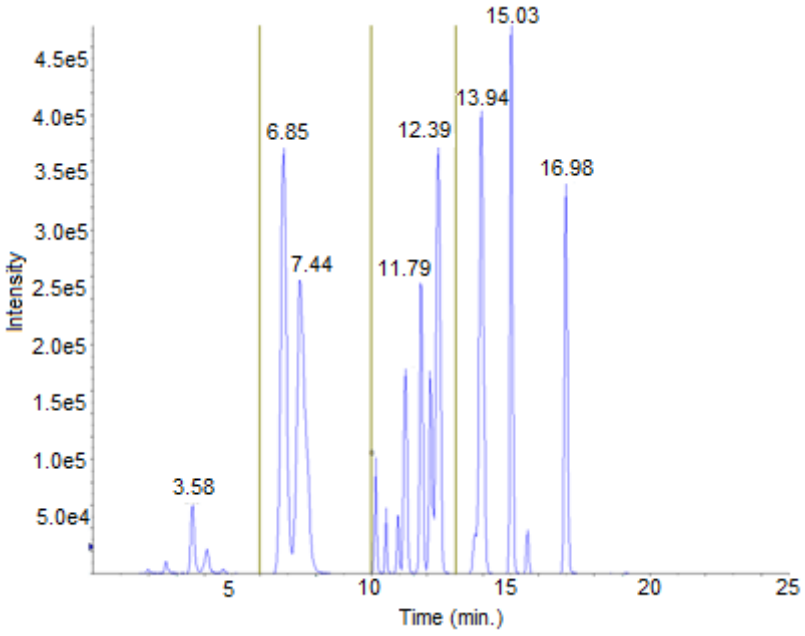
Mobile Phase programme H

Figure 23 – Graphical representation of Mobile Phase gradient programmes F (top), H (middle) and R (bottom). The red line shows the percentage composition of Mobile Phase A (H_2O with 2mM ammonium acetate and 0.1% formic acid); the blue line shows the percentage composition of Mobile Phase B (MeOH with 2mM ammonium acetate and 0.1% formic acid); and the green line shows the percentage composition of Mobile Phase C (ACN with 2mM ammonium acetate and 0.1% formic acid). These programmes were used in Methods 1.1, 1.2 and 2.1 (top, middle and bottom respectively).

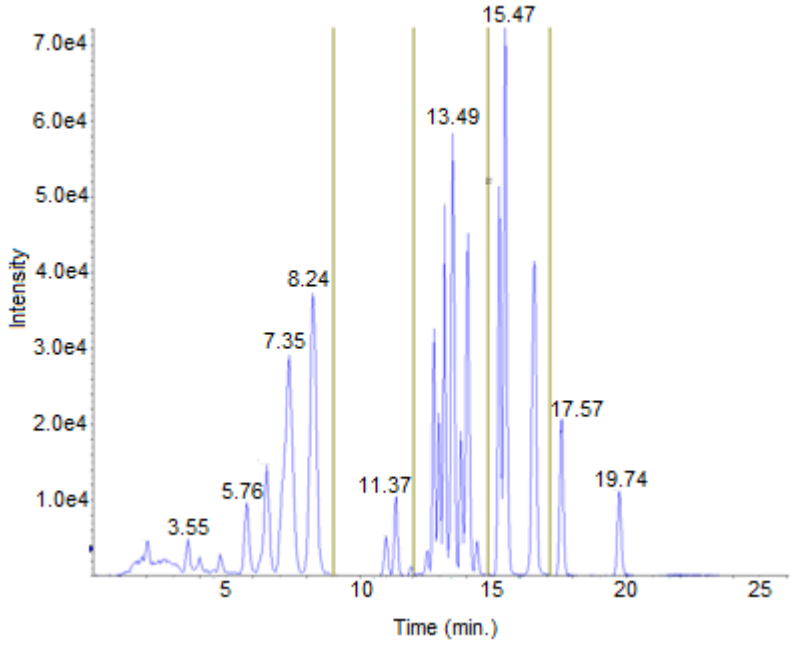


Mobile Phase programme R

Figure 23 – Graphical representation of Mobile Phase gradient programmes F (top), H (middle) and R (bottom). The red line shows the percentage composition of Mobile Phase A (H_2O with 2mM ammonium acetate and 0.1% formic acid); the blue line shows the percentage composition of Mobile Phase B (MeOH with 2mM ammonium acetate and 0.1% formic acid); and the green line shows the percentage composition of Mobile Phase C (ACN with 2mM ammonium acetate and 0.1% formic acid). These programmes were used in Methods 1.1, 1.2 and 2.1 (top, middle and bottom respectively).

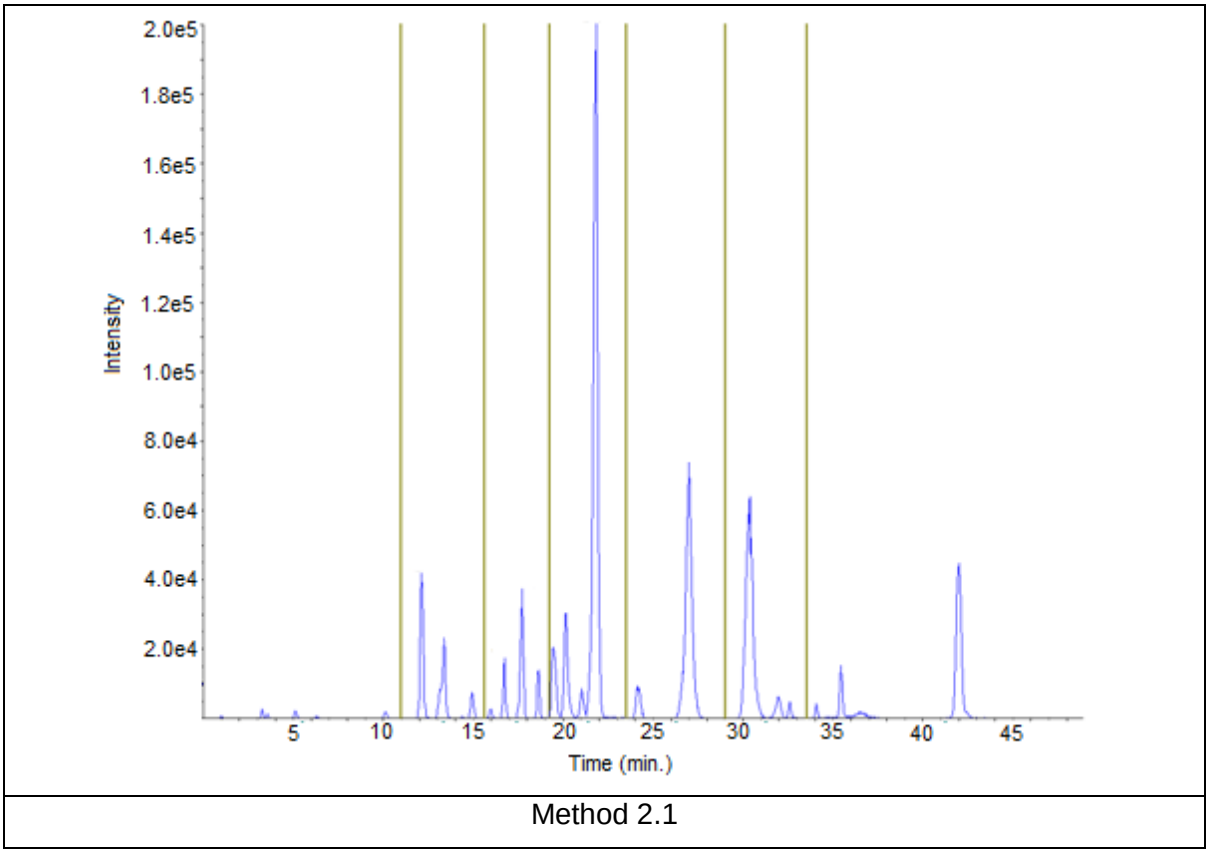


Method 1.1



Method 1.2

Figure 24 – Example chromatograms obtained from the Mobile Phase gradients employed in methods 1.1 (top), 1.2 (middle), and 2.1 (bottom)



Method 2.1

Figure 24 – Example chromatograms obtained from the Mobile Phase gradients employed in methods 1.1 (top), 1.2 (middle), and 2.1 (bottom)

Interrogation of the mixed standard injected and evaluated using these gradients showed baseline separation was achieved for all analytes with the same or similar (≤1 amu difference) ion transitions, with the exception of the shared BB-22/MDMB-CHMICA transition for method 1.1. The discussion around the interference between BB-22 and MDMB-CHMICA as presented in section 3.5.2.5 is relevant here also. Retention times were identified from injections of individual compounds and noted for each analyte. Analytes were therefore identifiable and distinctive by either ion transition or retention time, including the distinction between MDMB-CHMICA and BB-22 for methods 1.2 and 2.1.

4.4.3. Extraction of Analytes

4.4.3.1. Extraction from Blood

Experiments conducted in 4.3.4.1 identified experiment 10 as the optimal conditions for extraction of analytes from blood. This protocol is detailed in Table 19 and was used in the optimised and validated method 2.1 as applied to blood.

Table 19 – Optimised protocol for the extraction of selected Synthetic Cannabinoid Receptor Agonists from blood

Parameter	Conditions
Volume of blood (mL)	0.5
Buffer type	0.1M pH6.0 phosphate buffer
Volume of buffer (mL)	0.5
Extraction solvent	*t*BME
Volume of solvent (mL)	1
Mixing time (min.)	2

It was thought that the saline solution added to the packed red cells in the production of blank blood (see section 3.3.1.16) may be contributing towards unacceptable ME. It was therefore decided to prepare blank blood by mixing packed red cells 1:1 with deionised H_2O rather than saline solution and investigating the recovery, ME and process efficiency of the optimised extraction protocol. The recovery, ME and process efficiency of the default, optimised (with saline) and optimised (without saline) extraction protocols are given in Table 20, with green boxes showing the most desirable results. Figure 25 shows the process efficiencies for the original and optimised extraction protocols in the form of a bar chart. From this, it is clear to see the improvement for some compounds, and detrimental effects for others.

Table 20 – Recovery, matrix effects and process efficiency of original and optimised protocols for the extraction of selected Synthetic Cannabinoid Receptor Agonists from blood. Results are given for blank blood prepared with and without saline

Analyte	Original			Optimised (saline)			Optimised (no saline)		
	Rec (%)	ME (%)	PE (%)	Rec (%)	ME (%)	PE (%)	Rec (%)	ME (%)	PE (%)
AB-CHMINACA	56	164	93	88	119	105	99	92	91
AB-CHMINACA M1A	62	123	76	63	120	76	63	106	66
AB-CHMINACA M2	50	113	56	72	85	61	83	89	74
AB-FUBINACA	71	121	86	83	144	120	84	103	86
AB-FUBINACA M2B	14	106	15	5	106	5	11	98	10
AB-FUBINACA valine metabolite	56	126	70	72	137	99	66	101	67
MMB-FUBINACA	69	110	76	80	94	75	92	91	84
AKB48	31	40	12	70	19	13	33	40	13
AKB48 N5OH Pentyl	64	106	68	87	90	78	99	82	81
AKB48 N-Pentanoic Acid	63	101	63	83	84	70	97	92	89
5F-AKB48	47	76	36	97	36	34	45	90	41
5F-AKB48 N4OH Pentyl	66	96	63	78	74	57	101	84	84
PB-22	58	95	56	78	68	54	75	99	74
PB-22 N5OH Pentyl	73	91	66	77	101	79	91	97	88
PB-22 N-Pentanoic Acid	67	78	53	60	92	55	72	117	84
5F-PB-22	69	90	62	80	85	68	86	99	86
5F-NPB-22	70	91	63	67	96	64	84	101	85
MDMB-CHMICA	50	113	57	88	68	59	87	78	68
MDMB-CHMICA *O*-desmethyl Acid	74	130	96	71	80	56	74	85	63
MDMB-CHMINACA	49	85	41	100	41	41	50	92	46
BB-22	49	81	40	89	48	42	59	94	55
BB-22 3-carboxyindole	56	96	53	80	60	48	92	82	75
AM2201	60	98	59	86	71	61	82	88	73
AM2201 N4OH Pentyl	74	86	64	73	87	63	87	102	89
AB-PINACA	67	156	106	76	148	112	90	99	89
AB-PINACA N4OH pentyl	56	158	89	59	179	105	59	118	70
5F-AB-PINACA	73	175	128	75	163	123	81	119	96
5F-MDMB-PINACA	65	107	70	80	93	74	91	96	87
5F-MDMB-PINACA *O*-desmethyl Acid	59	167	99	60	147	88	72	100	73
APICA	39	57	23	102	23	23	36	80	29
APICA N4OH Pentyl	67	128	86	82	110	90	97	132	128
STS-135	50	92	46	88	60	53	82	80	65
STS-135 N4OH Pentyl	60	116	70	87	93	81	87	152	132
MMB2201	75	119	89	78	117	91	95	92	88
MAM2201 N4OH Pentyl	68	98	67	78	90	70	94	99	93

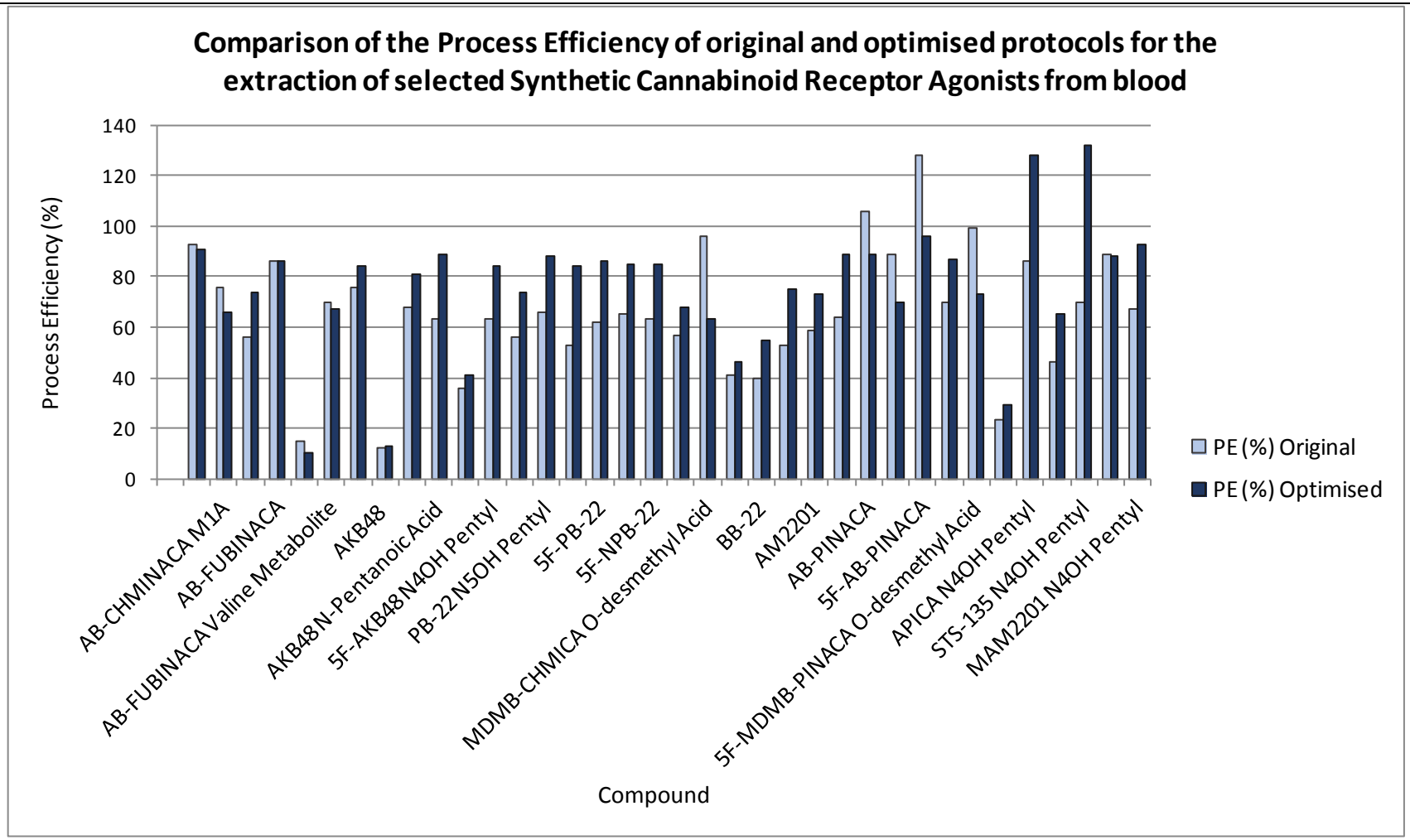


Figure 25 – Bar chart showing the comparison of the process efficiencies of the original and optimised protocols for the extraction of selected Synthetic Cannabinoid Receptor Agonists from blood.

In Table 20, the preferred values were considered ≥60% for recovery, ±20% for matrix effects and 60 – 120% for process efficiency. Acceptable but sub-optimal result were considered ≥40% for recovery, ±30% for matrix effects and 50 – 130% for process efficiency. Any result outside of these ranges should be considered when interpreting the results of analyses. It's clear from this table that optimisation of the extraction and subsequent use of saline-free blank blood has improved the performance of the extraction. The number of preferred values increased from 65 to 90 between the original method and the optimised method with no saline. Simultaneously, acceptable and undesirable results decreased from 26 to 7, and from 17 to 11 respectively. The results for AKB48 saw a decrease in recovery but improvement in ME from optimised with saline to the conditions without saline solution. The PE remained the same for both of these conditions so neither method is optimal.

The results that are outwith acceptable ranges relate to AB-FUBINACA M2B, AKB48, 5F-AKB48, MDMB-CHMINACA, APICA and STS-135 N4OH pentyl. Results for all of these drugs show values below acceptable ranges for recovery and/or process efficiency, with the exception of STS-135 N4OH pentyl which shows high levels of ion enhancement and therefore process efficiency. Caution should be taken when reporting negative results for the former compounds as their presence may be masked by ion suppression or poor recovery. The instrumental response for these compounds should be examined closely and that for the lowest calibrator should be multiplied by the process efficiency to determine whether low concentrations of the drug could be identified as positive in samples, taking sample condition into account.

Similar caution should be taken when reporting quantitative results for STS-135 N4OH pentyl: while ion enhancement will not cause false positives in negative samples, it will affect the accuracy of quantitative results.

These factors should be considered in determining whether the method is fit-for-purpose for these drugs and whether they are included in the overall method at all.

While further optimisation may allow for improvements in the recovery, matrix effects and, consequently, process efficiency, the extraction protocol is always going to be a compromise between all the analytes included. For this reason, the compounds that are known or suspected to be more common, such as MDMB-CHMICA, 5F-MDMB-PINACA, AB-FUBINACA, 5F-PB-22, and their metabolites

have been prioritised over others. 5F-AKB48 is also high on the priority list however the recovery is not critically low for this compound, and the instrumental response is relatively high (see Figure 26).

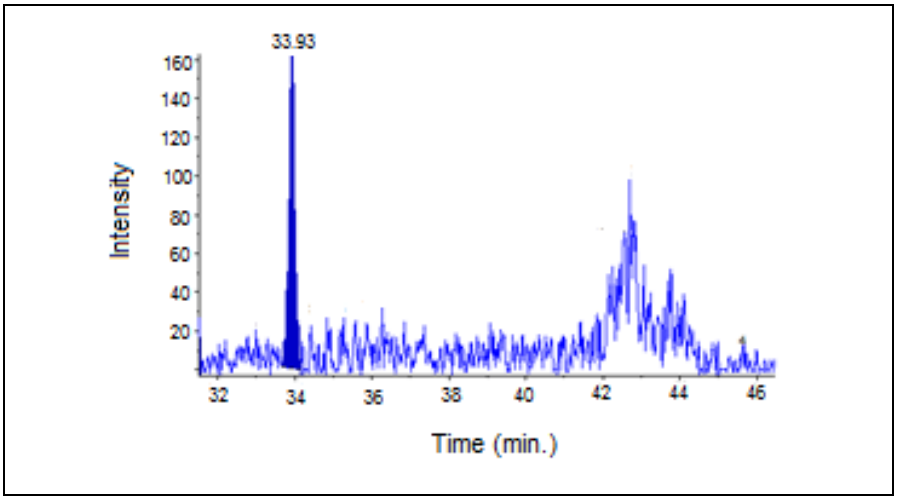


Figure 26 – Extracted ion chromatogram of 5F-AKB48 at 50% limit of detection (0.05 ng/mL) showing a recovery of 45% would still allow a concentration of the limit of detection (0.10 ng/mL) to be clearly seen above background noise. Intensity is given in counts per second

It can be seen that the panel of compounds used in the validated method contains additional analytes than were included in the optimisation experiments. This is due to the addition of these compounds latterly. It was therefore unknown whether the final extraction parameters are optimal for these compounds; however the performance of the extraction was determined for these during validation.

4.4.3.2. Extraction from Urine

Table 21 provides a summary of the optimum extraction conditions: experiment number 13 from Table 13. The results from the experiments conducted into extraction optimisation for urine in Section 4.3.4.2 are shown in Table 22 and Figure 27. The green boxes show the optimum results for recovery, process efficiency and matrix effects.

Table 21 – Optimised protocol for the extraction of selected Synthetic Cannabinoid Receptor Agonists from urine

Parameter	Conditions
Volume of urine (mL)	0.5
Buffer type	No buffer
Volume of buffer (mL)	
Extraction solvent	MeOH
Volume of solvent (mL)	2
Mixing time (min.)	5

Figure 27 shows clearly the improvement of the process efficiency between the original and optimised extraction protocols.

Table 22 – Recovery, matrix effects and process efficiency of original and optimised protocols for the extraction of selected Synthetic Cannabinoid Receptor Agonists from urine

Analyte	Original			Optimised		
	REC (%)	PE (%)	ME (%)	REC (%)	PE (%)	ME (%)
AB-CHMINACA	13	15	116	93	112	120
AB-CHMINACA M1A	9	12	130	98	68	70
AB-CHMINACA M2	13	12	91	77	88	114
AB-FUBINACA	11	17	149	99	114	115
AB-FUBINACA M2B	0	0	113	93	85	92
AB-FUBINACA Valine Metabolite	12	13	107	86	97	112
MMB-FUBINACA	13	13	100	79	62	78
AKB48	13	4	36	26	23	88
AKB48 N5OH Pentyl	14	13	91	77	72	94
AKB48 N-Pentanoic Acid	14	13	93	78	69	88
5F-AKB48	13	9	69	56	50	89
5F-AKB48 N4OH Pentyl	15	14	91	81	71	88
PB-22	1	1	90	66	44	66
PB-22 N5OH Pentyl	0	0	68	95	66	70
PB-22 N-Pentanoic Acid	8	6	73	98	78	79
5F-PB-22	0	0	96	76	53	70
5F-NPB-22	0	0	89	0	0	67
MDMB-CHMICA	15	12	79	71	72	101
MDMB-CHMICA O-desmethyl Acid	13	12	89	77	89	116
MDMB-CHMINACA	13	9	68	59	53	90
BB-22	1	0	74	65	43	66
BB-22 3-Carboxy Indole	17	16	95	85	63	74
AM2201	14	13	91	65	47	72
AM2201 N4OH Pentyl	13	13	95	84	55	66
AB-PINACA	14	17	126	91	113	124
AB-PINACA N4OH pentyl	10	13	138	102	80	78
5F-AB-PINACA	13	14	108	102	152	149
5F-MDMB-PINACA	14	14	104	78	63	81
5F-MDMB-PINACA O-desmethyl Acid	10	12	121	86	109	126
APICA	11	6	51	51	38	74
APICA N4OH Pentyl	14	15	108	80	71	89
STS-135	16	12	76	74	52	70
STS-135 N4OH Pentyl	14	13	94	78	70	90
MMB2201	14	15	107	94	80	85
MAM2201 N4OH Pentyl	14	13	93	96	66	69

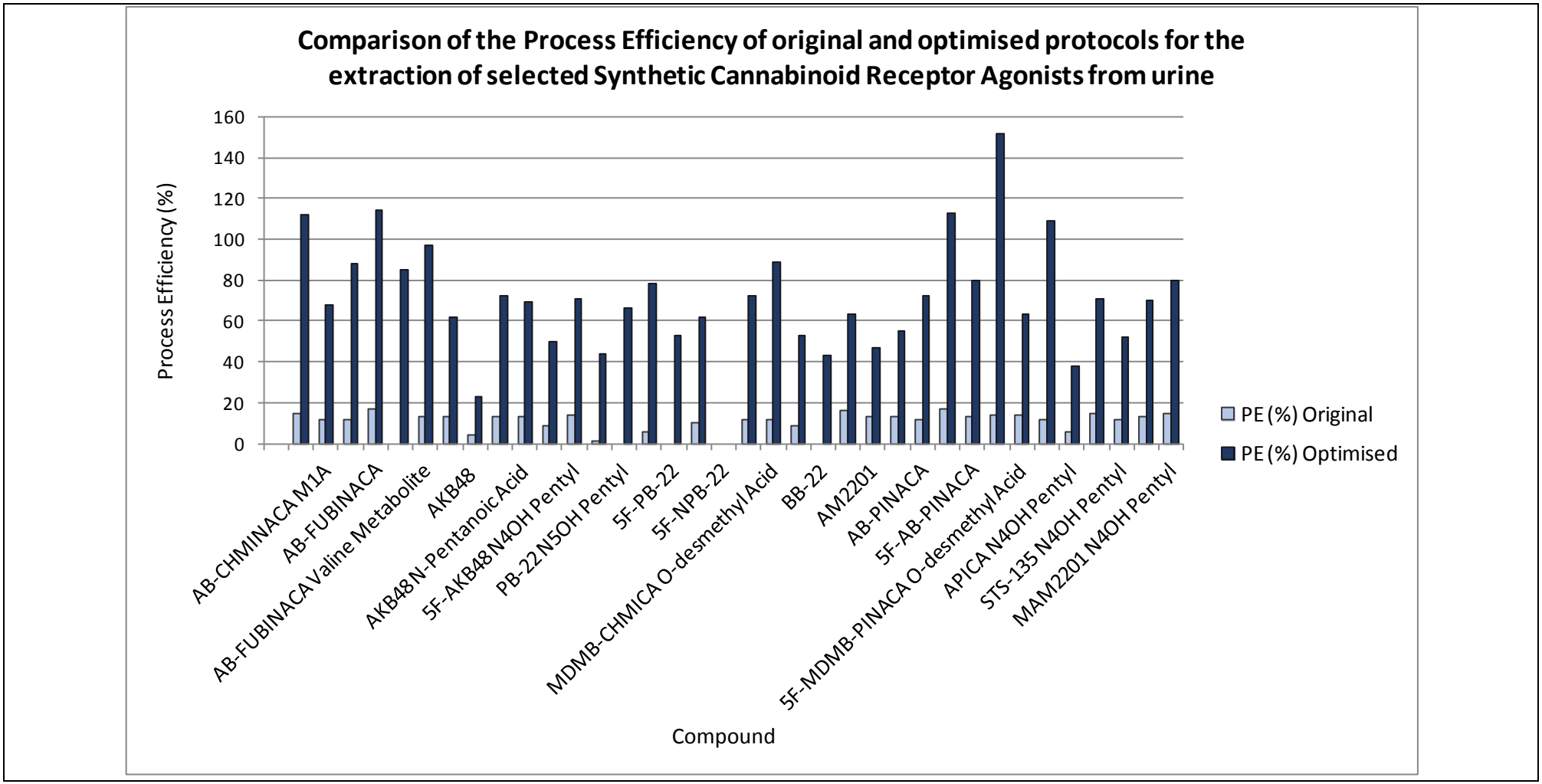


Figure 27 – Bar chart showing the comparison of the process efficiencies of the original and optimised protocols for the extraction of selected Synthetic Cannabinoid Receptor Agonists from urine.

While some of the results for the ME are better in the original protocol, the results for the recovery are significantly improved for all analytes with the exception of 5F-NPB-22 by using the optimised method. Overall, in the optimised method, 31, 24 and 17 compounds are in the preferred range for recovery, PE and ME respectively. The corresponding numbers for acceptable results are 3, 5 and 13 compounds for recovery, PE and ME. Only 2 compounds are outwith the acceptable range for recovery; 7 for PE and 6 for ME.

The compounds showing unacceptable results are AKB48, 5F-NPB-22 and APICA which show poor recovery and PE. 5F-AB-PINACA shows an unacceptably high degree of ME and, consequently, PE. While AKB48 is a high priority compound, based on the number of positive case samples observed, this and the other compounds giving unacceptable results are all parent molecules and are therefore unlikely to be encountered in authentic urine samples. As noted above, the optimisation process is a compromise to obtain the best results for the highest number of compounds and the conditions given in Table 21 were thought to provide sufficiently good results overall.

A more in-depth assessment of recovery, PE and ME will be conducted in the method validation.

The results for the experiments conducted into urine hydrolysis are given in Table 23. Numbers 1 – 6 in this table refer to the number of experiment as detailed in Table 14. Conditions for experiment number 4 were determined to be the optimum and were included in the optimised method as applied to urine samples. From Table 23 it is clear that experiment 4 did not give highest PE values for all compounds, however it should be borne in mind that experiments 5 and 6 did not include any β-glucuronidase enzyme. As a result, these conditions would not bring about hydrolysis if glucuronidated forms of the metabolites were present in genuine samples. Similarly, experiments 1, 3 and 5 were all conducted at RT, meaning that the β-glucuronidase enzyme would be unlikely to act effectively in these conditions. The experiments were intended to provide more information on how the presence of buffer, enzyme and heat affected the experimental results in terms of PE and compound stability. The highest result in Table 23, therefore, does not necessarily mean the best experimental conditions. It was accepted that β-glucuronidase would be required to actively hydrolyse conjugated compounds, and, in the absence of any compound loss through heating, that higher than

ambient temperatures would be required for this. It is clear then, from the results of these experiments, that the presence of the buffer used was detrimental to the PE. Therefore conditions detailed in experiment 4 were taken forward.

Table 23 – Results of the experiments into the hydrolysis of selected Synthetic Cannabinoid Receptor Agonists in urine. The conditions in experiment 4 were taken forward to induce satisfactory hydrolysis with acceptable process efficiency.

Analyte	Process Efficiency					
	1	2	3	4	5	6
AB-CHMINACA	63	57	101	93	86	79
AB-CHMINACA M1A	50	50	94	93	77	83
AB-CHMINACA M2	40	32	52	46	43	43
AB-FUBINACA	63	60	103	307	90	90
AB-FUBINACA M2B	0	0	0	0	0	0
AB-FUBINACA valine metabolite	25	18	36	30	21	22
MMB-FUBINACA	75	59	92	86	96	86
AKB48	28	8	16	6	40	11
AKB48 N5OH pentyl	61	51	85	82	83	78
AKB48 N-pentanoic Acid	52	47	72	66	68	71
5F-AKB48	57	22	42	30	77	44
5F-AKB48 N4OH pentyl	62	54	92	87	85	80
PB-22	65	39	50	49	85	68
PB-22 N5OH pentyl	60	58	81	79	83	81
PB-22 N-pentanoic Acid	27	19	31	24	21	31
5F-PB-22	68	57	72	74	90	82
5F-NPB-22	66	42	0	0	79	27
MDMB-CHMICA	60	36	63	52	83	67
MDMB-CHMICA *O*-desmethyl Acid	41	35	55	46	43	43
MDMB-CHMINACA	60	24	48	34	82	49
BB-22	53	26	32	31	75	53
BB-22 3-carboxyindole	74	62	90	86	95	75
AM2201	63	42	70	63	84	71
AM2201 N4OH pentyl	58	54	71	69	79	78
AM2201 N5OH indole	57	53	82	79	82	77
AB-PINACA	64	64	128	126	91	89
AB-PINACA N4OH pentyl	66	74	94	96	109	115
5F-AB-PINACA	62	60	122	117	85	94
5F-MDMB-PINACA	70	56	84	80	89	81
5F-MDMB-PINACA *O*-desmethyl Acid	27	19	45	36	24	22
APICA	42	15	23	16	60	30
APICA N4OH pentyl	58	51	96	92	84	82
STS-135	57	36	54	46	77	62
STS-135 N4OH pentyl	58	53	92	87	82	81
MMB2201	69	64	100	95	90	85
MAM2201 N4OH pentyl	60	55	82	77	80	76

A summary of the parameters used for methods 1.1, 1.2 and 2.1 is given for reference in Table 24. The compound panels for the methods, and the I.S. used for each analyte is given in Table 25.

Table 24 – Summary of extraction, hydrolysis (urine only), and instrumental parameters used in analytical methods applied to Emergency Department, post-mortem, Scottish Prison Service, Forensic Directorate, and Glasgow Drug Court cohorts.

Parameter	Method 1.1	Method 1.2	Method 2.1
Hydrolysis protocol (urine only)	50 µL β-glucuronidase, 60 °C for 1 H	50 µL β-glucuronidase, 60 °C for 1 H	50 µL β-glucuronidase, 60 °C for 1 H (no buffer)
Extraction protocol	0.5 mL blood/urine, 1 mL pH6.0 phosphate buffer, 2 mL *t*BME, *ca*. 30 second vortex mix	0.5 mL blood/urine, 1 mL pH6.0 phosphate buffer, 2 mL *t*BME, *ca*. 30 second vortex mix	0.5 mL blood, 0.5 mL pH6.0 phosphate buffer, 1 mL *t*BME, 2 min. flatbed mix 0.5 mL urine, 2 mL MeOH, 5 min. flatbed mix
MP Gradient A = dH2O* B= MeOH* C=ACN:dH2O (95:5)* *with 2mM ammonium acetate and 0.1% formic acid	F 0-5 min: 40 % A, 60% B 5-10 min: ramped to 10% A, 90% B 10-20 min: 10% A, 90% B 20-20.1 min: ramped to 40% A, 60% B 20.1-25 min:40% A, 60% B	H 0-5 min: 40% A, 60% B 5-8.5 min: ramped to 20% A, 80% B 8.5-18 min: ramped to 10% A, 90% B 18-20 min: 10% A, 90% B 20-20.1 min: ramped to 40% A, 60% B 20.1-25 min: 40% A, 60% B	R 0.-4 min: 60% A, 40% C 4-14 min: ramped to 40% A, 60% C 14-28 min: 40% A, 60% C 28-28.1 min: ramped to 20% A, 80% C 28.1-40 min: 20% A, 80% C 40-40.1 min: ramped to 60% A, 40% C 40.1-45 min: 60% A, 40% C

Table 25 – Compound panels and internal standards used for Methods 1.1, 1.2 and 2.1.

Compound	Method 1.1	Method 1.2	Method 2.1	I.S.
AB-CHMINACA	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-CHMINACA M1A	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-CHMINACA M2	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-FUBINACA	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-FUBINACA M2B	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-FUBINACA valine metabolite	Yes	Yes	Yes	AB-FUBINACA-d_4
AB-PINACA	No	Yes	Yes	AB-FUBINACA-d_4
AB-PINACA N4OH pentyl	No	No	Yes	AB-FUBINACA-d_4
ADB-FUBINACA	No	No	Yes	AB-FUBINACA-d_4
5F-AB-PINACA	No	Yes	Yes	AB-FUBINACA-d_4
5F-ADB-PINACA	No	No	Yes	AB-FUBINACA-d_4
AKB48	Yes	Yes	Yes	AKB48-d_{11}
AKB48 N5OH pentyl	Yes	Yes	Yes	AKB48 N5OH pentyl-d_4
AKB48 N-pentanoic acid	Yes	Yes	Yes	AKB48 N5OH pentyl-d_4
5F-AKB48	Yes	Yes	Yes	AKB48 N5OH pentyl-d_4
5F-AKB48 N4OH pentyl	Yes	Yes	Yes	AKB48 N5OH pentyl-d_4
AM-2201	No	Yes	Yes	AM2201-d_5
AM-2201 N4OH pentyl	No	Yes	Yes	AM2201-d_5
AM-2201 N5OH indole	No	Yes	Yes	AM2201-d_5
APICA	No	Yes	No	AKB48-d_{11}
APICA N4OH pentyl	No	Yes	Yes	AKB48 N5OH pentyl-d_4
FUB-PB-22	No	No	Yes	PB-22-d_9
BB-22	Yes	Yes	Yes	PB-22-d_9
BB-22 3-carboxy indole	Yes	Yes	Yes	PB-22-d_9
CUMYL-PeGACLONE	No	No	Yes	AB-FUBINACA-d_4
MAM-2201 N4OH pentyl	No	No	Yes	AM2201-d_5
MAB-CHMINACA	No	No	Yes	AB-FUBINACA-d_4
MAB-CHMINACA M1	No	No	Yes	AB-FUBINACA-d_4
MDMB-CHMICA	Yes	Yes	Yes	AB-FUBINACA-d_4
MDMB-CHMICA *O*-desmethyl acid metabolite	No	Yes	Yes	AB-FUBINACA-d_4
MDMB-CHMINACA	Yes	Yes	Yes	AB-FUBINACA-d_4
5F-MDMB-PINACA	No	Yes	Yes	AB-FUBINACA-d_4
5F-MDMB-PINACA *O*-desmethyl acid	No	No	Yes	AB-FUBINACA-d_4
MMB2201	No	No	Yes	AM2201-d_5
MMB-CHMICA	No	No	Yes	AB-FUBINACA-d_4
MMB-FUBINACA	No	No	Yes	AB-FUBINACA-d_4
5F-NPB-22	No	No	Yes	PB-22-d_9
PB-22	Yes	Yes	Yes	PB-22-d_9
PB-22 N5OH pentyl	Yes	Yes	Yes	PB-22-d_9

Table 25 – Compound panels and internal standards used for Methods 1.1, 1.2 and 2.1.

Compound	Method 1.1	Method 1.2	Method 2.1	I.S.
PB-22 N-pentanoic acid	Yes	Yes	Yes	PB-22-d_9
PB-22 N-pentanoic acid 3-carboxyindole	Yes	Yes	No	PB-22-d_9
5F-PB-22	Yes	Yes	Yes	PB-22-d_9
STS-135	No	Yes	No	AKB48-d_{11}
STS-135 N4OH pentyl	No	Yes	Yes	AKB48 N5OH pentyl-d_4
AB-FUBINACA-d_4	Yes	Yes	Yes	N/A
PB-22-d_9	Yes	Yes	Yes	N/A
AM2201-d_5	No	No	Yes	N/A
AKB48-d_{11}	No	No	Yes	N/A
AKB48 N5OH pentyl-d_4	No	No	Yes	N/A

4.4.4. Method Validation – Method 2.1 applied to blood

Due to the intended nature of the method and number of compounds included in its panel, it was decided to conduct a qualitative validation for all compounds with quantitative validation undertaken those compounds thought to be most likely encountered. Parameters relating to quantitation – linearity, accuracy and precision – were therefore not validated for all compounds.

4.4.4.1. Linearity

A linear calibration model using 1/χ-weighting was established for 26 compounds where quantitative validation was felt necessary, as demonstrated by correlation co-efficient values of ≥0.99 over 10 calibrations. The minimum values are given in Table 26. For all calibrations, 7 calibrators were used and the calculated concentrations for at least 6 of these were within ±20% of the expected value. Those calibration points outside ±20% were removed from the calibration. An example calibration curve for 5F-MDMB-PINACA *O*-desmethyl acid metabolite is shown in Figure 28.

Table 26 – Linearity of compounds selected for quantitative validation. All gave satisfactory correlation coefficients of ≥0.99.

Compound	Minimum R (n=10)
5F-MDMB-PINACA	0.9921
5F-MDMB-PINACA *O*-desmethyl acid	0.9939
MDMB-CHMICA	0.9919
MDMB-CHMICA *O*-desmethyl acid	0.9950
AB-FUBINACA	0.9995
MMB-FUBINACA	0.9956
AB-FUBINACA valine metabolite	0.9974
5F-PB-22	0.9906
PB-22	0.9959
PB-22 N5OH pentyl	0.9908
5F-AKB48	0.9942
5F-AKB48 N4OH pentyl	0.9939
AKB48	0.9973
AKB48 N5OH pentyl	0.9979
BB-22	0.9925
BB-22 3-carboxyindole	0.9902
AM2201	0.9996
AM2201 N4OH pentyl	0.9913
AB-PINACA	0.9965
AB-PINACA N4OH pentyl	0.9960
5F-AB-PINACA	0.9991
5F-ADB-PINACA	0.9975
MMB2201	0.9938
MAM2201 N4OH pentyl	0.9913
AB-CHMINACA	0.9984
AB-CHMINACA M2	0.9967

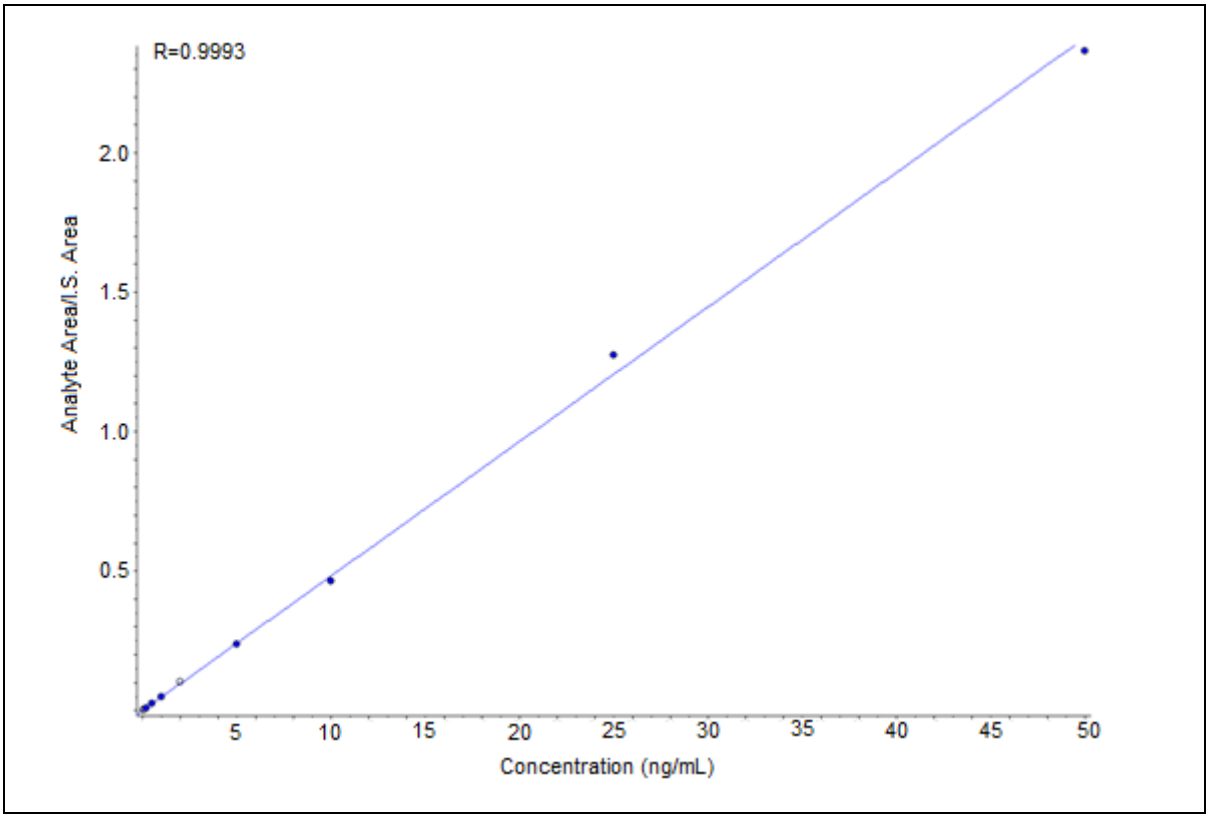


Figure 28 – An example calibration curve for 5F-MDMB-PINACA *O*-desmethyl acid metabolite from Method 2.1, with 1/χ weighting, giving a correlation coefficient of 0.9993. This is representative of 10 calibrations assessed for linearity.

4.4.4.2. Selectivity

Selectivity was demonstrated for all compounds. An example blank XIC for 5F-MDMB-PINACA *O*-desmethyl acid metabolite is given in Figure 29 (top), showing a lack of analyte response (left) and I.S. (right). An example of a low positive case sample (0.13 ng/mL, middle) and a higher positive case sample (7.4 ng/mL, bottom) is also given to demonstrate the difference to a blank sample.

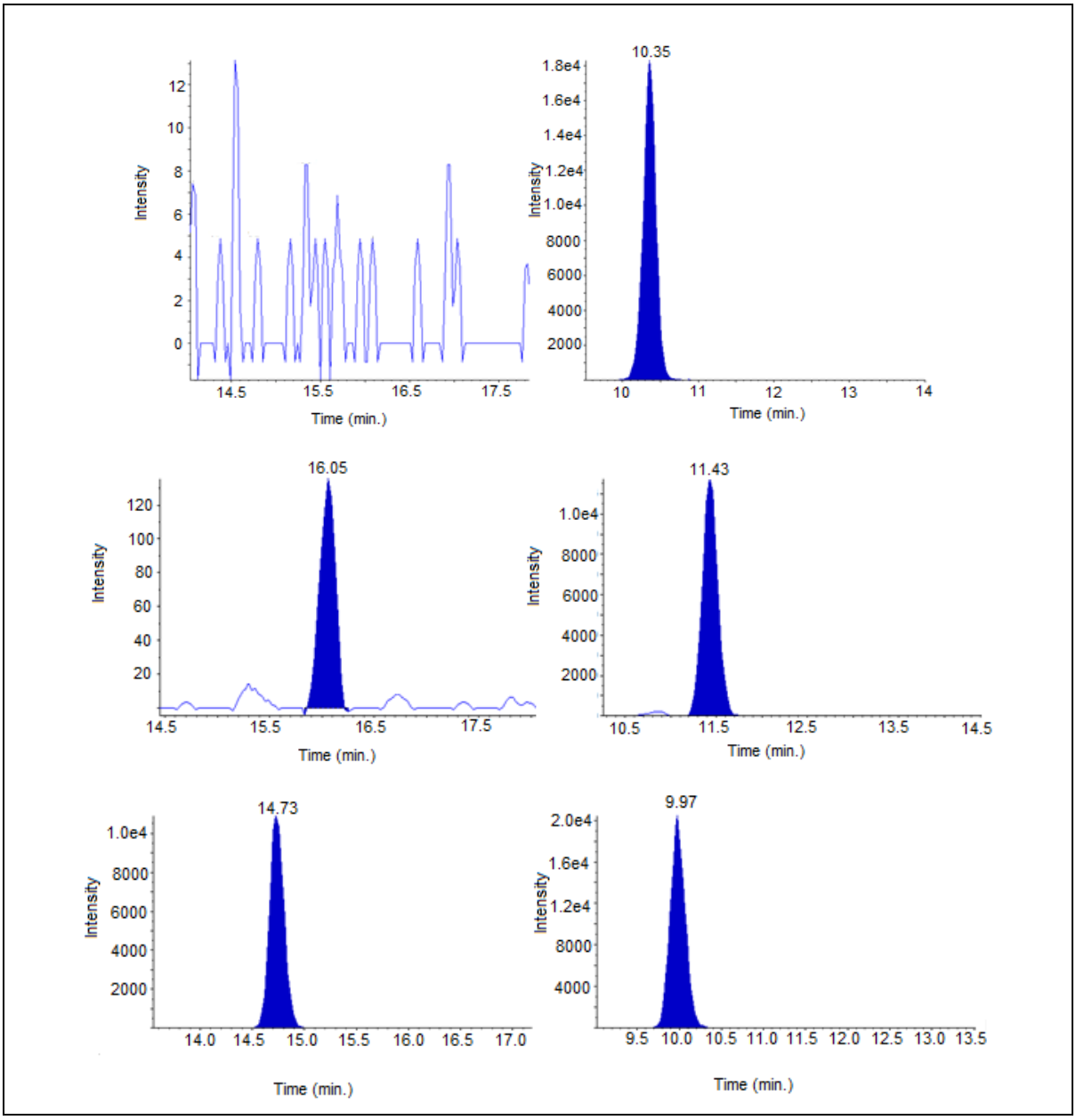


Figure 29 – An example blank (top), low positive case sample (middle) and higher positive case sample (bottom) chromatograms for 5F-MDMB-PINACA *O*-desmethyl acid metabolite (left) with internal standard (right), demonstrating selectivity. Note the internal standard is erroneously referred to as AB-FUBINACA-d_9 in the middle trace: AB-FUBINACA-d_4 was used. The variation in retention time is due to inter-batch variation, and the use of different analytical columns and mobile phase batches. Intensity is given in counts per second.

4.4.4.3. Sensitivity

The LODs and LLOQs for all compounds, determined as described in 4.3.5.3, are given in Table 27. The SNR are given in parenthesis for the LOD and an example of how these were calculated is given in Figure 30.

Table 27 – Limits of Detection and Lower Limits of Quantitation for all Synthetic Cannabinoid Receptor Agonist compounds included in Method 2.1, as applied to blood. The signal-to-noise ratios for the Limits of Detection are given in parenthesis.

Compound	LOD (ng/mL) (SNR)	LLOQ (ng/mL)
5F-MDMB-PINACA	0.02 (8)	0.10
5F-MDMB-PINACA *O*-desmethyl acid	0.05 (5)	0.20
MDMB-CHMICA	0.10 (9)	0.10
MDMB-CHMICA *O*-desmethyl acid	0.20 (10)	0.20
AB-FUBINACA	0.20 (11)	0.20
MMB-FUBINACA	0.02 (8)	0.10
AB-FUBINACA valine metabolite	0.10 (6)	0.20
5F-PB-22	0.01 (7)	0.10
PB-22	0.02 (6)	0.10
PB-22 N5OH pentyl	0.02 (13)	0.20
5F-AKB48	0.10 (5)	0.10
5F-AKB48 N4OH pentyl	0.05 (6)	0.20
AKB48	0.20 (5)	0.20
AKB48 N5OH pentyl	0.10 (7)	0.20
BB-22	0.05 (7)	0.10
BB-22 3-carboxyindole	5.00 (18)	5.00
AM2201	0.01 (6)	0.10
AM2201 N4OH pentyl	0.01 (5)	0.20
AB-PINACA	0.05 (8)	0.10
AB-PINACA N4OH pentyl	0.10 (6)	0.20
5F-AB-PINACA	0.10 (6)	0.10
5F-ADB-PINACA	0.10 (8)	0.10
MMB2201	0.02 (12)	0.10
MAM2201 N4OH pentyl	0.01 (8)	0.20
AB-CHMINACA	0.05 (7)	0.10
AB-CHMINACA M2	0.05 (8)	0.20
5F-NPB-22	0.02 (5)	0.10
AB-CHMINACA M1A	0.20 (4)	0.20
AB-FUBINACA M2B	5.00 (7)*	5.00
AKB48 N-pentanoic acid	0.10 (6)	0.20
APICA N4OH pentyl	0.05 (6)	0.20
FUB-PB-22	0.02 (8)	0.10
MDMB-CHMINACA	0.10 (8)	0.10
PB-22 N-pentanoic acid	0.20 (19)	0.20
STS-135 N4OH pentyl	0.05 (7)	0.20
CUMYL-PeGACLONE	0.05 (12)	0.10
MAB-CHMINACA	0.05 (14)	0.10
MAB-CHMINACA M1	0.10 (5)	0.20
MMB-CHMICA	0.10 (8)	0.10

* 1 source of blank blood for AB-FUBINACA M2B had a SNR <3.

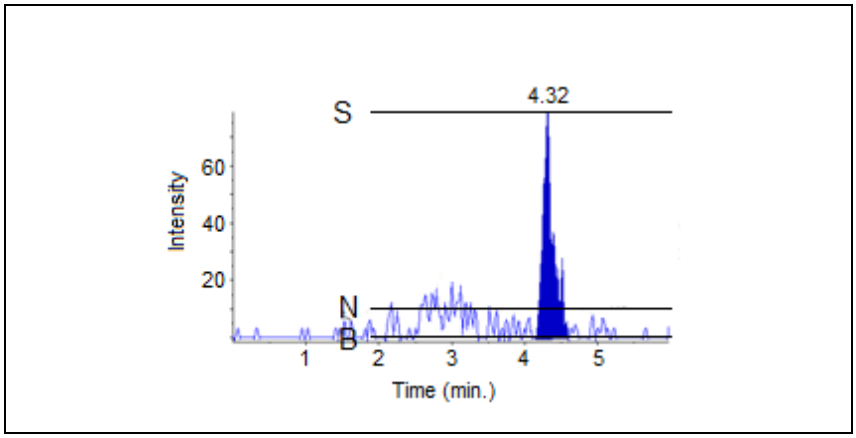


Figure 30 – Example of how the signal to noise ratio of the compounds was calculated. Intensity is given in counts per second.

While additional noise can be observed around 3.00 min. in the baseline in Figure 30, the compound peak would be baseline resolved from this so it was not deemed as an obstacle to accurate detection and thus the noise was measured closer to the retention time of interest.

The LLOQ was set as the lowest calibrator (0.10 or 0.20 ng/mL for parent compounds and metabolites respectively) or the LOD if the SNR of this was ≥4, whichever was higher.

Concentrations of SCRAs found in blood are typically very low due to their potency and the low dose required for effect. It is therefore essential that the method is sufficiently sensitive to detect these low concentrations. The LODs given in Table 27 show good sensitivity for the majority of compounds. LODs for BB-22 3-carboxyindole and AB-FUBINACA M2B are higher than ideal at 5.00 ng/mL. As these are metabolites the concentrations encountered in samples are likely to be higher, however an LOD of 5 ng/mL is too high to be able to say that this method is fit for the purpose of detecting AB-FUBINACA M2B and BB-22 3-carboxyindole. In addition to this, one of the sources of blank blood used to determine the LOD produced a SNR of <3 for AB-FUBINACA M2B.

It should be noted here that the use of whole blood, including plasma, rather than diluted packed red cells, may affect the sensitivity of the method as SCRAs may bind to plasma proteins. Whole blood was used for the assessment of matrix effects, process efficiency and recovery and discussed in Section 4.4.4.5.

4.4.4.4. Accuracy & Precision

Inter- and intraday accuracy and precision were determined for the 27 quantitative compounds. The data are shown in Table 28 and Table 29 for accuracy and precision respectively. All the values were within the criteria detailed in sections 4.3.5.4 and 4.3.5.5 with 1 exception: the interday precision for PB-22 at 0.1 ng/mL was high, with a %CV of 16.9. While this is close to the acceptable criteria of ≤15%, the LLOQ should be amended to 0.2 ng/mL for this compound, as precision and accuracy are within acceptable limits at this concentration and above. An LLOQ of 0.2 ng/mL is still acceptable for the purposes of this method. Data has not been included for BB-22 3-carboxyindole at 0.2 and 2.5 ng/mL as these concentrations are below the LOD for this compound (5 ng/mL).

Taking these exceptions into account, the data show that the method described is sufficiently accurate and precise to allow reporting of analyte concentrations. QC material at suitable concentrations should, however, be included with every batch to ensure continuing fitness-for-purpose.

Table 28 – Intra- and interday accuracy of compounds selected for quantitative validation for Method 2.1 applied to Blood

Compound	Accuracy (%)									
	Intraday (n=5)					Interday (n=5)				
	0.1 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL	0.1 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL
5F-MDMB-PINACA	104.1	86.2	93.5	112.4		96.0	94.0	94.0	92.7	
5F-MDMB-PINACA *O*-desmethyl acid		106.5	113.6		102.2		96.0	103.1		100.2
MDMB-CHMICA	80.0	95.2	107.6	115.6		108.0	97.0	101.0	91.0	
MDMB-CHMICA *O*-desmethyl acid		107.3	108.6		83.8		96.0	96.6		91.7
AB-FUBINACA	103.4	99.7	108.7	110.3		100.0	103.0	98.8	100.5	
MMB-FUBINACA	77.5	84.6	97.9	98.2		84.0	91.0	103.2	94.6	
AB-FUBINACA valine metabolite		109.2	102.2		98.0		101.0	96.8		98.7
5F-PB-22	98.3	88.1	89.9	95.3		94.0	94.0	107.0	103.2	
PB-22	102.4	97.0	108.9	107.1		96.0	96.0	101.9	100.5	
PB-22 N5OH pentyl		93.0	116.6		107.1		94.0	103.5		93.3
5F-AKB48	108.7	97.9	96.5	88.5		100.0	102.0	100.2	90.2	
5F-AKB48 N4OH pentyl		111.2	119.0		104.3		92.0	93.4		90.7
AKB48	90.2	91.2	115.9			90.0	102.0	100.3	97.6	
AKB48 N5OH pentyl		99.2	101.8		100.3		99.0	94.4		95.4
BB-22	112.8	90.9	102.8	102.3		98.0	98.0	102.1	101.9	
BB-22 3-carboxyindole		N/A	N/A		101.4		N/A	N/A		113.7
AM2201	99.0	89.2	97.5	97.6		98.0	104.0	102.0	99.5	
AM2201 N4OH pentyl		109.1	100.4		112.2		91.0	100.2		98.2
AB-PINACA	99.9	103.7	100.8	103.2		94.0	101.1	101.7	100.3	
AB-PINACA N4OH pentyl		87.5	103.3		103		98.0	98.7		98.0
5F-AB-PINACA	107.4	106.3	96.5	104.5		102.0	100.0	98.3	98.4	
5F-ADB-PINACA	104.6	110	112.5	114.8		98.0	104.0	104.0	106.9	
MMB2201	91	97.9	92.4	115.3		90.0	98.0	107.8	98.0	
MAM2201 N4OH pentyl		84.2	109.1		108.3		88.0	103.2		101.6
AB-CHMINACA	108.5	106.6	98	98.8		96.0	99.0	103.4	100.2	
AB-CHMINACA M2		109.6	102.4		87.1		110.0	98.0		92.8

'N/A' indicates the concentration is <LOD.

Table 29 – Intra- and interday precision of compounds selected for quantitative validation for Method 2.1 applied to Blood

Compound	Precision									
	Intraday (n=5)					Interday (n=5)				
	0.1 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL	0.1 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL
5F-MDMB-PINACA	1.7	2.6	3.7	4.4		10.6	7.9	3.0	9.5	
5F-MDMB-PINACA *O*-desmethyl acid		3.8	2.3		0.9		12.9	5.6		2.3
MDMB-CHMICA	8.5	4.2	6.5	3.5		10.8	7.0	2.6	10.0	
MDMB-CHMICA *O*-desmethyl acid		9.5	1.9		7.9		12.5	7.2		8.2
AB-FUBINACA	8.0	12.6	1.8	1.7		12.6	6.6	3.0	4.0	
MMB-FUBINACA	8.0	13.1	3.8	1.7		9.5	8.8	2.2	4.1	
AB-FUBINACA valine metabolite		9.7	3.8		2.9		6.6	3.5		3.9
5F-PB-22	2.2	7.2	12.8	10.6		10.8	11.4	7.9	5.8	
PB-22	3.5	7.9	3.3	4.1		16.9	6.9	6.7	3.4	
PB-22 N5OH pentyl		4.8	8.0		6.2		5.2	7.0		10.0
5F-AKB48	11.8	13.5	10	6.2		8.9	11.4	10.7	5.0	
5F-AKB48 N4OH pentyl		5.2	0.7		2.8		8.8	9.6		10.4
AKB48	13.8	8.2	4.3			9.9	7.3	5.1	3.6	
AKB48 N5OH pentyl		3.7	2.7		2.0		3.8	6.0		4.9
BB-22	2.5	9.8	6.2	2.2		14.9	6.1	5.6	9.0	
BB-22 3-carboxyindole		N/A	N/A		3.3		N/A	N/A		11.9
AM2201	4.4	3.6	1.5	1.6		10.0	5.6	3.1	6.2	
AM2201 N4OH pentyl		3.3	7.3		2.1		6.4	6.3		11.4
AB-PINACA	8.4	3.6	5	3.1		10.8	13.8	5.2	2.0	
AB-PINACA N4OH pentyl		7.9	4.9		4.7		6.1	3.7		6.5
5F-AB-PINACA	14.2	8.7	7.2	2.7		14.4	7.1	5.0	6.9	
5F-ADB-PINACA	10.1	8.8	4.9	2.6		13.5	9.8	9.4	6.3	
MMB2201	10.1	2.9	13.8	6.7		12.2	2.5	4.7	8.1	
MAM2201 N4OH pentyl		6.15	5.7		2.8		8.5	7.3		11.2
AB-CHMINACA	3.9	6.9	4.1	3.6		14.0	5.9	4.0	6.3	
AB-CHMINACA M2		10.2	7.2		4.0		9.5	12.6		8.1

'N/A' indicates the concentration is <LOD.

4.4.4.5. Recovery and Matrix Effects

The results for the recovery and ME experiments are given in Table 30 and Table 31 for 2.5 ng/mL and 15 ng/mL respectively. The term ‘absolute’ refers to the values obtained from using the peak areas in the calculation, whereas ‘I.S. compensated’ refers to the use of the peak area ratios. For these experiments, blank whole blood was used, rather than diluted packed red cells. The inclusion of components such as plasma here provides a relatively realistic account of the variation in samples this method was applied to.

As expected, given their structural diversity, the results of the recovery and ME experiments are varied. Absolute recoveries are low for 5F-MDMB-PINACA *O*-desmethyl metabolite, MDMB-CHMICA *O*-desmethyl metabolite, AB-FUBINACA valine metabolite, 5F-AKB48, AKB48, AB-CHMINACA M2, AB-FUBINACA M2B, AKB48 N-pentanoic acid, MDMB-CHMINACA and PB-22 N-pentanoic acid. These are improved to reasonable values when the I.S. is taken into account as a compensation for 5F-AKB48, AKB48, AB-CHMINACA M2, AKB48 N-pentanoic acid, BB-22, MDMB-CHMINACA and PB-22 N-pentanoic acid. AB-FUBINACA M2B is not recovered to any significant degree by the extraction employed in this method, leading to the high LOD exhibited in Table 27. As a result, analysis of this analyte using the proposed method does not meet acceptable criteria for quality.

As demonstrated by the LODs and accuracy and precision values for the remaining compounds with sub-optimal recoveries, the - albeit low - recovery is sufficient to allow adequate and reproducible sensitivity and quantitation as required by the nature of the analytes, *i.e.* differing concentrations of interest for parent and metabolite compounds.

Recoveries above 100% were observed for MDMB-CHMICA, PB-22, 5F-AKB48, BB-22, AM2201, MMB2201, 5F-NPB-22, APICA N4OH pentyl, FUB-PB-22, MDMB-CHMINACA, STS-135 N4OH pentyl, and CUMYL-PeGACLONE. Some instances of this could be due to the random error between different injections and indicate an almost complete recovery. Where the recovery is significantly over 100%, this may indicate retention and build up on the analytical column. The impact of this on quantitation could be monitored by injecting the QCs before and after samples, and ensuring resulting concentrations are consistent.

For many of the compounds including MDMB-CHMICA, 5F-PB-22, PB-22 N5OH pentyl, 5F-AKB48, BB-22, AM2201 N4OH pentyl, MMB2201, MAM2201 N4OH pentyl, 5F-NPB-22, APICA N4OH pentyl, MDMB-CHMINACA, STS-135 N4OH pentyl, CUMYL-PeGACLONE and MAB-CHMINACA, the recovery is significantly greater than 100% for the I.S. compensated calculation. The method used a relatively low number of I.S. for the number of analytes included, due largely to the limited number of deuterated forms of SCRAs available and the prohibitive cost of these. It is therefore possible that some of the I.S. do not behave in a sufficiently similar way chemically to the compound. As a result, variation between the peak areas of the compounds and the I.S. in different standards may be exhibited, leading to variations in the PAR. This may be the case where the recovery for the I.S. compensated ME values is significantly over 100%.

The ME results are equally variable, with both significant inhibition and significant enhancement observed. Significant inhibition was demonstrated by MDMB-CHMICA, PB-22, 5F-AKB48, AKB48, BB-22, AM2201, MDMB-CHMINACA and CUMYL-PeGACLONE when the absolute values are interrogated. All of these compounds, with the exception of AM2201, are eluted towards the end of the run time. This is indicative of a build-up of sample artifact on the column causing a decrease in the analyte signal, and may be improved by further development of the MP gradient or extraction process. The ion suppression observed for AKB48, PB-22 and AM2201 is markedly improved when the I.S. compensated results are considered as deuterated forms of these compounds are used. The use of alternative I.S. for the other affected compounds could, therefore, be examined in future work.

On the other hand, 5F-MDMB-PINACA *O*-desmethyl metabolite, AB-FUBINACA, 5F-AKB48 N4OH pentyl, AB-PINACA, AB-PINACA N4OH pentyl, 5F-ADB-PINACA, MMB2201, AB-CHMINACA, AB-CHMINACA M1A, APICA N4OH pentyl, PB-22 N-pentanoic acid, STS-135 N4OH pentyl, MAB-CHMINACA M1 and MMB-CHMICA have significantly enhanced signals in the absolute values. These effects are mitigated by I.S. use for all the compounds except AB-PINACA N4OH pentyl, 5F-ADB-PINACA, MMB2201, APICA N4OH pentyl, PB-22 N-pentanoic acid, STS-135 N4OH pentyl and MMB-CHMICA. However, for 5F-PB-22, PB-22 N5OH pentyl, BB-22 3-carboxyindole, AM2201 N4OH pentyl, MMB2201, MAM2201 N4OH pentyl, 5F-NPB-22, FUB-PB-22 and PB-22 N-pentanoic acid, the use of the I.S. makes ion enhancement more pronounced.

As well as looking at either ion suppression or enhancement, the general range of the ME values gave an indication of how variable these are for different sources of blank blood. As blood samples can vary drastically in condition, particularly post-mortem samples, a great deal of care should be taken when interpreting the results of this analysis, both in terms of the calculated concentration and the presence or absence of analyte. The inclusion of a blank run, or wash method in between samples was not conducted here, but could be investigated to clean the column and improve variation and extent of ME. The results from the accuracy and precision validation, however, do provide confidence in the ability of this method to determine the presence and quantity of SCRAs in a sample.

Table 30 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 2.5 ng/mL for Method 2.1 applied to blood.

Compound	2.5 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
5F-MDMB-PINACA[1]	64	96	71	117	83	115	67	109
5F-MDMB-PINACA *O*-desmethyl acid[1]	31	65	102	190	38	81	96	133
MDMB-CHMICA[1]	45	120	29	102	60	143	22	93
MDMB-CHMICA *O*-desmethyl acid[1]	23	43	62	93	30	109	40	93
AB-FUBINACA[1]	69	87	98	138	94	105	94	102
MMB-FUBINACA[1]	61	94	70	113	75	110	55	103
AB-FUBINACA valine metabolite[1]	21	54	91	117	25	69	82	100
5F-PB-22[2]	62	98	53	98	84	133	97	208
PB-22[2]	53	117	25	102	78	101	90	120
PB-22 N5OH pentyl[2]	74	89	101	137	69	134	108	494
5F-AKB48[3]	34	130	9	113	45	146	10	106
5F-AKB48 N4OH pentyl*[3]	63	96	69	147	85	120	87	116
AKB48[4]	19	94	4	91	92	120	85	96
AKB48 N5OH pentyl[3]	63	96	69	112	91	108	77	95
BB-22[2]	38	128	11	103	65	181	33	121
BB-22 3-carboxyindole[2]	40	80	45	105	61	125	99	163
AM2201*[5]	50	124	36	107	96	103	89	98
AM2201 N4OH pentyl[5]	72	89	94	124	71	159	95	279
AB-PINACA[1]	71	89	73	135	89	108	74	100
AB-PINACA N4OH pentyl[1]	58	68	105	242	70	87	105	187
5F-AB-PINACA[1]	69	84	90	149	93	101	89	106

* denotes where only 5 sources of blank blood were used due to retention time shift for compounds [1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 30 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 2.5 ng/mL for Method 2.1 applied to blood.

Compound	2.5 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
5F-ADB-PINACA[1]	69	83	104	206	90	104	103	145
MMB2201[5]	73	105	94	163	68	166	106	363
MAM2201 N4OH pentyl*[5]	70	89	101	125	71	135	97	257
AB-CHMINACA[1]	68	86	83	144	86	100	84	132
AB-CHMINACA M2[1]	37	70	70	96	45	83	49	87
5F-NPB-22[2]	71	102	67	116	88	128	105	267
AB-CHMINACA M1A[1]	53	72	93	142	73	93	93	109
AB-FUBINACA M2B[1]	0	3	0	98	0	4	68	96
AKB48 N-pentanoic acid[3]	37	75	78	138	58	111	80	124
APICA N4OH pentyl[3]	59	133	66	178	67	150	49	181
FUB-PB-22[2]	56	112	31	102	89	111	102	157
MDMB-CHMINACA[1]	34	125	13	112	46	148	10	102
PB-22 N-pentanoic acid[2]	32	54	89	155	44	87	96	388
STS-135 N4OH pentyl[3]	58	120	80	157	75	136	59	170
CUMYL-PeGACLONE[1]	52	119	26	103	66	197	12	94
MAB-CHMINACA[1]	57	108	61	124	75	128	52	117
MAB-CHMINACA M1[1]	66	81	99	178	85	102	98	127
MMB-CHMICA[1]	63	92	64	203	80	109	45	192

* denotes where only 5 sources of blank blood were used due to retention time shift for compounds [1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 31 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 15 ng/mL for Method 2.1 applied to blood

Compound	15 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
5F-MDMB-PINACA[1]	63	96	80	122	80	110	73	108
5F-MDMB-PINACA *O*-desmethyl acid[1]	31	65	101	173	38	78	98	123
MDMB-CHMICA[1]	41	123	32	113	55	135	26	93
MDMB-CHMICA *O*-desmethyl acid[1]	25	58	73	110	30	67	51	96
AB-FUBINACA[1]	70	86	100	134	93	105	94	104
MMB-FUBINACA[1]	59	101	76	120	75	115	57	112
AB-FUBINACA valine metabolite[1]	19	56	97	116	23	67	82	100
5F-PB-22[2]	62	98	65	107	84	141	101	209
PB-22[2]	46	118	30	111	88	101	91	105
PB-22 N5OH pentyl[2]	69	90	102	124	77	153	108	397
5F-AKB48[3]	33	138	10	114	46	148	10	95
5F-AKB48 N4OH pentyl[3]	64	91	81	142	91	113	82	106
AKB48[4]	16	119	5	99	90	101	91	100
AKB48 N5OH pentyl*[3]	66	91	81	116	92	102	84	96
BB-22[2]	37	114	12	104	68	123	37	97
BB-22 3-carboxyindole[2]	57	88	48	104	75	110	97	160
AM2201*[5]	44	117	42	113	93	97	93	99
AM2201 N4OH pentyl[5]	68	91	101	131	75	164	100	253
AB-PINACA[1]	68	90	78	135	84	103	76	115
AB-PINACA N4OH pentyl[1]	55	68	106	226	69	82	103	180
5F-AB-PINACA[1]	72	89	81	146	94	110	79	108

* denotes where only 5 sources of blank blood were used due to retention time shift for compounds [1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 31 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 15 ng/mL for Method 2.1 applied to blood

Compound	15 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
5F-ADB-PINACA[1]	72	88	105	185	94	102	101	139
MMB2201[5]	71	91	107	155	76	172	108	316
MAM2201 N4OH pentyl*[5]	65	92	104	124	77	142	104	253
AB-CHMINACA[1]	69	86	96	146	87	99	87	128
AB-CHMINACA M2[1]	36	74	77	111	60	84	55	97
5F-NPB-22[2]	74	108	71	116	92	147	102	237
AB-CHMINACA M1A[1]	53	72	96	145	70	87	90	115
AB-FUBINACA M2B[1]	1	6	93	105	1	7	69	95
AKB48 N-pentanoic acid[3]	46	74	91	143	66	116	74	117
APICA N4OH pentyl[3]	53	122	74	178	67	150	49	181
FUB-PB-22[2]	54	109	40	108	93	108	99	150
MDMB-CHMINACA[1]	33	138	15	120	43	158	12	99
PB-22 N-pentanoic acid[2]	36	66	92	144	56	100	104	345
STS-135 N4OH pentyl[3]	55	110	90	154	92	135	58	142
CUMYL-PeGACLONE[1]	49	126	28	106	62	144	22	94
MAB-CHMINACA[1]	50	107	77	135	63	122	60	116
MAB-CHMINACA M1[1]	63	78	99	177	83	93	97	130
MMB-CHMICA[1]	64	93	67	198	80	106	47	184

* denotes where only 5 sources of blank blood were used due to retention time shift for compounds [1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

4.4.4.6. Interference Testing

None of the compounds tested produced any responses in the XICs of compounds of interest at a concentration of 1 mg/L.

4.4.4.7. Autosampler Stability

The results of the experiments into autosampler stability over *ca*. 46 hours fell roughly into 3 categories: upwards trend, stable/downward trend and no discernible trend. No significant differences were observed between experiments conducted at 2.5 and 15 ng/mL.

The plot in Figure 31 for 5F-MDMB-PINACA is an example of an upwards trend and the majority of compounds exhibited this. An upwards trend was diagnosed by observing a sustained increase in peak areas which reached ≥120% of the t_0 value and did not return to an area below this within the duration of the experiment. For all the compounds in which this trend was observed, the I.S. compensated for these effects, bringing the change much closer to within 100 ±20%, although sometimes still *ca.* 5 percentage points outside this range. The plots for 5F-ADB-PINACA, AB-PINACA, AB-PINACA N4OH pentyl, AM2201 OH, MAB-CHMINACA and MAM-2201 N4OH pentyl still showed instability in the I.S.-compensated trends. This may be an indication that the I.S. chosen for these was not as chemically similar as it could be, and consideration should be made about selecting a different deuterated compound. As mentioned previously, the availability and cost of deuterated SCRAs is prohibitive to this, but expense may be justified if significant detrimental effects are observed.

As seen in the previous section, the majority of ME observed are enhancing in nature. It was thought that the additive enhancement effects of the build-up of proteins on the column over time may have contributed towards the upward trend observed for the compounds. This would affect the deuterated compounds in the same way and thus the PAR would maintain a more stable trend than the compound peak areas taken alone. It is also possible that evaporation of the solvent took place in the autosampler, which was not temperature controlled (although RT was controlled and monitored). This would cause a concentration in analytes over time and lead to an observable upward trend, compensated by I.S.

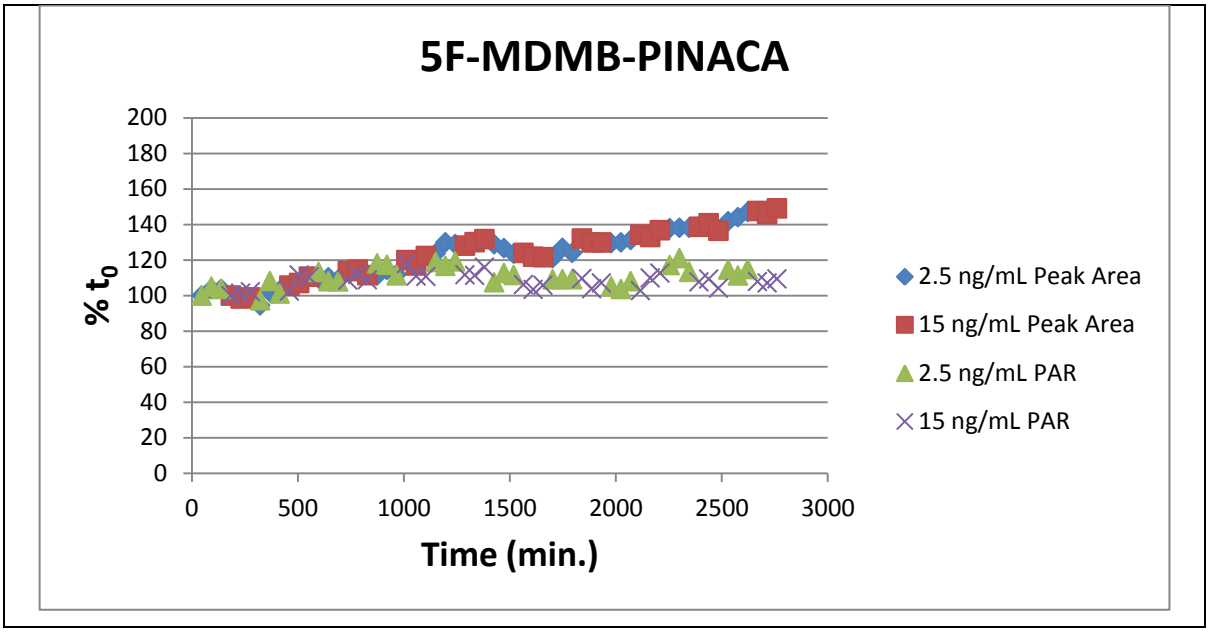


Figure 31 – Stability on the autosampler, at room temperature, of 5F-MDMB-PINACA over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

The stable/downward trend was observed in 5F-NPB-22, 5F-PB-22, AB-FUBINACA M2B, AB-FUBINACA valine metabolite, MDMB-CHMICA *O*-desmethyl acid metabolite, MMB-CHMICA and PB-22 N-pentanoic acid. This trend was characterized by peak areas remaining within (or exiting and then returning to) the 100 ±20% range while PAR values decrease. An example is given by PB-22 N-pentanoic acid in Figure 32. In this example the PAR decreases outwith the 100 ±20% range, but this was not the case for all compounds showing this trend. It was not possible to determine whether the compounds were truly stable, or whether the ion enhancement and/or evaporation observed in compounds with an upward trend was counter-acted by decrease in analyte concentration through instability, giving the appearance of stability. None of the compounds that fall within this category used deuterated forms of themselves as I.S. so if the I.S. peak area was increasing, through ME for example, and the analyte peak area was either stable or decreasing this would show as a decrease in PAR over time. While the peak areas of the compounds exhibiting this trend were largely within the stable range (100 ±20%), with some outliers, a slight upwards trend was observed in some compounds, with peak areas increasing over time, albeit not above 120%. It is therefore possible that over a longer experimental period an increase would have been observed. Although PAR were observed to decrease over time, they

largely stayed within the stable range and so the effect of this on sample results should be minimal, however it is still a factor to consider in interpretation.

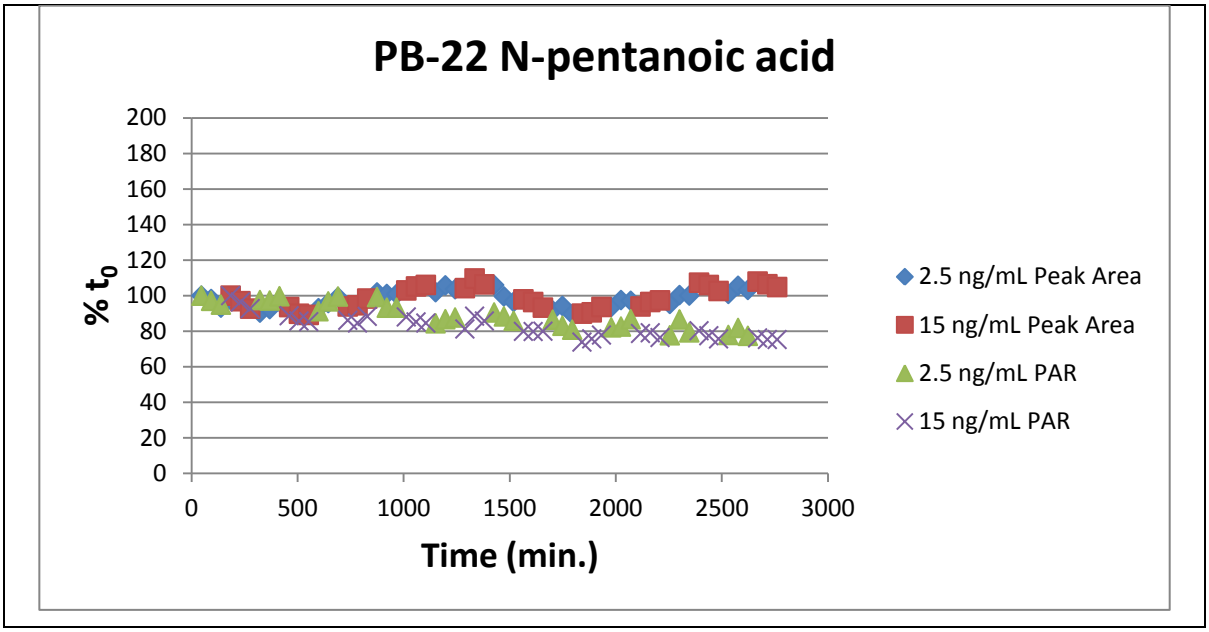


Figure 32 – Stability on the autosampler, at room temperature, of PB-22 N-pentanoic acid over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

The final observation category is increased variation with no discernible trend, as exemplified by 5F-AKB48 in Figure 33. While a slight upward and a slight downward trend may be observed in the peak areas and PAR respectively, the spread of the data (*i.e.* random error) was too great to say with any certainty whether the compounds were stable or not.

In addition to 5F-AKB48, this was the case for AKB48 N5OH pentyl, BB-22, BB-22 3-carboxyindole, and MDMB-CHMINACA. This may be an artifact of the significant ion suppression and large variability observed for ME in Table 30 and Table 31.

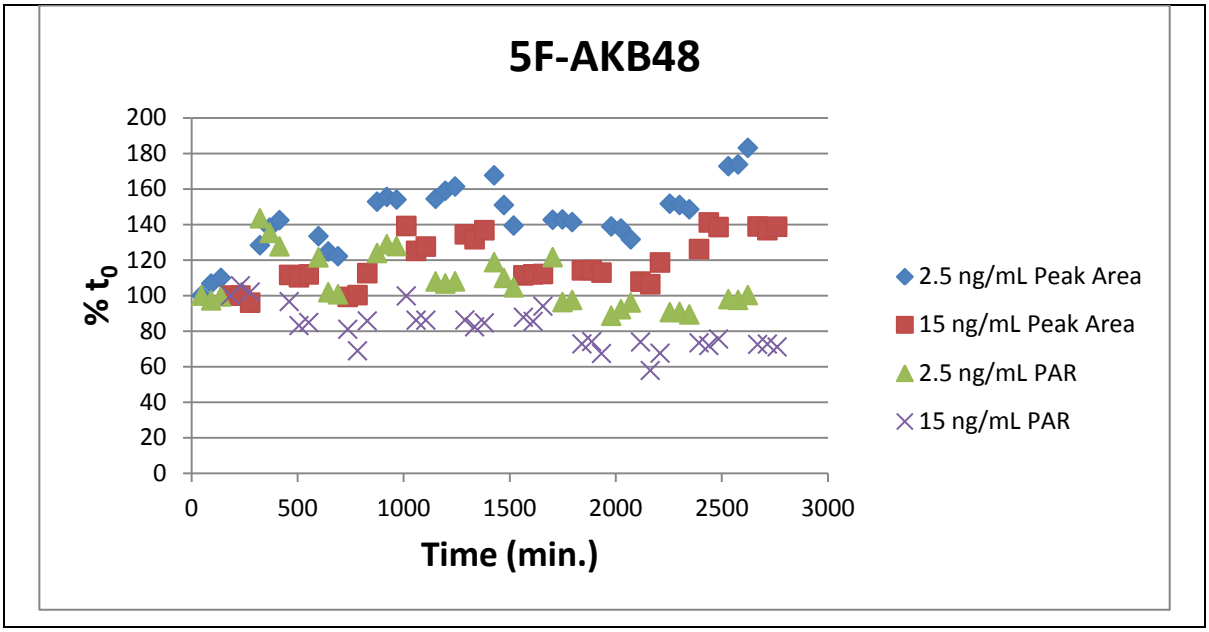


Figure 33 – Stability on the autosampler, at room temperature, of 5F-AKB48 over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

While the experiment was designed so as to be an extreme example of how the assay may be run in a real-world situation, *i.e.* a sample being injected 40 hours after the beginning of the run, it is important to consider the stability of compounds when interpreting both positive and negative results. Re-injecting a calibrator or QC at the end of the sequence for longer runs is recommended to provide assurances that no significant changes have taken place to the concentration of analytes during the sequence duration.

4.4.5. Method Validation – Methods 1.1, 1.2 and 2.1 applied to urine

4.4.5.1. Linearity

A linear calibration model using 1/χ-weighting was established for 5F-MDMB-PINACA metabolite, MDMB-CHMICA metabolite and AB-FUBINACA valine metabolite, as demonstrated by correlation co-efficient values of ≥0.99 over 10 calibrations. For all calibrations, a minimum of 6 calibrators were used between 0.20 and 50 ng/mL, and the calculated concentrations of these were within ±20% of the expected value.

An example calibration curve for AB-FUBINACA valine metabolite is given in Figure 34.

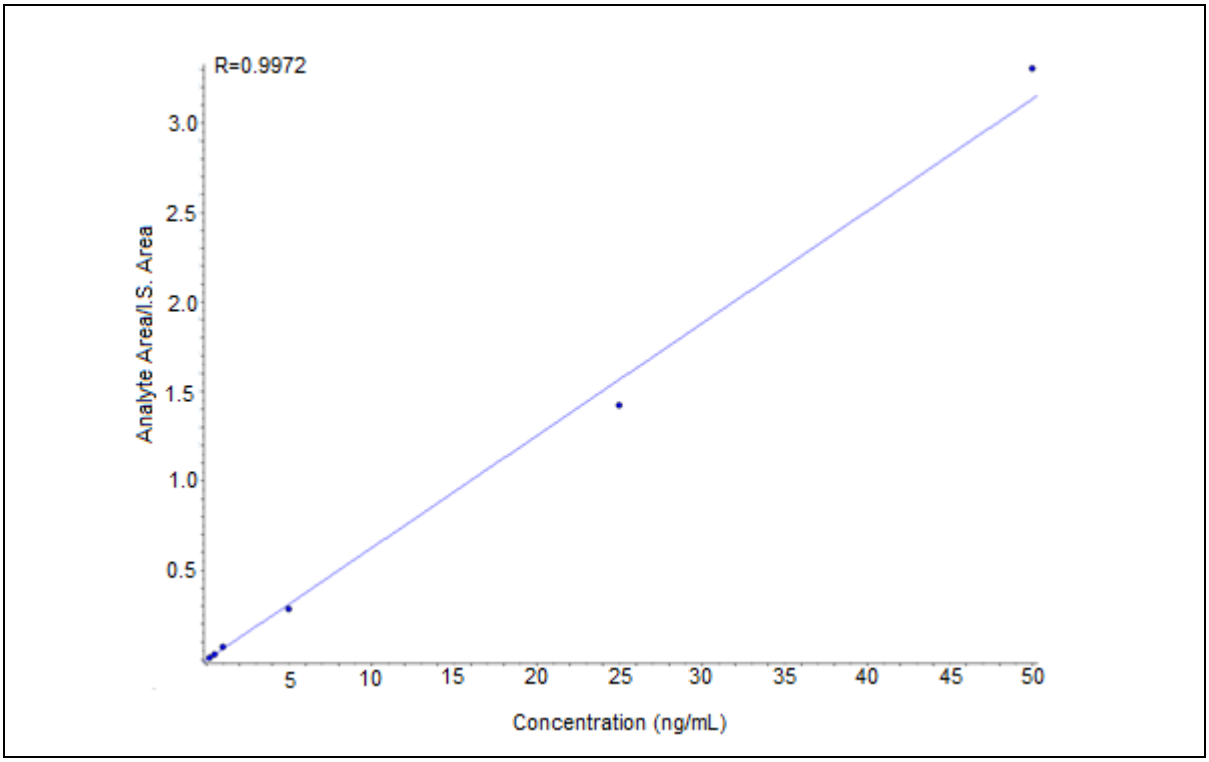


Figure 34 – Example calibration curve of AB-FUBINACA valine metabolite using Method 2.1 applied to blood, using 1/χ weighting, giving a correlation coefficient of 0.9972.

4.4.5.2. Selectivity

Selectivity was demonstrated for all methods by the observation of no response at a t_R of interest in the XIC of analytes where the analyte was not present.

Examples of blank chromatograms exhibiting selectivity for all methods are given in Figure 35. Figure 36 shows examples of low (<0.20 ng/mL) and higher (11 ng/mL) positive case samples for AB-FUBINACA valine metabolite using method 2.1.

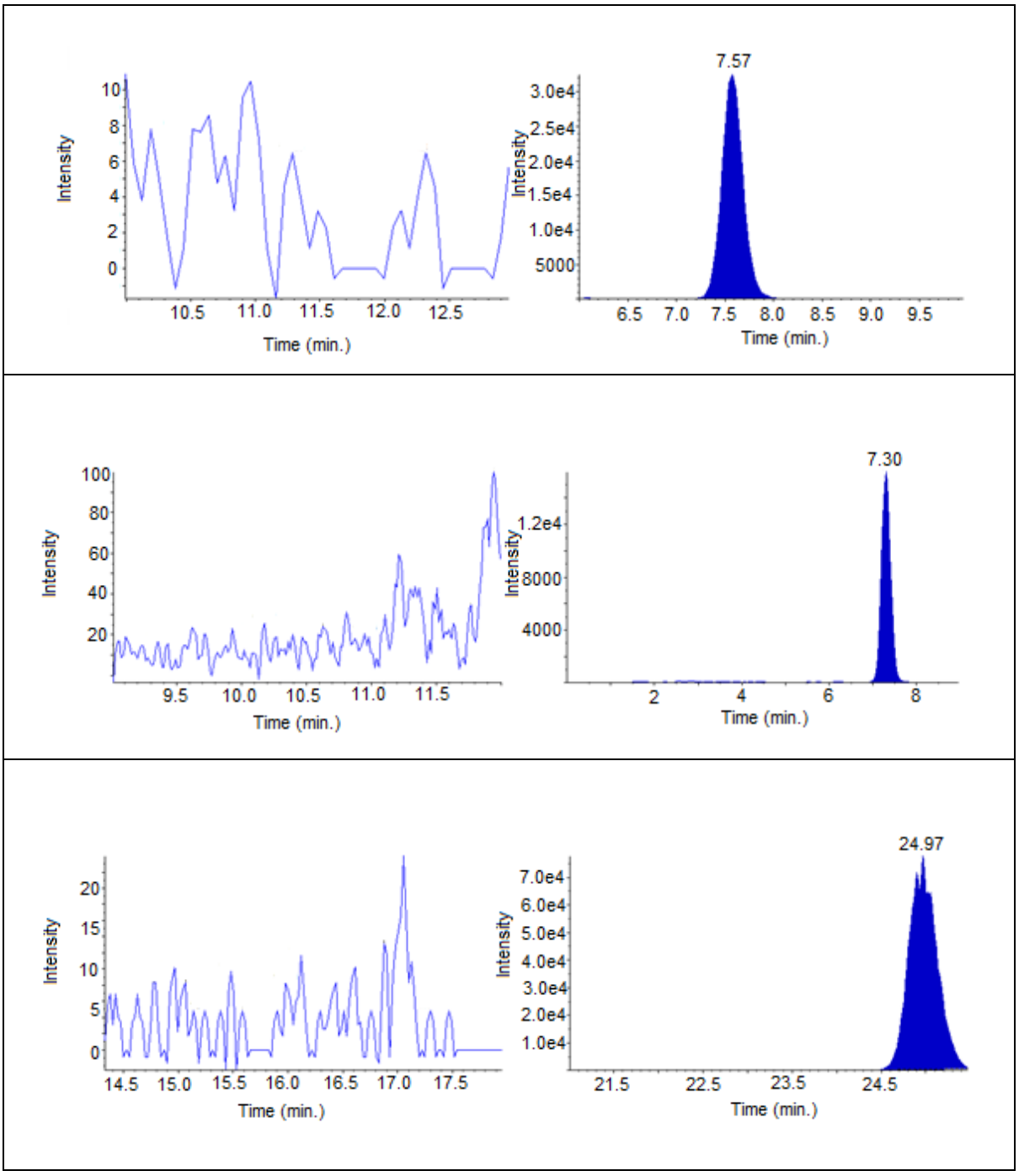


Figure 35 – Example chromatograms from a blank standard, demonstrating selectivity from method 1.1 for AB-CHMINACA (top), method 1.2 for AB-FUBINACA valine metabolite (middle), and method 2.1 for AM2201 N4OH pentyl (bottom). Analytes are shown on the left and internal standards on the right and intensity is given in counts per second.

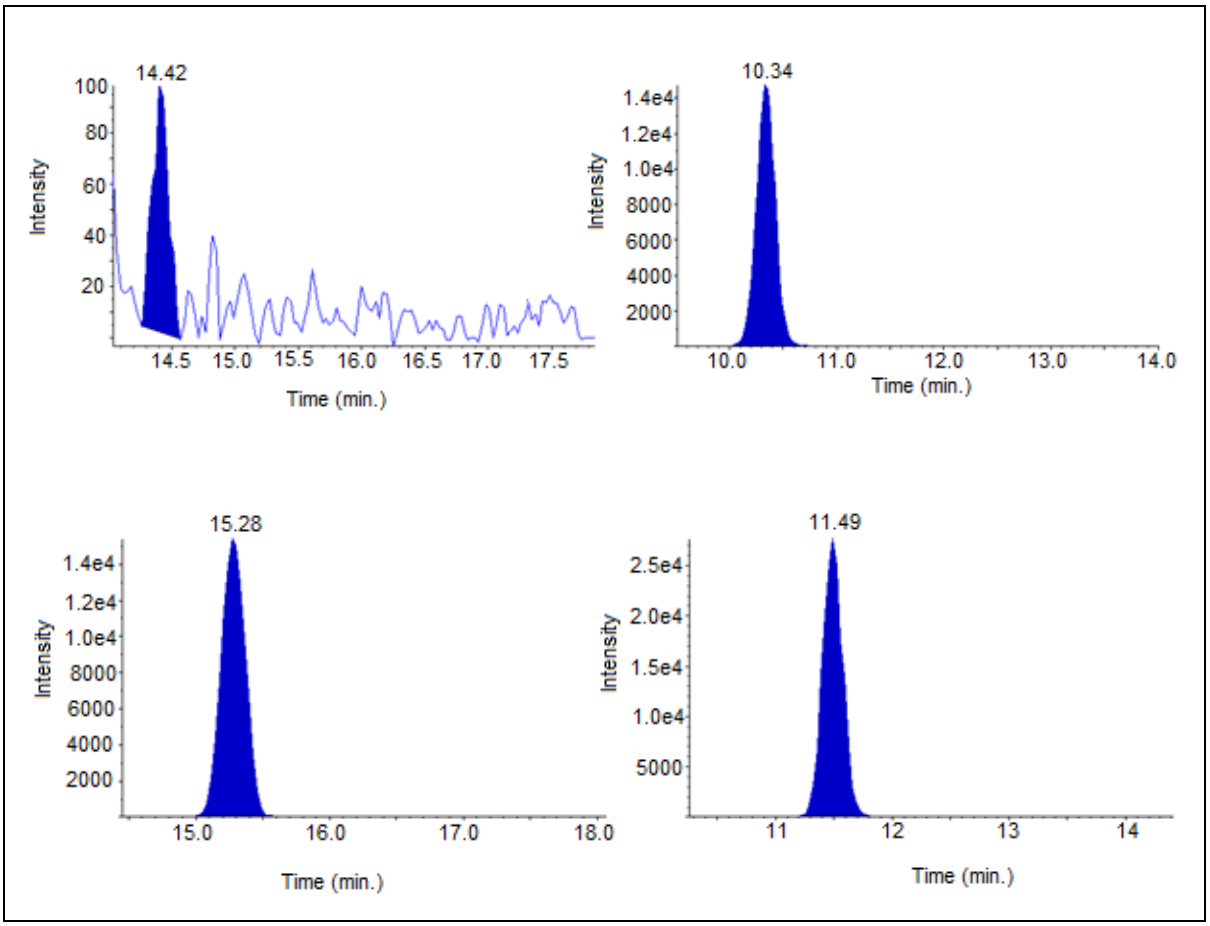


Figure 36 – Example chromatograms from a low positive case sample (top) and a higher positive case sample (bottom) for AB-FUBINACA valine metabolite using method 2.1. The difference in retention times is due to inter-batch variation, different batches of mobile phase and different analytical columns. Intensity is given in counts per second.

4.4.5.3. Sensitivity

Limits of detection for methods 1.1, 1.2 and 2.1 are shown in Table 32 with the LLOQ and the SNR at the LOD for method 2.1 shown.

Regarding methods 1.1 and 1.2, the purpose was to identify the presence of SCRAs in urine and a LOD of 0.2 ng/mL was deemed acceptable for this. For this reason, no standards of lower concentration were ran, although it is likely that the method could detect concentrations <0.2 ng/mL for most compounds. The only compound for which the LOD was determined to be significantly above an acceptable concentration was PB-22 N-pentanoic acid 3-carboxyindole, with an LOD of 25 ng/mL. While SCRAs are known to be present in the urine at higher concentrations than in blood, particularly metabolites, it is likely that concentrations of the compound would be <25 ng/mL in urine (80). Therefore it was decided that

this compound would not be reported, as it could not be certain that the compound was absent in samples. It was removed from standard solutions for subsequent method development.

Optimisation work was conducted on the extraction protocol (see Section 4.3.4.2) and consequently the LODs for method 2.1 were generally improved. LODs for AB-CHMINACA M1A, AB-FUBINACA M2B and BB-22 3CI were increased to 2, 1 and 1 ng/mL respectively. While this is not ideal, the optimisation process is a compromise, and the majority of compounds saw improvements in sensitivity through this. In addition, all of these compounds are metabolites, which are likely to be present in higher concentrations in urine, and for all but BB-22 other metabolites are included within the method to further the chances of detection.

As the lowest calibrator was selected as the LLOQ for most compounds, it was decided to use a SNR of ≥4 for the LOD to ensure a clear distinction from noise. It is clear from Table 32 that some SNRs are much higher than 4, for example FUB-PB-22, MAM2201 N4OH pentyl, and PB-22. This is because the standard at the next lowest concentration gave a SNR that was not consistently ≥4 for duplicate results in 3 sources of blank blood.

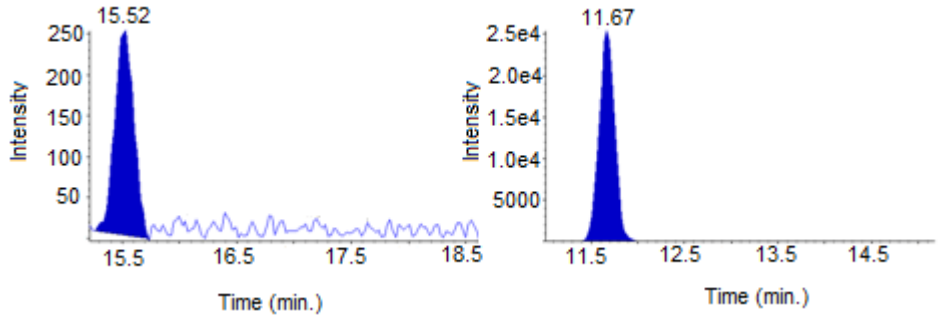
Table 32 – Limits of Detection and Quantitation for Methods 1.1, 1.2 and 2.1 in Urine. The mean signal-to-noise ratio at the Limit of Detection is also given.

Compound	LOD Methods 1.1 and 1.2 (ng/mL)	Method 2.1		
		LOD (ng/mL)	LLOQ (ng/mL)	Mean SNR at LOD
5F-AB-PINACA	0.2	0.50	0.50	6
5F-ADB-PINACA	N/A	0.10	0.20	7
5F-AKB48	0.2	0.10	0.20	6
5F-AKB48 N4OH pentyl	0.2	0.10	0.20	6
5F-MDMB-PINACA	0.2	0.01	0.20	6
5F-MDMB-PINACA *O*-desmethyl acid metabolite	N/A	0.10	0.20	10
5F-NPB-22	N/A	0.02	0.20	10
5F-PB-22	0.2	0.01	0.20	9
AB-CHMINACA	0.2	0.10	0.20	6
AB-CHMINACA M1A	0.2	2.00	2.00	5
AB-CHMINACA M2	0.2	0.10	0.20	14
AB-FUBINACA	0.2	0.20	0.20	5

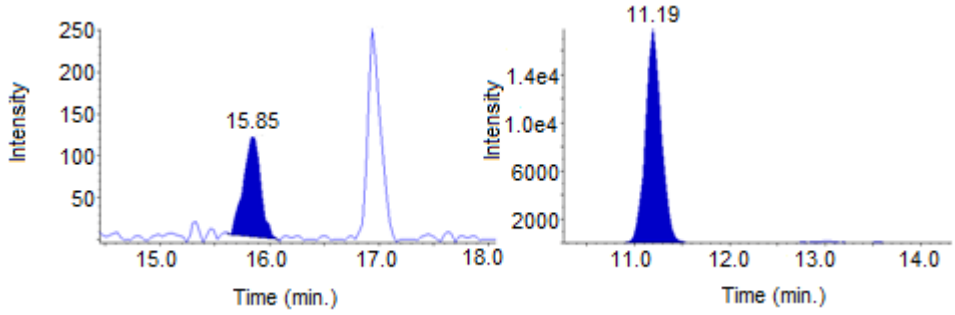
Table 32 – Limits of Detection and Quantitation for Methods 1.1, 1.2 and 2.1 in Urine. The mean signal-to-noise ratio at the Limit of Detection is also given.

Compound	LOD Methods 1.1 and 1.2 (ng/mL)	Method 2.1		
		LOD (ng/mL)	LLOQ (ng/mL)	Mean SNR at LOD
AB-FUBINACA M2B	0.5	1.00	1.00	8
AB-FUBINACA valine metabolite	0.2	0.20	0.20	8
AB-PINACA	0.2	0.20	0.20	9
AB-PINACA N4OH pentyl	N/A	2.00	2.00	6
AKB48	0.2	0.10	0.20	5
AKB48 N5OH pentyl	0.2	0.20	0.20	9
AKB48 N-pentanoic acid	0.2	0.05	0.20	4
AM2201	0.2	0.01	0.20	7
AM2201 N4OH pentyl	0.2	0.02	0.20	6
AM2201 N5OH indole	0.2	N/A	N/A	N/A
APICA	0.2	N/A	N/A	N/A
APICA N4OH pentyl	0.2	0.20	0.20	8
BB-22 3-carboxyindole	0.2	1.00	1.00	5
BB-22	0.2	0.02	0.20	12
FUB-PB-22	N/A	0.02	0.20	15
MAM2201 N4OH pentyl	N/A	0.02	0.20	14
MDMB-CHMICA	0.2	0.10	0.20	10
MDMB-CHMICA *O*-desmethyl acid metabolite	0.2	0.20	0.20	14
MDMB-CHMINACA	0.2	0.05	0.20	6
MMB2201	N/A	0.01	0.20	7
MMB-FUBINACA	N/A	0.02	0.20	8
PB-22	0.2	0.02	0.20	13
PB-22 N5OH pentyl	0.2	0.05	0.20	9
PB-22 N-pentanoic acid 3-carboxyindole	25	N/A	N/A	N/A
PB-22 N-pentanoic acid	0.2	0.05	0.20	6
STS-135	0.2	N/A	N/A	N/A
STS-135 N4OH pentyl	0.2	0.10	0.20	6
MAB-CHMINACA	N/A	0.01	0.20	7
MAB-CHMINACA M1	N/A	1.00	1.00	6
CUMYL-PeGACLONE	N/A	0.02	0.20	5
MMB-CHMICA	N/A	0.05	0.20	6

Examples of compounds present at their assigned LOD in Method 2.1 are given Figure 37. These show that the analyte response is easily distinguishable from baseline noise.



AB-FUBINACA valine metabolite (left) @ 0.2 ng/mL with I.S. (right)



5F-MDMB-PINACA metabolite (left) @ 0.1 ng/mL with I.S. (right)

Figure 37 – Example extracted ion chromatograms of selected compounds (left) present at their assigned Limit of Detection with internal standard (right) in Method 2.1 applied to urine. The signal-to-noise ratios are ≥4. Intensity is given in counts per second.

4.4.5.4. Accuracy and Precision

The results for 5F-MDMB-PINACA *O*-desmethyl acid, AB-FUBINACA valine metabolite and MDMB-CHMICA *O*-desmethyl acid metabolite for intra- and interday accuracy and precision are shown in Table 33 and Table 34 respectively.

These show that the quantitation is both suitably accurate and shows good precision at a range of concentrations. The intraday precision for the MDMB-CHMICA *O*-desmethyl acid metabolite at 0.2 ng/mL is slightly outside the acceptable limit of ±15%, and as such QC standards near to the LLOQ should be run with batches, and caution taking when reporting low concentrations.

It was thought that the interday results would show more variation than intraday, but that was not the case for the 5F-MDMB-PINACA and MDMB-CHMICA *O*-

desmethyl acid metabolites. It is known that these compounds suffer from a significant degree of ion enhancement when the PAR is considered, so it is possible that the main source of variation for these compounds is due to matrix variation and not random error.

Table 33 – Intra- and interday accuracy for 5F-MDMB-PINACA *O*-desmethyl acid, AB-FUBINACA valine metabolite, and MDMB-CHMICA *O*-desmethyl acid for Method 2.1 applied urine. All compounds gave satisfactory results, within 100±20%.

Compound	Accuracy							
	Intraday (n=5)				Interday (n=5)			
	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL
5F-MDMB-PINACA *O*-desmethyl acid	113	106	105	110	100	105	101	105
AB-FUBINACA valine metabolite	100	104	101	107	100	105	97	104
MDMB-CHMICA *O*-desmethyl acid	95	117	118	102	96	110	109	105

Table 34 – Intra- and interday precision for 5F-MDMB-PINACA *O*-desmethyl acid, AB-FUBINACA valine metabolite, and MDMB-CHMICA *O*-desmethyl acid for Method 2.1 applied urine. With the exception of MDMB-CHMICA *O*-desmethyl acid, all compounds gave results ≤15%.

Compound	Precision							
	Intraday (n=5)				Interday (n=5)			
	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL	0.2 ng/mL	2.5 ng/mL	15 ng/mL	42 ng/mL
5F-MDMB-PINACA *O*-desmethyl acid	5.9	2.1	4.8	2.9	3.2	8.6	5.5	7.8
AB-FUBINACA valine metabolite	6.2	2.5	5.2	3.5	5.5	6.0	9.3	4.4
MDMB-CHMICA *O*-desmethyl acid	15.5	1.7	1.9	5.2	11.6	7.1	5.5	8.3

4.4.5.5. Recovery and Matrix Effects

Table 35 and Table 36 show the recovery and matrix effects at 2.5 and 15 ng/mL respectively.

Recoveries are higher and more consistent compared to those found with the blood extraction. All are within acceptable ranges, with the exception of the absolute values for 5F-AKB48 and AKB48 at 2.5 ng/mL, and these plus 5F-NPB-22 and MMB-CHMICA at 15 ng/mL. For AKB48 and MMB-CHMICA, the compensation by the I.S. improves these, with a less significant improvement seen for I.S compensation for 5F-AKB48. Compensation by the I.S. for 5F-NPB-22 at 15 ng/mL actually makes the recovery significantly more variable, suggesting the I.S. is not very suitable.

With regards to ME, values are, again, more consistent and acceptable than the values for blood. Significant enhancement was seen for MDMB-CHMICA, 5F-AKB48, BB-22, MDMB-CHMINACA and CUMYL-PeGACLONE. This was compensated by the I.S. for BB-22, to some extent, but not the other compounds.

On the other hand, AM2201 N4OH pentyl, AB-PINACA N4OH pentyl, AB-CHMINACA M1A and APICA N4OH pentyl suffered from significant inhibitory ME. The I.S. compensated slightly for the inhibition of AM2201 N4OH pentyl but did not do so for the other compounds.

The absolute values for AKB48 show significant variation in the ME between sources of urine. As the I.S. used for this compound is a deuterated form of AKB48, a significant compensation is made, to bring the ME values within an acceptable range.

Generally, the recoveries and ME observed were acceptable, however caution should be exercised with the more affected compounds, and in samples that are visibly dark, cloudy and/or viscous in appearance.

Table 35 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 2.5 ng/mL in Method 2.1 applied to urine

Compound	2.5 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max	Min.	Max.	Min.	Max
5F-MDMB-PINACA[1]	85	104	75	122	89	112	84	132
5F-MDMB-PINACA *O*-desmethyl acid[1]	85	103	88	113	87	109	93	130
MDMB-CHMICA*[1]	68	97	67	98	81	132	55	106
MDMB-CHMICA *O*-desmethyl acid[1]	88	108	81	145	90	110	88	161
AB-FUBINACA[1]	82	102	85	102	92	107	97	110
MMB-FUBINACA[1]	90	107	82	97	91	121	73	107
AB-FUBINACA valine metabolite[1]	83	101	71	99	91	106	79	112
5F-PB-22**[2]	90	105	83	100	90	112	84	123
PB-22[2]	75	100	83	117	94	102	96	108
PB-22 N5OH pentyl[2]	84	106	59	89	79	121	50	153
5F-AKB48*[3]	23	80	70	132	56	82	79	142
5F-AKB48 N4OH pentyl[3]	84	111	68	104	94	112	75	117
AKB48[4]	23	55	46	176	88	102	88	107
AKB48 N5OH pentyl*[3]	80	109	79	97	92	106	93	100
BB-22[2]	70	91	74	152	82	106	72	119
BB-22 3-carboxyindole[2]	76	110	71	91	90	118	69	105
AM2201*[5]	82	98	64	81	96	104	92	99
AM2201 N4OH pentyl[5]	86	100	41	71	65	120	53	138
AB-PINACA**[1]	94	104	87	106	90	106	92	115
AB-PINACA N4OH pentyl[1]	85	106	42	67	90	113	47	71

* denotes where only 9 sources of blank blood were used due to retention time shift for compounds ** denotes where only 6 sources of blank blood were used due to retention time shift for compounds

[1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 35 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 2.5 ng/mL in Method 2.1 applied to urine

Compound	2.5 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max	Min.	Max.	Min.	Max
5F-AB-PINACA[1]	86	107	87	127	93	107	96	144
5F-ADB-PINACA[1]	77	101	77	122	86	108	87	149
MMB2201[5]	87	99	66	96	84	113	85	143
MAM2201 N4OH pentyl**[5]	94	104	61	78	99	109	74	110
AB-CHMINACA[1]	83	111	82	102	87	112	91	111
AB-CHMINACA M2[1]	82	108	83	110	92	111	85	129
5F-NPB-22[2]	52	108	74	102	14	86	80	114
AB-CHMINACA M1A[1]	80	102	44	66	77	104	50	70
AB-FUBINACA M2B[1]	86	113	72	97	91	112	86	105
AKB48 N-pentanoic acid[3]	82	107	64	90	93	116	67	101
APICA N4OH pentyl[3]	85	114	48	95	92	118	54	96
FUB-PB-22[2]	80	104	76	87	97	109	68	97
MDMB-CHMINACA[1]	57	80	72	143	55	86	74	148
PB-22 N-pentanoic acid[2]	82	102	58	90	94	120	49	106
STS-135 N4OH pentyl[3]	82	106	53	114	83	116	55	127
CUMYL-PeGACLONE[1]	75	96	75	131	81	97	77	133
MAB-CHMINACA[1]	78	100	90	116	86	106	92	129
MAB-CHMINACA M1[1]	85	102	63	93	95	104	71	104
MMB-CHMICA[1]	83	101	80	109	86	111	80	124

* denotes where only 9 sources of blank blood were used due to retention time shift for compounds ** denotes where only 6 sources of blank blood were used due to retention time shift for compounds

[1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 36 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 15 ng/mL for Method 2.1 applied to urine

Compound	15 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max	Min.	Max.	Min.	Max
5F-MDMB-PINACA[1]	79	94	72	94	83	100	87	105
5F-MDMB-PINACA *O*-desmethyl acid*[1]	84	99	95	120	88	105	101	150
MDMB-CHMICA*[1]	68	89	73	116	71	96	87	118
MDMB-CHMICA *O*-desmethyl acid[1]	48	95	84	187	80	98	91	232
AB-FUBINACA[1]	82	97	79	98	86	100	100	115
MMB-FUBINACA[1]	89	94	79	98	90	101	90	107
AB-FUBINACA valine metabolite[1]	85	99	90	102	85	104	90	123
5F-PB-22**[2]	86	93	81	107	93	111	89	107
PB-22[2]	74	91	75	128	88	94	86	113
PB-22 N5OH pentyl[2]	83	105	61	95	90	124	59	119
5F-AKB48[3]	28	84	71	139	66	87	83	143
5F-AKB48 N4OH pentyl[3]	74	96	85	100	81	97	87	111
AKB48[4]	28	55	53	191	83	96	94	110
AKB48 N5OH pentyl*[3]	73	95	80	111	79	100	91	110
BB-22[2]	72	87	75	161	82	94	91	136
BB-22 3-carboxyindole[2]	76	99	72	97	88	117	69	114
AM2201*[5]	75	91	62	92	88	99	97	103
AM2201 N4OH pentyl[5]	83	97	50	74	94	104	65	110
AB-PINACA**[1]	91	93	83	98	89	100	96	112
AB-PINACA N4OH pentyl[1]	75	100	43	77	78	98	52	84

* denotes where only 9 sources of blank blood were used due to retention time shift for compounds ** denotes where only 6 sources of blank blood were used due to retention time shift for compounds

[1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

Table 36 – Ranges for absolute and internal standard-compensated recovery and Matrix Effects at 15 ng/mL for Method 2.1 applied to urine

Compound	15 ng/mL (n=10)							
	Absolute				I.S. Compensated			
	Recovery (%)		Matrix Effects (%)		Recovery (%)		Matrix Effects (%)	
	Min.	Max.	Min.	Max	Min.	Max.	Min.	Max
5F-AB-PINACA[1]	80	100	84	120	84	101	99	138
5F-ADB-PINACA[1]	82	99	70	116	87	101	90	144
MMB2201[5]	84	101	72	97	90	105	91	148
MAM2201 N4OH pentyl**[5]	92	100	63	79	99	109	74	112
AB-CHMINACA[1]	82	102	85	104	85	102	97	117
AB-CHMINACA M2[1]	79	95	82	121	84	99	92	150
5F-NPB-22[2]	46	93	71	111	10	80	77	117
AB-CHMINACA M1A[1]	76	99	39	66	79	100	47	72
AB-FUBINACA M2B[1]	84	105	71	87	87	109	81	108
AKB48 N-pentanoic acid[3]	80	102	72	97	83	113	78	101
APICA N4OH pentyl[3]	84	99	65	96	88	102	67	97
FUB-PB-22[2]	80	94	74	93	94	105	79	107
MDMB-CHMINACA[1]	64	83	81	145	66	120	88	152
PB-22 N-pentanoic acid[2]	84	100	60	94	92	116	52	115
STS-135 N4OH pentyl*[3]	80	98	75	122	84	103	75	117
CUMYL-PeGACLONE[1]	76	92	69	139	80	100	83	146
MAB-CHMINACA[1]	77	89	90	131	80	95	101	145
MAB-CHMINACA M1[1]	81	103	64	89	84	102	77	105
MMB-CHMICA*[1]	44	99	85	114	88	98	79	129

* denotes where only 9 sources of blank blood were used due to retention time shift for compounds ** denotes where only 6 sources of blank blood were used due to retention time shift for compounds

[1] Used AB-FUBINACA-d_4 as I.S. [2] Used PB-22-d_9 as I.S. [3] Used AKB48 N5OH pentyl-d_4 as I.S. [4] Used AKB48-d_{11} as I.S. [5] Used AM2201-d_5 as I.S.

4.4.5.6. Interference Testing

The experiments into potential interferences conducted in 4.3.5.7 and discussed in 4.4.4.6 were done so using unextracted standards. As the instrumental method for blood and urine analyses is the same, these experiments show there is no response from any of the compounds tested.

4.4.5.7. Autosampler Stability

The graphs shown in Figure 38 – Figure 40 are representative of the trends seen for all compounds.

Figure 38 is an example of the autosampler stability observed for all compounds with the exceptions of 5F-AKB48, AKB48, BB-22 and MDMB-CHMINACA. The peak areas for both 2.5 ng/mL and 15 ng/mL standards decreased over time to ≥40% of the t_0 value. For these compounds, the I.S. was observed to compensate well, keeping the change from t_0 to within ± 20%. The I.S.-compensated plot for the MDMB-CHMICA metabolite did show an increase outwith ± 20% towards the latter stages of the experiment, which is certainly something to consider where sequence run times exceed 30 h.

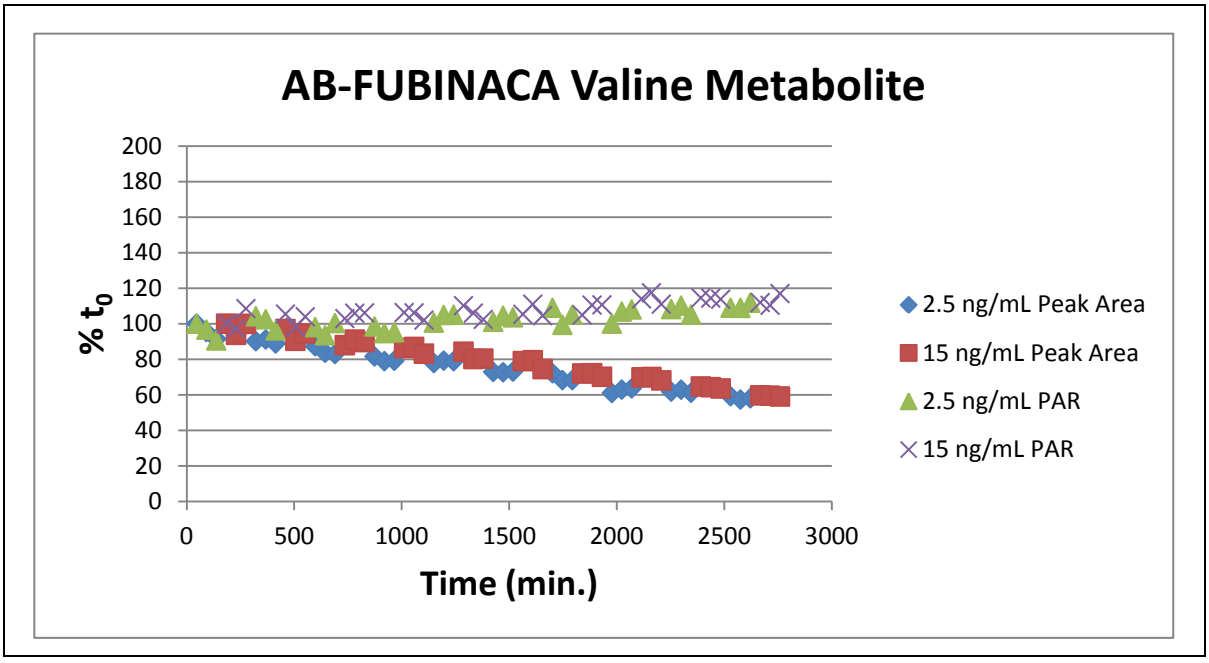


Figure 38 – Stability on the autosampler, at room temperature, of AB-FUBINACA valine metabolite over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

Figure 39 shows the trend seen for the autosampler stability of AKB48. This shows a high level of variation between the peak areas at both 2.5 and 15 ng/mL concentrations over time. This is possibly due to the low and relatively variable recovery of this compound. As the I.S. used for this compound is AKB48-$d_{11,}$ this compensated well for the variation to bring the change from t_0 to within ± 20% until around 38 h, where it increases outside these limits. This increase is could be due either to degradation of AKB48-d_{11} or an increase in response from the AKB48, which did show significant ion enhancement effects. It is possible that matrix components are building up on the column during long runs and contributing towards AKB48 ion enhancement which does not have such a significant effect on the I.S.

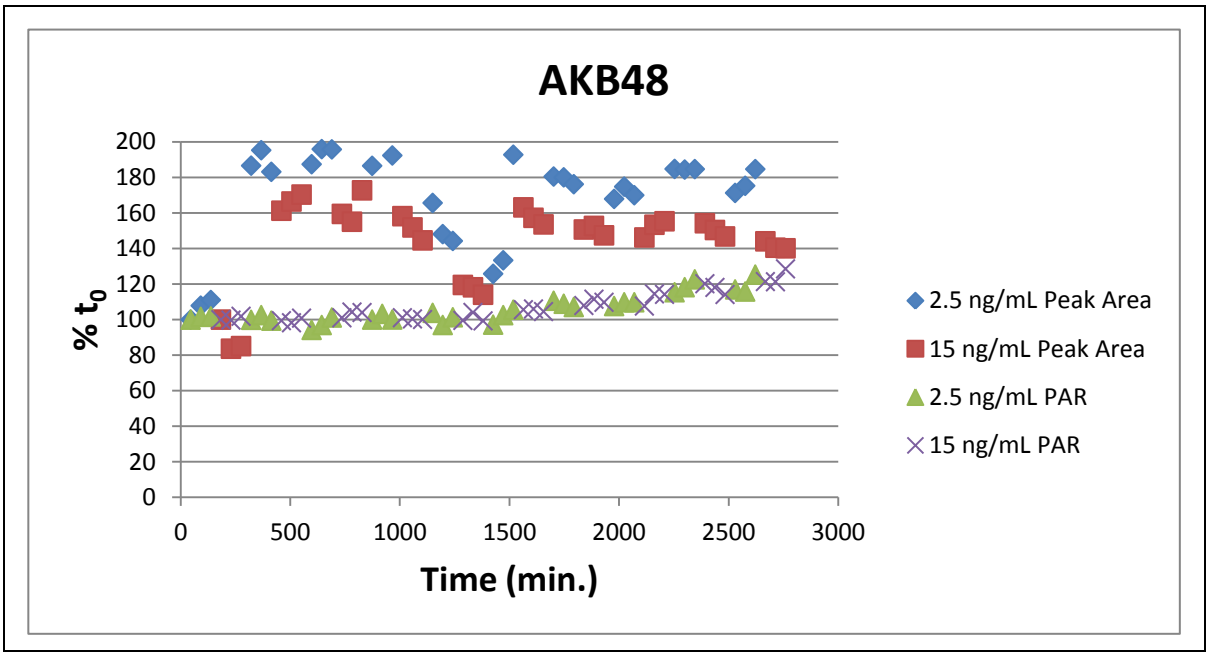


Figure 39 – Stability on the autosampler, at room temperature, of AKB48 over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

Figure 40 shows the results of the autosampler stability experiments for 5F-AKB48. This shows a high level of variation between both the 2.5 and 15 ng/mL experiments for both the peak area and I.S. compensated plots. As discussed in 0, this suggests that the I.S. used for 5F-AKB48 (AKB48 N5OH pentyl-d_4) is not greatly suited for this compound. The variation in the peak areas for this compound and AKB48 is likely due to the variation in recovery and ME associated with these compounds. Quantitative validation for these compounds was not

undertaken in urine as they are no longer thought to be prevalent in use, and they are parent compounds which are rarely seen in urine. Given that the variation observed in the autosampler stability experiments suffers from a positive bias, the LOD should not be adversely affected by extended sequence run times.

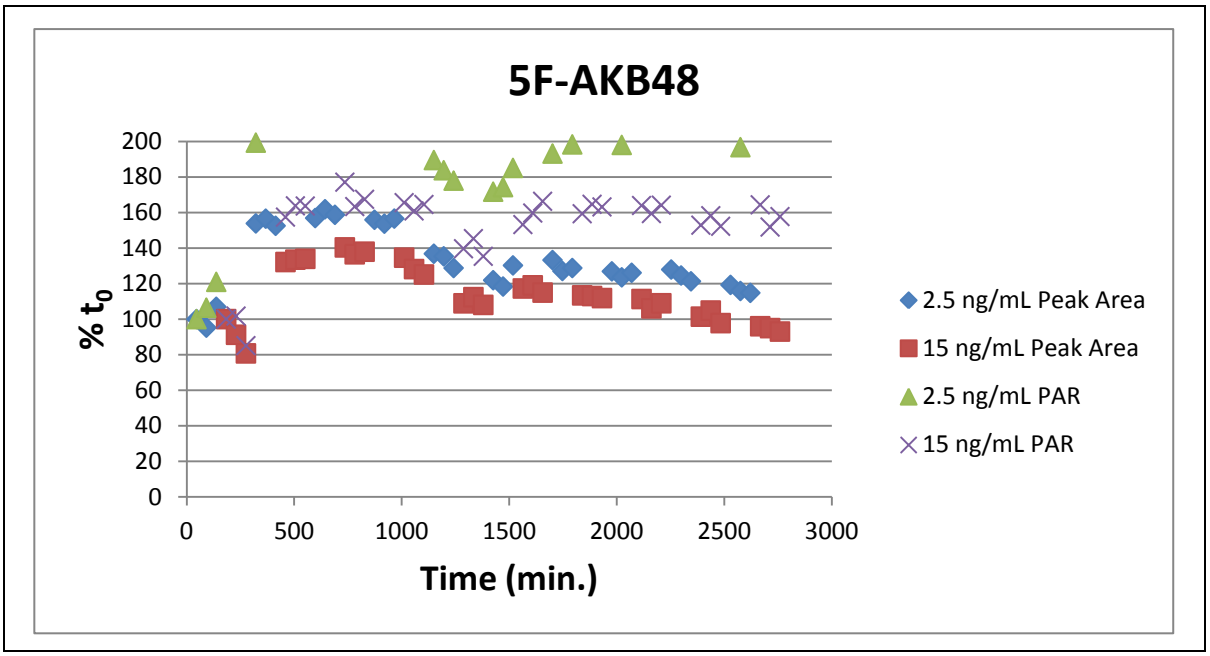


Figure 40 – Stability on the autosampler, at room temperature, of 5F-AKB48 over *ca.* 46 hours. Trends for 2.5 and 15 ng/mL concentrations are shown, along with the stability for the compound alone, and the peak area ratio.

As stated earlier regarding autosampler stability in blood extracts, injecting QC standards at the end of the sequence should ensure qualitative and quantitative results are valid where long run times are required.

4.4.6. Comparison of Prison 'A' and 'B' Samples

The comparison of results of analysis for prison 'A' and 'B' samples is given in Table 37. The 'B' results for cases where a defined value was reported (*i.e.* not >50 ng/mL) were plotted against the 'A' results and this is shown in Figure 41.

From the results shown in Table 37, it appears there was a slight positive bias compared to the 'A' results initially, which then shifted to a slight negative bias from Case 5 onwards. From looking at the dates of receipt of Cases 4 and 5, it is clear that the extraction method changed from the *t*BME extraction to MeOH (see Section 4.3.4.2) between these two cases, explaining this shift.

From Case 5 onwards, the difference is minimal, with the exceptions of cases 10 and 12, which are more significant. The original date of the 'A' analysis is unknown, as are the storage conditions during transfer to FMS. It is therefore possible that stability could have played a role in the decrease in concentration between 'A' and 'B' sample analysis, and that the period between 'A' and 'B' sample analysis is longer for cases 10 and 12.

Table 37 – Comparison of results obtained from original 'A' laboratory and 'B' testing using methods developed in Section 4 for Synthetic Cannabinoid Receptor Agonist-positive urine samples obtained from prison mandatory drug testing.

Case No	'A' Sample Result		'B' Sample Result		Difference (%)
	Compound	Conc. (ng/mL)	Compound	Conc. (ng/mL)	
1	AB-FUBINACA metab.	46	AB-FUBINACA valine metab.	>50 *(ca.* 56)	N/A
2	MDMB-CHMICA *O*-desmethyl acid metab.	7	MDMB-CHMICA *O*-desmethyl acid metab.	10	143
3	AB-FUBINACA	>100 (*ca*. 380)	AB-FUBINACA valine metab.	460	N/A
4	AB-FUBINACA metab.	43	AB-FUBINACA valine metab.	63	147
5	AB-FUBINACA	>100 (*ca*. 1000)	AB-FUBINACA valine metab.	>1250	N/A
6	AB-FUBINACA	6	AB-FUBINACA valine metab.	4.7	78
	5F-MDMB-PINACA	9	5F-MDMB-PINACA *O*-desmethyl acid metab.	6.1	68
7	AB-FUBINACA metab.	59	AB-FUBINACA valine metab.	56	95
8	Synthetic Cannabinoids	not given	5F-MDMB-PINACA *O*-desmethyl acid metab.	31	N/A
9	ADB	13	5F-MDMB-PINACA *O*-desmethyl acid metab.	13	100
10	5F-ADB desmethyl metabolite	37	5F-MDMB-PINACA O-desmethyl acid metab.	18	49
11	5F-ADB desmethyl metabolite	8	5F-MDMB-PINACA *O*-desmethyl acid metab.	5.4	68
12	AB-FUBINACA metab.	26	AB-FUBINACA valine metab.	14	54

Because of the unknowns related to inter-analysis period and compound stability, the accuracy is not calculated on reporting results, but is shown here as a measure of agreement.

The information contained within the ‘A’ Sample Result column in Table 37 is provided with the ‘B’ sample when it is delivered from the ‘A’ laboratory. The detail of information contained within varies and frequently contains the name of the parent drug rather than the specific metabolite. This is possibly due to the fact that it is the parent drug which is prohibited and the metabolite is simply used to prove ingestion of the parent drug. It should be noted that ADB is a synonym for MDMB-PINACA, with 5F-ADB being a synonym for 5F-MDMB-PINACA.

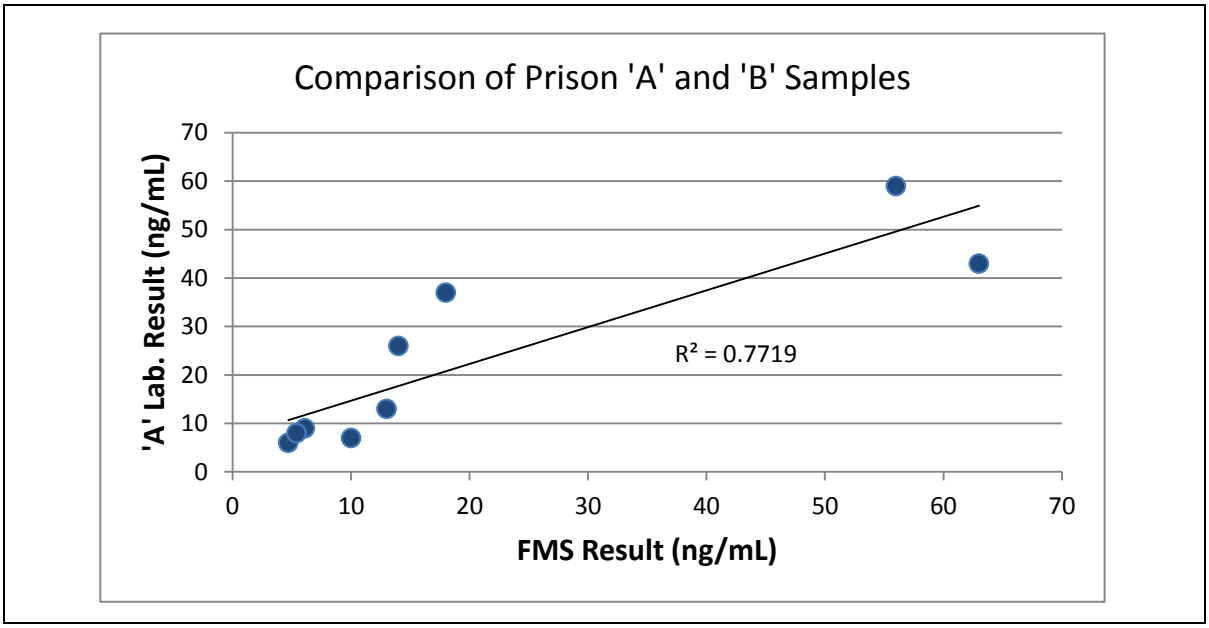


Figure 41 – Results of ‘A’ sample analysis (y-axis) plotted against results of ‘B’ sample analysis (x-axis) using methods developed in Section 4. A coefficient of determination of 0.7719 indicates good correlation of results.

The plot of results shown in Figure 41 shows a relatively good correlation between ‘A’ and ‘B’ results, with a linear correlation co-efficient of 0.7719. It should be noted that the top calibrator is 50 ng/mL so the two samples quantified above this concentration would have the additional error associated with a dilution. The correlation appears to be tighter at lower concentrations, which is possibly due to the higher number of calibrators at the lower end of the calibration curve and a more accurate quantitation as a result.

It is acknowledged that the result of the ‘A’ sample analysis did not necessarily provide the true value, taking all errors that may be associated with this method into account. As the ‘A’ method was validated and provides evidence for legal proceedings, however, the comparison of results obtained from the method discussed here to the ‘A’ result provided valuable information regarding method performance. Overall, all of the results of the ‘B’ sample analysis were consistent with the ‘A’ sample analysis results, both in terms of compounds identified and concentrations detected.

4.4.7. Analysis of Drug Packets

The results from the analysis of drug packets, shown in Table 38, show the discrepancy in the compounds listed as ingredients on packaging and what the material actually contains. It is possible that some of the additional compounds detected were present from the re-use and poor maintenance of production equipment, poor quality control, or inaccurate description of starting materials. From the chemical structure it does not seem likely that any of the un-labeled compounds are present from degradation or transformation of any of the labeled compounds.

Table 38 – Ingredients listed on packaging versus analytical findings for products suspected of containing Synthetic Cannabinoid Receptor Agonists

Product	Active Ingredients on Labeling	Analytical Findings
Afghan Black Ultra (Formula 2A)	5F-AKB48, STS-135	5F-AKB48, STS-135, 5F-MDMB-PINACA, 5F-PB-22
Blueberry Hazel Ultra (Formula 4A)	5F-AKB48, 5F-PB-22	5F-AKB48, STS-135, 5F-PB-22
Kuber Khaini	Tobacco	No SCRAs detected
Lunar Diamond	5F-AKB48	5F-AKB48, 5F-MDMB-PINACA, 5F-PB-22
Pandora’s Box Unleashed	5F-AKB48, 5F-PB-22, BB-22	5F-NPB-22, MMB2201, 5F-MDMB-PINACA, 5F-PB-22
Tribal Warrior Ultimate	5F-AKB48, 5F-PB-22	5F-AKB48, STS-135, 5F-PB-22

4.5. Conclusion

Method 2.1 as discussed in Section 4 was validated and deemed to be of sufficient quality to allow sensitive and selective detection, and accurate and precise quantitation of the SCRAs detailed in blood and urine, with a few exceptions. The LODs for BB-22 3CI and AB-FUBINACA M2B in whole blood were too high to detect the low concentrations that SCRAs are known to be present at after use. Similarly, the urine LODs for these compounds plus MAB-CHMINACA M1, AB-CHMINACA M1A and AB-PINACA N4OH pentyl were higher than optimal. Concentrations of SCRAs in urine samples, however, do tend to be higher than in blood.

The use of packed red cells for the calculation of LOD and LLOQ in place of whole blood is acknowledged as a limitation, as the extent of the effect of plasma protein binding with SCRAs is unknown.

No interferences were observed either between SCRAs in the method or from other compounds likely to be present in forensic samples, at suitable concentrations.

The calibration model was determined to be linear over the range assessed and linearity was acceptable for all compounds for which this was measured.

Accuracy and precision, where calculated, were found to be within the acceptable ranges.

Matrix Effects showed variation and some significant enhancement and inhibition in blood, particularly for CUMYL-PeGACLONE, MDMB-CHMICA, BB-22, 5F-AKB48, MDMB-CHMINACA, 5F-PB-22, AM2201 N4OH pentyl, AB-PINACA N4OH pentyl, MMB2201, MAM2201 N4OH pentyl and 5F-NPB-22. Further optimisation to the extraction protocol or MP gradient could improve these, however with the number of compounds included in the method, it will always be a compromise to obtain the best results. Investigation into more suitable I.S. may compensate for the ME, but will add expense to the assay.

ME for the urine method were much less significant and variable, likely due to the nature of urine as a less complex matrix.

Stability under autosampler conditions was generally acceptable for up to *ca*. 46 h. The compounds for which variation was outside ±20% tended to be the compounds for which significant ME were observed. Due to this, and as expected, variation over time was lower in urine than in blood.

The analysis of drug packets provides evidence both that this method is suitable for application to non-biological matrices, and also of the inaccurate nature of ingredients listings on product packaging. While limited in the scope of products tested, this work shows the drugs included in this method at the time of development are relevant to the compounds available to potential users and again highlights its fitness-for-purpose.

Overall, the result from Section 4 is a powerful method for the detection and quantitation of the most commonly available compounds on the UK market in whole blood and urine samples.

5. Synthetic Cannabinoid Receptor Agonists in Scottish Sub-Populations

5.1. Summary

This chapter reports the application of the methods developed in Chapters 3 and 4 to case samples from various cohorts representing various sub-populations of Scotland. As samples were received at various times throughout the process of method development, not all samples were analysed by the optimised and validated method, Method 2.1. Table 39 andTable 40, and Figure 42 provide a summary of the methods applied to each cohort, including extraction and instrument parameters and compounds included in each method. Table 39 also summarises the results of the analysis of each cohort in terms of participant numbers and positivity rate. These results are discussed in more detail in Sections 5.2 – 5.6.

Table 39 – Summary of analytical method used, participant numbers and results in terms of number of positive cases for the Emergency Department, post-mortem, Scottish Prison Service, Forensic Directorate, and Glasgow Drug Court cohorts

Cohort	Analytical Method Used	Number of Participants	% Positive
Emergency Department	See Figure 42	34	56
Post-Mortem	See Figure 42	250	11
Scottish Prison Service – overall	Method 1.1 (see Table 40 and Figure 42)	725	3
Scottish Prison Service – admission		432	5
Scottish Prison Service - liberation		285	0
Forensic Directorate	Method 1.2 (see Table 40 and Figure 42)	95	0

Table 39 – Summary of analytical method used, participant numbers and results in terms of number of positive cases for the Emergency Department, post-mortem, Scottish Prison Service, Forensic Directorate, and Glasgow Drug Court cohorts

Cohort	Analytical Method Used	Number of Participants	% Positive
Glasgow Drug Court	Method 1.2 (see Table 40)	73	1

Table 40 – Summary of extraction, hydrolysis (urine only), and instrumental parameters used in analytical methods applied to Emergency Department, post-mortem, Scottish Prison Service, Forensic Directorate, and Glasgow Drug Court cohorts

Parameter	Method 1.1	Method 1.2	Method 2.1
Hydrolysis protocol (urine only)	50 µL β-glucuronidase, 60 °C for 1 H	50 µL β-glucuronidase, 60 °C for 1 H	50 µL β-glucuronidase, 60 °C for 1 H (no buffer)
Extraction protocol	0.5 mL blood/urine, 1 mL pH6.0 phosphate buffer, 2 mL *t*BME, *ca*. 30 second vortex mix	0.5 mL blood/urine, 1 mL pH6.0 phosphate buffer, 2 mL *t*BME, *ca*. 30 second vortex mix	0.5 mL blood, 0.5 mL pH6.0 phosphate buffer, 1 mL *t*BME, 2 min. flatbed mix 0.5 mL urine, 2 mL MeOH, 5 min. flatbed mix

Table 40 – Summary of extraction, hydrolysis (urine only), and instrumental parameters used in analytical methods applied to Emergency Department, post-mortem, Scottish Prison Service, Forensic Directorate, and Glasgow Drug Court cohorts

Parameter	Method 1.1	Method 1.2	Method 2.1
MP Gradient A = dH2O* B= MeOH* C=ACN:dH2O (95:5)* *with 2mM ammonium acetate and 0.1% formic acid	F 0-5 min: 40 % A, 60% B 5-10 min: ramped to 10% A, 90% B 10-20 min: 10% A, 90% B 20-20.1 min: ramped to 40% A, 60% B 20.1-25 min:40% A, 60% B	H 0-5 min: 40% A, 60% B 5-8.5 min: ramped to 20% A, 80% B 8.5-18 min: ramped to 10% A, 90% B 18-20 min: 10% A, 90% B 20-20.1 min: ramped to 40% A, 60% B 20.1-25 min: 40% A, 60% B	R 0.-4 min: 60% A, 40% C 4-14 min: ramped to 40% A, 60% C 14-28 min: 40% A, 60% C 28-28.1 min: ramped to 20% A, 80% C 28.1-40 min: 20% A, 80% C 40-40.1 min: ramped to 60% A, 40% C 40.1-45 min: 60% A, 40% C

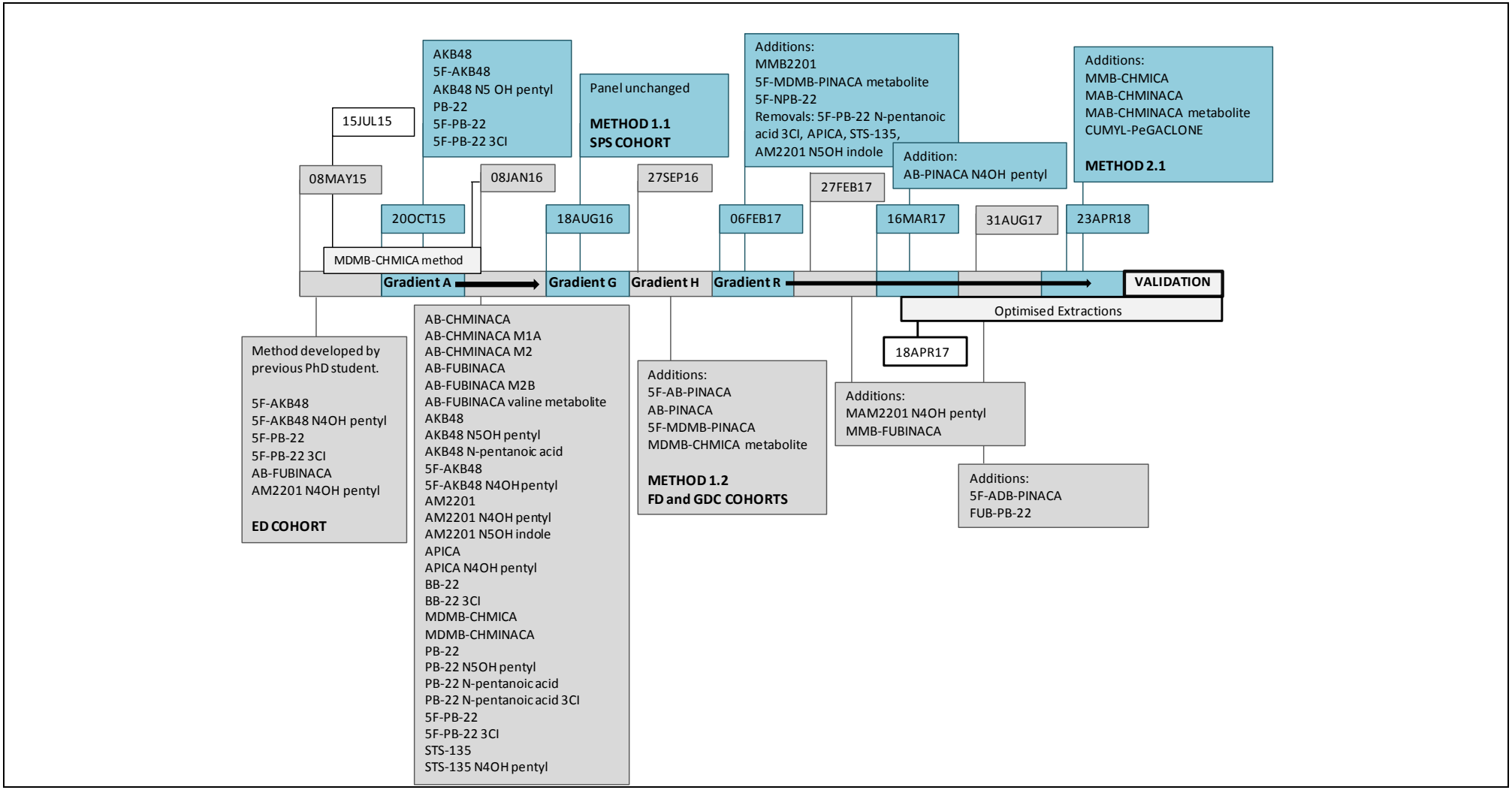


Figure 42 – Chronological flow diagram of details of methods employed to detect Synthetic Cannabinoid Receptor Agonists in post-mortem samples between May 2015 and April 2019

5.2. Individuals Undergoing Emergency Department Treatment

5.2.1. Introduction

From May to November 2015 FMS was involved in a collaborative research project with the emergency department (ED) of Glasgow Royal Infirmary (GRI) to analyse clinical samples from individuals treated for suspected drug toxicity. The priority for this project was to analyse samples for NPS, but alcohol and traditional drugs of abuse were included in the battery of analyses if noted case circumstances indicated use of these substances, and sufficient sample volume was available.

Ethical approval was not required as the work was deemed service development. Similarly, no consent was required from the sample donors.

5.2.2. Method

Samples of blood and/or urine were collected by clinicians at GRI ED in cases where an individual presented with symptoms indicative of drug toxicity. The original purpose of these samples was for clinical testing and diagnosis. No separate sample was collected for this project as it was not possible to get informed consent from impaired individuals. The remainder of the samples was sent, along with clinical observations and any available case history, by courier to FMS, where they were stored between 2 – 8 °C. As the sample received was what remained of the clinical sample, volume was sometimes limited and urine not always available. Samples were analysed within 21 days of receipt.

SCRA analysis was assigned if case circumstances were indicative of SCRA use or if SCRA product(s) were noted as having been taken on sample documentation. SCRA analysis was conducted according to a previously developed method which included the analytes 5F-AKB48, AKB48 N5OH pentyl, 5F-PB-22 and 5F-PB-22 3-carboxyindole (131). The 5F-PB-22 3-carboxyindole included in this method was from a different source than the faulty batch discussed in Section 4.4.1, and thus the results are valid for this compound in this cohort. If sample volume allowed, samples were also extracted and analysed for MDMB-CHMICA using the validated method detailed in 3.4. In cases where sample volume was limited, a decision was made as to whether to analyse for MDMB-CHMICA only or the wider panel. Additional analyses, conducted by laboratory staff, were assigned based on case

circumstances and sufficient sample volume, and these are detailed in Table 55, in Appendix C, Section 9.2.

The results of all toxicological analyses were reported to clinicians at GRI ED on completion of individual case work.

More comprehensive clinical details, such as heart rate, blood pressure, Glasgow Coma Score (GCS), Poison Severity Score (PSS), treatment and results of clinical tests were received after toxicological results had been obtained and were noted with toxicological findings. Due to the individual nature of each case, the clinical details available were not standardised.

5.2.3. Results and Discussion

Between 8th May and 27th November 2015, 98 cases in total were received from GRI ED for analysis. Of these, 34 (35%) cases were submitted for SCRA analysis. All cases included a blood sample and 11 cases contained blood and urine samples, both of which were analysed. Nineteen out of 34 cases (56%) showed use of SCRAs, with 14 (41%) cases negative for all SCRAs in all matrices available. Blood samples for 2 cases gave inconclusive results for the wider SCRA analysis, due to ion suppression from the matrix and insufficient volume to repeat, but were positive for MDMB-CHMICA. Urine samples gave negative results for the wider SCRAs analysis and MDMB-CHMICA in these cases, but the cases overall were designated positive as one drug had been detected in one matrix. One case, for which only one sample was received, was analysed for MDMB-CHMICA as the priority, and insufficient sample volume remained after this analysis for the wider SCRA analysis. This case was treated as inconclusive overall. The MDMB-CHMICA method was only developed after the first 12 cases submitted for SCRA analysis had been completed and thus no information on the presence or absence of this drug in these initial samples is available. The results obtained are summarised in Table 41.

The details of the MDMB-CHMICA analysis for this cohort have been published (72), and a copy of this paper is given in Appendix D, Section 9.4.

Table 41 – Summary of results for Emergency Department samples tested for Synthetic Cannabinoid Receptor Agonists

Result	No. Cases
Positive	**19**
in 1 of 2 matrices	3
in all matrices	16
Negative	**14**
Inconclusive	**1**

Detailed information regarding specific SCRAs, concentrations found, other drugs detected and clinical observations found in SCRA-positive cases can be found in Table 42.

The cases positive for one or more SCRA comprised samples from 17 males (89%) and 2 females (11%). The ages ranged from 14 – 55 years with a mean and median of 24.9 and 21 years respectively. These data are in-keeping with other studies which have found younger males the most likely to use SCRAs (14, 23, 133-135). In data collated from 35 studies into acute/sub-acute cases of SCRA intoxication conducted mostly in the USA, but also Germany, Hong Kong and Switzerland, the mean age of the affected individual was 22.6 years, with the median and mode both 20 years and the range 14 – 59 years (14). The split by sex was 79% male to 21% female (14).

The detection of SCRAs in individuals as young as 14 in both the current study and other studies is concerning as the effects of these compounds on physiological and psychological development are not fully understood. O'Shea *et al.* found impairment in working memory and social interaction in adolescent rats treated with CP 55,940; effects which were absent in the adult rat control group (136). While far from conclusive, this indicates a difference in effect between the age groups. Negative pre-natal physiological and adolescent psychosocial development effects associated with cannabis use have also been reported (137).

The number of compounds present in positive ED cases in this study is presented in Figure 43. This shows the majority of positive cases contained 1 compound only, and none were found positive for more than 4 compounds.

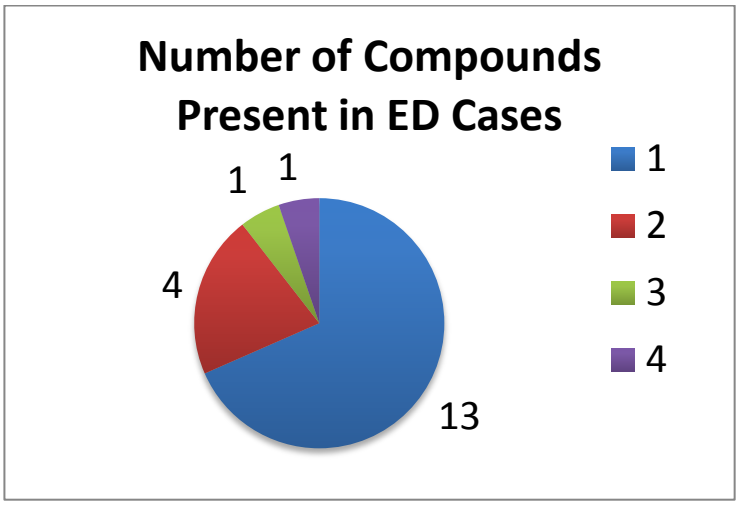


Figure 43 – Number of Synthetic Cannabinoid Receptor Agonists detected in samples within the Emergency Department cohort.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
1*	5F-AKB48 (2 ng/mL)	5F-PB-22 3-carboxyindole (22 ng/mL)	Alcohol, Basic Drugs, DOA screen, Benzodiazepines 1	Lorazepam (0.022 mg/L, administered in ED)	24 y.o. male, reported to have taken 'Vertex' and 'Cherry Bomb' products within 6 hours of presentation at ED. Sinus tachycardia and GCS of 6. Unresponsive initially, extremely combative, incontinent of urine, dilated pupils, recurrent hypoglycaemia, severe serotonin toxicity, rhabdomyolysis and febrile. Duration of hospitalisation was 10 days.	CNS 3, CVS 2, MS. 2, Liver 1, Kidney 0, Blood 0, Muscle 3
2*	5F-AKB48 (<1 ng/mL)	None available	Alcohol, Basic Drugs and DOA screen	Alcohol (237 mg/100mL), DOA screen POS for benzodiazepines (insufficient for confirmation)	18 y.o. male, reported to have taken 'Damnation' product within 6 hours of presentation at ED. Sinus tachycardia and GCS of 14. Violent, incontinent of urine and recurrent hypoglycaemia. Duration of hospitalisation was 2 days. Same individual as cases 10, 14 and 17.	CNS 3, CVS 0, MS 1, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
3*	5F-AKB48 (<1 ng/mL)	5F-AKB48 N4OH pentyl (5 ng/mL), 5F-PB-22 3-carboxyindole (<1 ng/mL)	Alcohol, Basic Drugs and DOA screen	None	14 y.o. male, reported to have smoked an unspecified 'legal high' with cannabis within 3 hours of presentation at ED. GCS of 15. Exhibited agitation and paranoia. Duration of hospitalisation was 3 hours.	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
4*	5F-AKB48 (3 ng/mL)	None available	Alcohol, Basic Drugs, DOA screen, Cannabinoids	THC-COOH (<4 ng/mL), Alcohol (200 mg/100 mL)	22 y.o. male, reported to have taken 'Black Mamba' product with alcohol within 2 hours of presentation at ED. GCS of 14. Exhibited abusive, aggressive behaviour and syncope. Duration of hospitalisation was 2 hours.	CNS 2, CVS 2, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
5*	NEG	5F-PB-22 3-carboxyindole (Present)	Alcohol, Basic Drugs and DOA screen	None	18 y.o. male, reported to have taken 'Exodus' product with alcohol within 6 hours of presentation at ED. Found collapsed but on arrival at ED GCS was 15. Duration of stay was 3 hours.	All 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
6*	5F-PB-22 3-carboxyindole (Present)	5F-AKB48 N4OH pentyl (present), 5F-PB-22 3-carboxyindole (Present)	Alcohol, Basic Drugs, DOA screen, Benzodiazepines 1	Desmethyldiazepam (<0.10 mg/L), Chlordiazepoxide (Present)	55 y.o. male, reported to have taken 'Exodus Damnation' product within 2 hours of presentation at ED. Exhibited a dissociative state with GCS of 13. Prescribed chlordiazepoxide.	CNS 2, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
7	MDMB-CHMICA (5 ng/mL)	None available	Alcohol, Basic Drugs, DOA screen, Benzodiazepines 1	Diazepam (0.075 mg/L), Alcohol (130 mg/100 mL)	20 y.o. female, reported to have taken 'Sweet Leaf' product within 6 hours of presentation at ED. Found unconscious, exhibited acute behavioural disturbance with sinus tachycardia and a GCS of 10. Duration of hospitalisation was 1.5 days.	CNS 2, CVS 1, MS 1, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
8	5F-AKB48 (Present), 5F-PB-22 (Present)	None available	Alcohol, Basic Drugs, DOA screen, Cannabinoids	DOA screen POS for cannabinoids (NEG in confirmation)	41 y.o. male, reported to have taken 'Black Mamba' product within 4 hours of presentation at ED. Unresponsive, hypothermic (temp. <33 °C), with sinus bradycardia and a GCS of 10. Duration of hospitalisation was 1 day. Same individual as case 9.	CNS 2, CVS 3, MS 3, Liver, Kidney, Blood and muscle unknown
9	5F-AKB48 (Present), 5F-PB-22 (Present), 5F-PB-22 3-carboxyindole (Present), MDMB-CHMICA (22 ng/mL)	None available	Alcohol, Basic Drugs and DOA screen	None	41 y.o. male, reported to have taken 'Black Mamba' product. Unresponsive, exhibited recurrent hypoglycaemia, hypothermia (temp <33 °C). Same individual as case 8.	Unknown

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
10	5F-AKB48 (Present)	None available	Alcohol, Basic Drugs and DOA screen	Diphenhydramine (0.20 mg/L), Alcohol (93 mg/100 mL)	18 y.o. male, reported to have taken 'Exodus Damnation' and 'Annihilation' products within 4 hours of presentation at ED. Presented with sinus tachycardia, vomiting and dizziness, GCS of 15. Duration of hospitalisation 2 hours. Same individual as cases 2, 14 and 17	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
11	5F-AKB48 (Present), MDMB-CHMICA (<5 ng/mL)	None available	Alcohol, Basic Drugs, DOA screen, Cannabinoids	THC-COOH (4 ng/mL), Alcohol (80 mg/100 mL)	25 y.o. male, reported to have taken 'Sweet Leaf' and 'Saint Row' products with alcohol within 4 hours of presentation at ED. Exhibited syncope, possible seizures and confusion, with a GCS of 14. Duration of hospitalisation was 3.5 hours.	CNS 2, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
12	MDMB-CHMICA (<2 ng/mL)	None available	Alcohol, Basic Drugs and DOA screen	None	15 y.o. male, reported to have taken 'Red Exodus' product within 6 hours of presentation at ED. Exhibited profuse vomiting with GCS of 13. Duration of hospitalisation was 1 day.	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
13	5F-AKB48 (Present)	None available	Alcohol, Basic Drugs and DOA screen	Methadone (0.27 mg/L), DOA screen POS for benzodiazepines and cannabinoids (insufficient for confirmation)	43 y.o. male, reported to have taken a 'mixed cannabinoid' product within 6 hours of presentation at ED. Found unconscious and febrile with hypoglycaemia and a GCS of 6. Treatment was ongoing 5 months after incident.	CNS 3, CVS 0, MS 1, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
14	AKB48 OH pentyl metabolite (Present)	None available	Alcohol, Basic Drugs and DOA screen	Alcohol (138 mg/100 mL)	18 y.o. male, reported to have taken 'Exodus Damnation' product within 4 hours of presentation at ED. Exhibited seizures prior to, and acute behavioural disturbance within ED. Sinus tachycardia and a GCS of 15. Duration of hospitalisation was 1.5 hours. Same individual as cases 2, 10 and 17	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
15	MDMB-CHMICA (<1 ng/mL)	None available	Alcohol, Basic Drugs, DOA screen, Benzodiazepines 2	DOA screen POS for benzodiazepines (confirmation NEG)	21 y.o. male, inmate at SPS facility, reported to have taken a 'legal high' product within 24 hours of presentation at ED. Exhibited drug-induced psychosis and acute behavioural disturbance with a GCS of 14. Duration of hospitalisation was 3 hours.	CNS 3, CVS 0, MS 1, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
16	MDMB-CHMICA (<1 ng/mL)	NEG (tested for MDMB-CHMICA only)	Alcohol, Basic Drugs, DOA screen, Cannabinoids	THC-COOH (9 ng/mL), Alcohol (225mg/100 mL)	16 y.o. female, reported to have taken 'Red X' with alcohol within 6 hours of presentation at ED. Exhibited severe disorientation, acute behavioural disturbance (barking noises), sinus tachycardia, a GCS of 14 and mild hypothermia. Duration of hospitalisation was 1 day.	CNS 3, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
17	MDMB-CHMICA (2 ng/mL)	5F-PB-22 3-carboxyindole (Present)	Alcohol, Basic Drugs, DOA screen, Benzodiazepines 2	Alcohol (229 mg/100 mL)	19 y.o. male, reported to have taken 'Damnation' product within 6 hours of presentation at ED. Exhibited acute behavioural disturbance, increased limb tone, sinus tachycardia and a GCS of 12. Duration of hospitalisation was 1 day. Same individual as case 2, 10 and 14.	CNS 3, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

Table 42 – Clinical and toxicological findings in Emergency Department samples which are positive for Synthetic Cannabinoid Receptor Agonists.

Case No.	SCRA Findings Blood	SCRA Findings Urine	Additional Analyses	Additional Toxicological Findings	Clinical Observations	Poison Severity Score
18	MDMB-CHMICA (1 ng/mL)	None available	Alcohol, Basic Drugs, DOA screen, Cannabinoids, Benzodiazepines 2, Opiates	Morphine (<0.05 mg/L), THC (5 ng/mL), THC-COOH (35 ng/mL), Diazepam (0.2 mg/L), Desmethyldiazepam (0.13 mg/L)	24 y.o. male, reported to have taken 'Obliteration' product within 4 hours of presentation at ED. Was found unconscious. GCS of 13. Duration of hospitalisation was 1 day.	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0
19	MDMB-CHMICA (4 ng/mL)	NEG	Alcohol, Basic Drugs, DOA screen, Cannabinoids, Benzodiazepines 2	THC-COOH (23 ng/mL), Diazepam (0.28 mg/L), Desmethyldiazepam (0.34 mg/L),	22 y.o. male, reported to have taken 'K2' within 4 hours of presentation at ED. Exhibited dissociative state and syncope with sinus tachycardia and a GCS of 13. Duration of hospitalisation was 12 hours.	CNS 1, CVS 0, MS 0, Liver 0, Kidney 0, Blood 0, Muscle 0

* MDMB-CHMICA analysis not performed. DOA = Drugs of Abuse. ED = Emergency Department. y.o. = years old. GCS = Glasgow Coma Scale. CNS = Central Nervous System. CVS = Cardio-Vascular System. MS = Metabolic System. POS = Positive. THC = Tetrahydrocannabinol. THC-COOH = 11-Nor-Δ9-THC-carboxylic acid. NEG = Negative. SPS = Scottish Prison Service.

The most commonly encountered compounds in this cohort were 5F-AKB48 and MDMB-CHMICA (n=9), followed by 5F-PB-22 3-carboxyindole (n=6), 5F-AKB48 metabolite (n=3) and 5F-PB-22 (n=2). The times since sample collection, windows of detection and analyte stability should all be borne in mind when interpreting the significance of these findings. The more frequent presence of the 5F-PB-22 metabolite, rather than the parent drug, in both blood and urine suggests 5F-PB-22 is broken down more rapidly in the body than 5F-AKB48, which was detected in parent form. It is also possible that 5F-PB-22 continues to breakdown in the stored sample, while 5F-AKB48 does not - at least not to the same extent. The dose and mechanism of action of 5F-PB-22 should also be considered when interpreting the concentrations and absence of parent drug. As no metabolite for MDMB-CHMICA was included in the method, no corresponding observations can be made for this drug. Overall, results are indicative that 5F-AKB48 was taken in 11 cases, MDMB-CHMICA in 9 cases and 5F-PB-22 in 6 cases. Due to the lack of information on SCRA pharmacodynamics generally, and for these compounds specifically, no interpretation of their contribution to adverse effects was made. SCRAs detected in blood were much more likely to be exerting a – potentially significant – physiological effect at the time of collection, compared to compounds detected in urine only. That being said, undetected active metabolites could have been present in the blood in cases where the urine only was found positive.

The most commonly co-administered substance was alcohol (n=8), followed by benzodiazepines (n=5 confirmed, including 1 administered in hospital, plus 2 presumptive positive) and cannabis (n=5). The notes received for two cases (Case 1 and Case 6) highlighted that lorazepam was administered during hospital treatment (Case 1) or chlordiazepoxide was prescribed (Case 6). Indeed benzodiazepines are mentioned in the NEPTUNE document on SCRA harms and their management as having been reported as some value in SCRA treatment (108). However, ED staff in this project noted specifically that no diazepam had been administered to these patients so use must be through GP prescription or illicit.

The presence of alcohol as a co-administered substance in 42% of cases is not surprising, given its ubiquity in Scottish culture. The concentrations at

which it was found in cases, however, were moderately high: the mean and median concentrations were 167 and 169 mg/100mL respectively. Given the commonality of alcohol in the Scottish population, it is likely that the individuals possess some degree of tolerance to this. This tolerance would affect the physiological effects of alcohol on the individuals in question.

Similarly, 5 (26%) cases were positive for the inactive Δ9-THC metabolite 11-nor-Δ9-THC-carboxylic acid, and one case was positive for Δ9-THC itself. This is indicative of co-administration of cannabis and SCRAs; if not acutely co-administered then at least taken together over a broader period of weeks to months (as the Δ9-THC metabolite is known to be sequestered in fat, and be released over time). This finding is also reinforced in the literature (106). It is difficult, therefore, to separate the effect that might have been exerted by the SCRA from the effect of alcohol, any co-administered substances or the potentially synergistic effect of these.

The GCS allows standardised communication regarding the consciousness of an individual. In order to calculate this, scores from 1-4 are given for ocular responsiveness; scores from 1-5 are given for verbal responsiveness; and scores from 1-6 are given for motor responses. These are summed to provide the overall score, with the maximum of 15 representing a completely conscious and alert subject (138).

The GCS range found in the SCRA-positive cases described here was 6 - 15, with the mean and median being 12.6 and 13.5 respectively. While this indicates a variety in the severity of effects on consciousness, it suggests the majority of individuals' consciousness was not significantly affected. The same range was found in a series of analytically confirmed SCRA-positive cases detailed by Abouchedid *et al.* with a mean of 13.9 and a median of 15 (134).

Similarly, the PSS was developed to categorise the severity of poisoning in qualitative and translatable terms. It assigns a number from 0 (asymptomatic) to 4 (fatal), with 1 (minor), 2 (moderate) and 3 (severe) in between (139). Individual scores can be given to different systems and organs: Central Nervous System (CNS), Cardiovascular System (CVS), Metabolic System (MS), Liver, Kidney, Blood and Muscle.

In the cases positive for SCRAs presented here, 7 cases (37%) exhibited no or mild symptoms on the PSS. With regards to CNS symptoms, 6 cases (32%) were categorised as severe and 5 cases (26%) were categorised as moderate.

The second most commonly raised score on the PSS was for the CVS, with 2 cases scoring at 2 and one each for 1 and 3. The majority of noted cardiovascular symptoms were sinus tachycardia (n=8, 42%), with only 1 where sinus bradycardia was noted.

Where noted and symptomatic, the MS was scored as 1 four times, and 2 or 3 once each. The muscle and liver were scored as 3 once and 1 once respectively. The kidneys and blood showed no symptoms where scores were noted.

With regards to behavioural symptoms, syncope, collapse or unresponsiveness was noted in 10 cases (53%); behavioural disturbance was noted in 5 cases (26%); combative, aggressive or violent behaviour was noted in 3 cases; seizures were noted in 2 cases; and agitation and paranoia was noted in 1 case. More physiological symptoms included hypoglycaemia (n=4), hypothermia (n=3) and severe serotonin toxicity (n=1).

It was suspected that the hypothermia exhibited by some individuals was an action of the drug, as clinical staff treating the individuals in the ED noted the outside temperatures around the time of presentation to be ambient (*ca.* 12 °C). Lowered body temperatures were also present in the majority (61%) of cases studied by Abouchedid *et al.* (134).

The relationship between SCRAs and blood glucose concentration is not so clear. The hypoglycaemia presented here is not mirrored in the literature, with some studies describing hyperglycaemia as a symptom of SCRA toxicity (25). It is possible that the symptom described here is either coincidental, or present due to the action of additional drugs or generally poorer wellbeing in the affected individuals.

The symptoms observed in this case series tend to agree with the findings of Tait *et al.,* who conducted an analysis of adverse events associated with SCRA use in the literature (23). They also found that tachycardia was a

common presentation in cases of SCRA toxicity, in addition to the presence of seizures and vomiting in patients. Agitation, new onset psychosis, paranoia and hallucinations were noted as psychological effects presented in their study. The authors also warned that a non-specific toxidrome associated with SCRA use could lead to drug toxicity being overlooked as the aetiology of the symptoms.

In another presentation of adverse health effects van Amsterdam, Brunt and van den Brink reported effects such as anxiety, panic attacks, agitation, delusions as well as nausea/vomiting, seizures, tachycardia, hypertension, hypokalaemia and hyperglycaemia (140). They were, however, clear to highlight the variation in adverse effects from the use of different SCRA compounds.

Similarly, tachycardia, seizure, agitation and psychosis are described in SCRA-positive cases in Abouchedid's study, and these symptoms plus vomiting are noted by Bäckberg *et al.* (134, 141).

The interval between drug ingestion and presentation at ED was noted in 18 cases and ranged from 2 – 24 hours (mean and median 5.7 and 5 respectively). It is important to bear this in mind when considering the SCRA concentrations and clinical observations for each case. It is acknowledged that the number of SCRAs detected by this method is low, and that additional compounds – including additional, active metabolites of the parent compounds included and others – may contribute to the effects observed. Maintaining a panel of drugs which is up-to-date with current trends was a noted challenge of this project.

The duration of hospitalisation ranged from 1.5 hours to > 5 months, with a mean and median of 30 and 7.8 hours (when the case with treatment ongoing after 5 months was excluded). The variation in length of treatment time is to be expected given the diversity of psychological and physiological effects described, and the relative novelty of SCRA toxicity to medical professionals. Guidelines such as those provided by NEPTUNE should allow a framework for ED staff to build experience in treating individuals exhibiting SCRA toxicity (108). This being said, the diagnosis of SCRA toxicity in this case series was confirmed weeks after presentation, and as yet, there is no

commercially available point of care test (POCT) which can detect all the SCRA compounds, or even those most likely to be encountered in the UK. As a result, the diagnosis of the symptoms as SCRA-related may be reliant on patient communication, which may not be possible if the patient is unconscious or suffering from psychological disturbances. It was not disclosed what treatment the individual in Case 13 was receiving 5 months after initial presentation, or whether this was linked to SCRA use.

5.2.4. Conclusion

Whilst this data set is limited in size, and does not measure prevalence of SCRA use, the results show that these drugs are being used by individuals requiring hospital treatment.

The effects of co-administered substances cannot be ruled out as contributing factors in the attendance at the ED. However, the detection of very low concentrations of SCRAs in the blood and associated physiological and psychological harms is indicative of highly potent substances. Of particular interest is Case 3 where a sub-nanogramme concentration of 5F-AKB48 was detected in the blood sample along with a sub-nanogramme concentration of 5F-PB-22 3-carboxyindole and 5 ng/mL of 5F-AKB48 N4OH pentyl metabolite which were detected in the urine, with no co-administered substances detected. Whilst little interpretation of concentrations of SCRAs is possible, due to very limited pharmacodynamic data, it is important to consider the potentially significant contribution of SCRAs present in the blood to intoxication. While urinary concentrations will no longer be pharmacologically active, their presence does indicate SCRA use, and the possibility of undetectable active metabolites present in the blood remains.

The time since sampling and possible instability should be borne in mind when considering the compounds detected, and the concentrations at which these were found, relative to the LOD.

Further research with a larger sample size and including a broader range of analytes would be beneficial to gain a deeper insight into the medical consequences and demographics of SCRA use.

5.3. Post-Mortem Casework

5.3.1. Introduction

Forensic Medicine and Science (FMS) at the University of Glasgow (UG) provides a post-mortem toxicology service covering Greater Glasgow and Clyde (GGC), Lothian, Tayside, Ayrshire and Arran (AA), Dumfries and Galloway (DG) and Borders NHS regions. This encompasses the cities of Edinburgh, Glasgow and Dundee, and has a caseload in excess of 3800 cases per year.

Due to the constantly shifting trends relating to recreational drug use, the analyses required to cover this diverse group of compounds are particularly dynamic. The emergence of Novel Psychoactive Substances (NPS) made it necessary for FMS to develop methods to detect and quantify SCRAs in post-mortem case samples. The methods detailed in Chapter 4 were applied to the case samples described below, depending on the sample type and period of receipt.

Ethical approval for mining data from the FMS case database was sought and received from the UG College of Medical Veterinary and Life Sciences (MVLS) REC. See Section 9.4, Appendix E, for details.

5.3.2. Method

5.3.2.1. Sample Collection

Samples of blood and urine were collected by pathologists or mortuary technicians during PM. As standard, blood was collected from the femoral vein by dissecting the vessel and collecting the blood in a universal container as it empties. Depending on the condition of the cadaver, or circumstances of death, blood from different sites such as the axillary vein, chest or abdominal cavities may have been collected. Where available, urine was extracted from the bladder using a syringe and transferred into a universal container. Where a sample is referred to as preserved, this relates to the use of a container containing a pre-loaded mass of sodium fluoride and potassium oxalate.

After collection, samples are delivered securely by courier to FMS where they are stored in a temperature-monitored fridge (maintained between 2 – 8 °C) for up to 3 months before transfer to a temperature-monitored freezer (≤ -18

°C). Generally, all analyses were conducted prior to sample freezing, but a low number of samples may have been frozen and thawed once prior to analysis.

5.3.2.2. Sample Analysis

SCRAs analysis was conducted on unpreserved blood and preserved urine where available and where sample volume allowed.

Due to the nature of the project, and the time span covered (2015 – 2019), the specifics of the methods used to analyse samples varied. Either the original or optimised extraction protocol, detailed in Table 19, was used. The instrumental method adhered to the parameters detailed for methods 1.1, 1.2 or 2.1. The only additional variation within methods was the panel of compounds covered, which was frequently updated to include additional compounds when they became potentially available on the NPS market. These were added at various stages throughout method development. For a small number of very early samples, the previously validated method used in Section 5.2 was employed. For this method, the 5F-PB-22 3-carboxyindole metabolite was included in the panel, and all samples positive for this compound were detected using the valid reference standard. Figure 42 provides a summary of the details of different methods applied to PM samples over the period of study.

Additional analyses for commonly encountered prescription and abused drugs, as well as alcohol and β-hydroxybutyrate (BHB) were conducted by laboratory staff, based on case circumstances.

5.3.3. Results and Discussion

The results from the analysis conducted in Section 5.3.2.2 are shown in Table 43. This table provides the date of case receipt, toxicological results, case circumstances and causes of death determined by the pathologist. Out of 250 cases tested for SCRAs between Summer 2015 and Spring 2019, 28 cases were found positive for at least 1 SCRA in at least 1 biological sample, a positivity rate of 11.2%.

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
1	15 June 2015	MDMB-CHMICA (1 ng/mL) (sub-clavian blood)	5F-PB-22 3-carboxyindole (Present)	Amitriptyline (0.12 mg/L)	44 y.o. male, found hanged	1a - Hanging
2	28 July 2015	MDMB-CHMICA (<1 ng/mL)	NEG	Alcohol (237 mg/dL) BHB (249 mg/L) Acetone (<100 mg/L)	38 y.o. male, alcoholic, found unresponsive at home	1a - Complications of acute and chronic alcoholism
3	25 September 2015	5F-AKB48 (Present)	5F-PB-22 3-carboxyindole (Present)	Sertraline (1.1 mg/L) Olanzapine (0.08 mg/L) Zopiclone (<0.05 mg/L)	44 y.o. male, previous mental health issues, found unresponsive after bouts of vomiting	1a - Coronary artery thrombus 1b - Coronary artery atherosclerosis 2 - Hypertensive heart disease
4	08 January 2016	5F-PB-22 (<0.5 ng/mL) AKB48 N5OH pentyl (<0.5 ng/mL) MDMB-CHMICA (<0.5 ng/mL)	None available	Tramadol (0.94 mg/L) Citalopram (0.55 mg/L)	52 y.o. male collapsed and became unresponsive after bouts of vomiting	1a – Bronchopneumonia 1b - Chronic Bronchitis and Emphysema 2 Atherosclerotic Coronary Artery Disease and Synthetic Cannabinoid Intoxication

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
5	04 March 2016	AKB48 N5OH pentyl (Present)	NEG	Morphine (0.06 mg/L) (6MAM in the urine)	31 y.o. male found collapsed in prison cell	1a - Adverse effects of heroin and AKB48 N5OH pentyl
6	16 March 2016	AKB48 N5OH pentyl (Present) MDMB-CHMICA (Present) AB-FUBINACA valine metabolite (Present) AB-FUBINACA M2B (Present)	AKB48 N5OH pentyl (Present) AB-FUBINACA (Present) AB-FUBINACA valine metabolite (Present) AB-FUBINACA M2B (Present)	Alcohol (12 mg/100mL) COHb (51%) Methadone (1.2 mg/L) Etizolam (0.11 mg/L) THC-COOH (6 ng/mL)	31 y.o. male found unresponsive in a house fire, “legal highs” and needles found at scene. Same scene as Case 8.	1a - Smoke Inhalation and Carbon Monoxide Poisoning 1b - House Fire 2 Acute Drug Misuse
7	16 March 2016	AB-FUBINACA valine metabolite (Present)	None available	COHb (48%) Etizolam (0.016 mg/L) Morphine (<0.025 mg/L)	56 y.o. female, found unresponsive in a house fire, “legal highs” and needles found at scene. Same scene as Case 7.	1a - Inhalation of Smoke and Carbon Monoxide Toxicity 1b - House Fire 2 Drug Misuse
8	25 May 2016	5F-PB-22 (<0.50 ng/mL) MDMB-CHMICA (<0.50 ng/mL)	None available	Alcohol (11 mg/100mL) BHB (39 mg/L) Flubromazepam (0.013 mg/L) THC-COOH (8 ng/mL)	50 y.o. male found unresponsive at home, history of mental health issues, drug use and alcohol	1a - Ischaemic heart disease and possible drug toxicity 2 - Fatty degeneration of the liver

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
9	08 June 2016	MDMB-CHMICA *O*-desmethyl acid metabolite (Present)	NEG	Alcohol (114 mg/100mL)	33 y.o. male, found collapsed in homeless accommodation	1a - Complications of drug toxicity
10	04 July 2016	5F-MDMB-PINACA (Present)	None available	None	37 y.o. male suspected of heroin and "legal high" use. Found collapsed on the toilet with the hood of his sweatshirt caught on the tap, appearing to have strangled him. "Cherry Bomb" found near body.	1a - Hanging

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
11	19 October 2016	NEG	AB-FUBINACA valine metabolite (Present)	Morphine (0.08 mg/L) Codeine (0.14 mg/L) (6MAM in the urine) Pregabalin (7 mg/L) Etizolam (0.006 mg/L) Diazepam (0.76 mg/L) Desmethyldiazepam (0.38 mg/L) Oxazepam (<0.05 mg/L) Temazepam (<0.05 mg/L) Tramadol (0.14 mg/L) THC-COOH (2 ng/mL)	28 y.o. male with history of drug use and mental health issues, found unresponsive after injecting heroin	1a - Adverse Effects of Heroin, Pregabalin, Tramadol, Diazepam and Etizolam
12	06 January 2017	5F-MDMB-PINACA *O*-desmethyl acid metabolite (Present) AB-FUBINACA valine metabolite (Present) MDMB-CHMICA *O*-desmethyl acid metabolite (Present)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (Present) AB-FUBINACA valine metabolite (Present) MDMB-CHMICA *O*-desmethyl acid metabolite (Present)	Alcohol (311 mg/100mL) Fluoxetine (0.18 mg/L) Norfluoxetine (0.39 mg/L)	49 y.o. male alcoholic, "addicted" to legal highs, found unresponsive at home	1a - Acute and chronic alcoholism in association with synthetic cannabinoid use

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
13	30 May 2017	5F-MDMB-PINACA *O*-desmethyl acid metabolite (<0.50 ng/mL) (ante-mortem blood)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (1.1 ng/mL) (ante-mortem urine)	Methadone (0.11 mg/L) Amitriptyline (0.19 mg/L) Zopiclone (0.012 mg/L)	46 y.o. male found unresponsive in prison cell after witnessed strange behaviour. Died in hospital	1a - Hypoxic brain injury 1b - cardiac arrest 1c - suspected drug toxicity
14	21 September 2017	MDMB-CHMICA *O*-desmethyl acid metabolite (2.5 ng/mL)	MDMB-CHMICA *O*-desmethyl acid metabolite (1.1 ng/mL)	Fluoxetine (0.52 mg/L) Norfluoxetine (0.42 mg/L) COHb (<10%) THC-COOH (20 ng/mL)	21 y.o. female with a history of mental health issues, found unresponsive in bed	1a - Complications of anorexia nervosa
15	06 October 2017	5F-MDMB-PINACA (Present) 5F-MDMB-PINACA *O*-desmethyl acid metabolite (7.4 ng/mL)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (24 ng/mL)	Zopiclone (0.043 mg/L)	30 y.o. male found unresponsive in prison room	1a - Adverse effects of 5F-MDMB-PINACA
16	24 November 2017	5F-MDMB-PINACA *O*-desmethyl acid metabolite (0.05 ng/mL) AB-FUBINACA valine metabolite (0.11 ng/mL)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (<0.10 ng/mL) AB-FUBINACA valine metabolite (<0.10 ng/mL)	BHB (>500 mg/L) Acetone (298 mg/L)	23 y.o. male found unresponsive in supported accommodation surrounded by bags of "psychoactive substances"	1a - diabetic ketoacidosis 1b – Insulin dependent diabetes mellitus

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
17	21 February 2018	5F-MDMB-PINACA (Present) 5F-MDMB-PINACA *O*-desmethyl acid metabolite (Present) AB-FUBINACA valine metabolite (Present)	5F-MDMB-PINACA (Present) 5F-MDMB-PINACA *O*-desmethyl acid metabolite (Present) AB-FUBINACA valine metabolite (Present)	Methadone (0.2 mg/L) Mirtazapine (0.02 mg/L) Lignocaine (0.30 mg/L)	46 y.o. male stabbed in prison	1a - stab wounds of trunk
18	04 April 2018	AB-FUBINACA valine metabolite (0.29 ng/mL)	AB-FUBINACA valine metabolite (1.5 ng/mL)	Methadone (0.6 mg/L) Morphine (0.09 mg/L) Etizolam (0.06 mg/L) Pregabalin (30 mg/L)	36 y.o. male found unresponsive 2 days after prison release, witnessed behaviour under the influence	1a - multiple drug toxicity 2 Coronary artery atheroma
19	02 May 2018	5F-MDMB-PINACA *O*-desmethyl acid metabolite (3.0 ng/mL) AB-FUBINACA valine metabolite (0.19 ng/mL)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (14 ng/mL) AB-FUBINACA valine metabolite (0.90 ng/mL)	Alcohol (11 mg/100mL) Methadone (1.1 mg/L) Mirtazapine (0.19 mg/L)	49 y.o. male found unresponsive in prison cell	1a - Methadone and synthetic cannabinoid receptor agonist intoxication
20	21 June 2018	5F-MDMB-PINACA *O*-desmethyl acid metabolite (1.1 ng/mL) AB-FUBINACA valine metabolite (0.56 ng/mL)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (2.7 ng/mL) AB-FUBINACA valine metabolite (1.4 ng/mL)	None	33 y.o. male found hanged in prison cell	1a - Hanging

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
21	11 July 2018	5F-MDMB-PINACA (0.18 ng/mL) 5F-MDMB-PINACA *O*-desmethyl acid metabolite (19 ng/mL) (ante-mortem blood)	None available	Methadone (0.67 mg/L) Mirtazapine (0.02 mg/L)	47 y.o. male found collapsed in prison cell after drugs being found concealed on his body	1a - Myocardial infarction 1b - Coronary artery atherosclerosis
22	05 October 2018	NEG	5F-MDMB-PINACA *O*-desmethyl acid metabolite (<0.20 ng/mL)	6MAM (0.02 mg/L) Morphine (1.3 mg/L) Codeine (0.08 mg/L) THC-COOH (34 ng/mL)	38 y.o. male of no fixed abode, released from prison 4 days prior, found collapsed in car park	1a - Heroin intoxication
23	27 December 2018	5F-MDMB-PINACA *O*-desmethyl acid metabolite (<0.10 ng/mL) (ante-mortem blood)	NEG	Diazepam (<0.05 mg/L) Lorazepam (0.021 mg/L)	25 y.o. male collapsed while using prison gym, died in hospital	1a - Basilar artery dissection
24	31 January 2019	5F-MDMB-PINACA *O*-desmethyl acid metabolite (0.13 ng/mL) AB-FUBINACA valine metabolite (Present)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (0.24 ng/mL) AB-FUBINACA valine metabolite (11 mg/L)	Alcohol (15 mg/100mL) Methadone (0.98 mg/L) Amitriptyline – 0.37 mg/L	43 y.o. male found hanged in prison cell	1a - Hanging

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

Table 43 – Circumstances and findings in Synthetic Cannabinoid Receptor Agonist-positive Post-Mortem casework

Case No.	Date of Receipt	SCRA Findings in Blood	SCRA Findings in Urine	Additional Toxicological Findings in Blood	Case Circumstances	Cause of Death
25	04 February 2019	4F-MDMB-BINACA (0.07 ng/mL)	NEG	Mirtazapine (0.02 mg/L)	36 y.o. male found hanged in prison cell	1a - Hanging
26	12 February 2019	5F-MDMB-PINACA *O*-desmethyl acid metabolite (0.12 ng/mL) AB-FUBINACA valine metabolite (Present)	None available	None	27 y.o. male in prison, circumstances undisclosed	Undisclosed
27	04 April 2019	AB-FUBINACA valine metabolite (5.3 ng/mL)	5F-MDMB-PINACA *O*-desmethyl acid metabolite (<0.20 ng/mL) AB-FUBINACA valine metabolite (11 ng/mL)	EME (<0.25 mg/L), BZE (0.05 mg/L), Morphine (1.2 mg/L), Codeine (0.10 mg/L) (6MAM in the urine)	40 y.o. male found unresponsive in homeless accommodation the day after prison release	1a - Heroin and cocaine intoxication

y.o. = year old NEG = Negative BHB = β-hydroxybutyrate 6MAM = 6-monoacetyl morphine COHb = carboxyhaemoglobin
THC-COOH = 11-Nor-Δ9-THC-carboxylic acid

From this table it is clear that the toxicological picture is often very complex, with a variety of different drugs, with different mechanisms of action, present in cases positive for SCRAs.

Figure 44 shows the number of SCRA compounds detected in PM casework over the study period. The most commonly identified SCRAs, as identified from parent drug or metabolite, were 5F-MDMB-PINACA (n=14), MDMB-CHMICA (n=8) and 5F-PB-22 (n=4). The AB-FUBINACA valine metabolite was present in 12 cases, but this compound is a shared metabolite of AB-FUBINACA and MMB-FUBINACA so, with no parent compound detected, it is unclear which compound was ingested. The windows of detection for SCRAs have not been widely reported. Franz *et al.,* have, however, found that the AB-FUBINACA valine metabolite was still detectable 13 days after a single ingestion of AB-FUBIANCA, and found evidence to indicate heavy consumption could lead to a period of elimination lasting for months (142).

The high turnaround and short lifetime of SCRAs could account for the variety of compounds seen. For example 4F-MDMB-BINACA was only identified in 1 case, but this wass a relatively novel compound (at the time of writing) and if the time period of the study was extended, it is potentially the case that more incidences of this compound would be identified. The 5F-PB-22 3-carboxyindole metabolite was not tested for cases received from early 2016 onwards due to the faulty reference standard being used, and this only being discovered after the conclusion of practical work. The parent drug and another metabolite, PB-22 N-pentanoic acid, were included in the panel so the detection of 5F-PB-22 was possible. The relative abundances of the metabolites should be considered, though, and the fact that PB-22 N-pentanoic acid was not seen where 5F-PB-22 3-carboxyindole was could be indicative that the latter is a much better identifier of use. The inadvertent use of a defective reference standard was unfortunate but unavoidable given the point at which the fault was discovered by the producer and communicated to the researcher. This highlights another challenge associated with working with compounds of such similar structures.

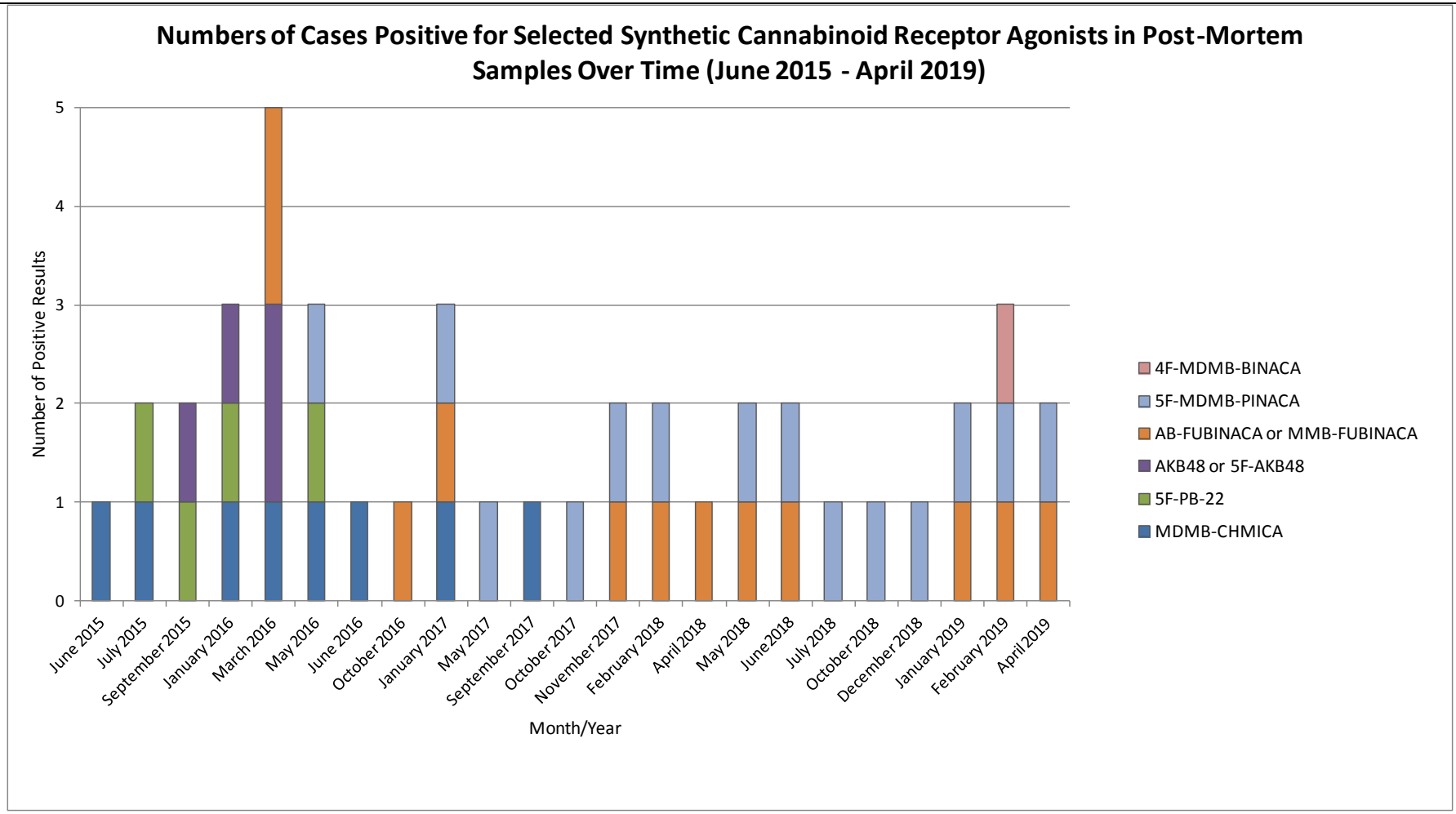


Figure 44 – Bar chart showing the number of cases Synthetic Cannabinoid Receptor Agonist compounds were detected in in post-mortem samples by month and year over the study period (June 2015 – April 2019)

From Table 43, it is clear that SCRAs were not quantified in every case. This was for a variety of reasons. For some batches, time constraints meant that a full calibration and QCs could not be run. As little pharmacological data exists regarding the interpretation of concentrations of SCRAs in PM samples it was determined that a qualitative result was sufficient for the case. In other batches, a calibration and QCs were run but calibrator removal and/or QC failure meant quantitative results could not be reported. After method optimisation and validation, the vast majority of results were reported as quantitative. For these reasons, and the complex toxidrome likely caused by co-administered substances, it is not possible to interpret the concentrations observed. It is clear to see, however, that the concentrations are generally very low, mostly in the sub-nanogramme per millilitre range for blood especially, highlighting the need for sensitive methods of detection. Table 44 shows the concentrations of different compounds detected where quantitative results have been reported. The widest range was observed for the 5F-MDMB-PINACA metabolite at 0.05 – 19 ng/mL. This is one of the most commonly seen SCRAs in PM casework since the method was validated, and as such has the highest number of quantitatively reported results.

As little information is available regarding what might constitute a ‘significant concentration’ of SCRA compounds in PM samples, it is of limited value to report concentrations. Having said that, by providing quantitative information, an understanding of what concentrations might be present in certain circumstances, *i.e.* where no other cause of death is determined, can begin to be formed. If, for example, the concentration range detected in the blood for 5 cases where 5F-MDMB-PINACA has been mentioned as potentially contributing or causing death is available to a pathologist, they may be more confident in certifying additional deaths as related to this drug, where similar concentrations are detected. In order to collate this information, quantitative analysis is required. The concentrations reported in the publication relating to MDMB-CHMICA in the ED cohort (72) (see Appendix D, Section 9.4) have gone some way to remedy this, as they appear in the reference book Disposition of Toxic Drugs and Chemicals in Man (143). This book is referred to frequently by forensic toxicologists when interpreting concentrations of drugs.

Table 44 – Concentrations of Synthetic Cannabinoid Receptor Agonists detected in PM casework, in nanogrammes per litre, where reported quantitatively

Compound	N=	Blood Concentrations (ng/mL)
MDMB-CHMICA	4	<0.5 - 1
MDMB-CHMICA *O*-desmethyl acid metabolite	1	2.5
5F-PB-22	2	<0.5
AKB48 N5OH pentyl	1	<0.5
AB-FUBINACA valine metabolite	5	0.11 - 5.3
5F-MDMB-PINACA	1	0.18
5F-MDMB-PINACA *O*-desmethyl acid metabolite	9	0.05 - 19
4F-MDMB-BINACA	1	0.07

The most commonly co-administered substances were alcohol and methadone, observed in 7 cases each, at median concentrations of 15 mg/100mL (range: 11 – 311 mg/100mL) and 0.67 mg/L (range: 0.11 – 1.2 mg/L) respectively. Cannabis metabolites (THC, THC-COOH) were detected in 5 cases, a similar proportion to the ED cohort discussed in Section 5.2.3 (19% in PM cases compared with 26% in ED cases). The high incidence of alcohol, methadone and cannabis metabolites in this cohort is not unexpected for various reasons. As mentioned in Section 5.2.3, alcohol use is widespread in both Scottish culture and Western culture more generally, and is commonly detected in the PM casework conducted in this laboratory. Similarly, cannabis is the most widely used drug worldwide (144). The effects of cannabis as similar to SCRAs make it a likely candidate to be used by the same individuals, either at the same or different times. Methadone is commonly present in PM casework, having been implicated in 47% of drug-related deaths in 2018 (114). The combined effects of SCRA and alcohol and/or methadone use are not characterised. It is possible that the CNS depressant effects of both these substances could counteract certain stimulant-type effects reported in SCRA use such as tachycardia and aggressive behaviour. Alternatively, alcohol could exacerbate psychoactive effects of SCRAs and cause further behavioural disturbances in the form of aggression and agitation. Similarly, the co-administration of methadone may

cause CNS depression such as bradycardia, as was observed in the ED cohort. In addition to this, the presence of methadone may be problematic if emergency medical professionals are required to provide benzodiazepines to treat SCRA toxicity.

As a drug group, antidepressants (amitriptyline, sertraline, citalopram, mirtazapine, fluoxetine (and metabolite norfluoxetine)) are present in 11 cases. This could either suggest pre-existing mental health conditions in SCRA users, or could indicate a detrimental effect of SCRA use on mental health. Antidepressants are also an unfortunately common feature in PM casework and it is possible that their detection in SCRA-positive cases is simply an artifact of their commonality.

The cohort consisted predominantly of males (93%), in the age range 23 – 52 years (mean and median: 38 years). The 2 females included in the cohort were 21 and 56 years old. Abouchedid *et al.* found an age range of 18 – 44 years (median: 31 years) in a cohort of 18 individuals found positive for SCRA from a group presenting to hospital with acute recreational drug toxicity (134). In 4 case studies, discussing 8 fatalities involving various SCRAs, by Shanks, Behonick *et al.*, the ages ranged from 17 – 41 years (mean: 27, median: 28) and 63% were male (93, 145-147). The demographic identified in this study therefore conforms to the general trend seen in the literature of a relatively wide age range of predominantly male users.

Individuals in 14 cases (52%) were either currently incarcerated or had been liberated from prison in the few days preceding death. While SCRA use is known to be a problem within prisons (103, 104, 111), it should be noted that samples from individuals who died whilst within or recently released from prisons were preferentially analysed for SCRAs. The potential effects of this preferential testing should also be borne in mind when considering the demographics of the SCRA-positive PM cases, with respect to the demographics of the prison population. The use of SCRAs in prison is covered in more detail in Section 5.4.

A number of different causes of death were reported for this cohort. For the purposes of this discussion, these have been split into the following headings: drug-related, alcohol-related, medical/natural, hanging, house fire,

and homicide. Out of the 27 cases positive for SCRAs, the majority of the causes of death fall within the drug-related category, with 8 cases (30%). Of these, 3 cases (38%) name a SCRA in the cause of death. The cause of death for Case 15 is 'adverse effects of 5F-MDMB-PINACA', to the author's knowledge the only case in Scotland which is purely SCRA-related. The other drugs included in these cases were heroin (inferred from the detection of metabolites, n=4), methadone (n=1), pregabalin (n=1), diazepam (n=1), etizolam (n=1), and tramadol (n=1) These are all drugs that are commonly detected in drug-related deaths in Scotland.

Medical/natural deaths account for 7 of the 27 deaths (26%). Case 4 falls into this category, but does mention synthetic cannabinoid intoxication in section 2 (detailing other significant conditions contributing to the death but not related to the disease or condition causing it). Five cases (19%) had hanging noted as the cause of death, with 4 of these (80%) being hangings in prison. As discussed previously, the preferential nature of testing samples from individuals in prison should be borne in mind when considering these numbers. Hanging appears to be quite highly represented in this cohort, so further investigation into the role SCRAs play in cases of hanging deaths would provide more information. For example, whether SCRAs have a negative psychological impact on users, or whether their use is more prevalent in individuals with pre-existing psychological conditions. Alcohol-related causes of death and house-fires were noted on the death certificates in 2 cases each, with homicide by stab wounds noted for 1 case.

Overall SCRAs are mentioned in the cause of death for 5 cases (19%) in this cohort. While there have been reports of fatal cases of SCRA intoxication (93, 145-148), information about the pharmacodynamics and clinical symptoms for SCRAs in general as well as specific compounds is still very limited. Particularly as there are often underlying medical conditions or co-administered substances in fatal cases making the determination of the mechanism of death highly complex.

It should be noted that Case 5 actually names a SCRA metabolite in the cause of death. It is unclear whether the pathologist meant that the metabolite itself contributed specific toxicity to the individual, or whether this was taken as a marker of presence of the parent compound, which

contributed towards the death. There is evidence that the metabolite in question, AKB48 N5OH pentyl, does retain efficacy at the cannabinoid receptors (97), but even less is known about the pharmacodynamics and toxicity of SCRA metabolites.

Case 26 is subject to a Fatal Accident Inquiry (FAI) still ongoing at time of submission (September 2019), as such, circumstances and cause of death are not disclosable.

The causes of death for all cases tested for SCRAs are shown, by percentage, in Figure 45. Causes of death for 4 cases were undisclosed and do not feature in these data. From this it is clear that drugs played a role in the deaths of half of the cohort. Causes of death were unascertained in the second largest group (17%). It should be noted, however, that pathologists would often resubmit cases for additional analyses for less-commonly encountered compounds if a cause of death could not be determined from initial pathological, histological or toxicological findings. Consequently, an artificially large proportion of cases tested for SCRAs would be unascertained. The third and fourth most common cause of death in cases tested for SCRAs were medical/natural (15%) and suicide (11%). It is possible that the preferential testing of individuals who died in prison is responsible for this, as these are common causes of death among incarcerated individuals. These medical/natural deaths, for example, may not have been subjected to PM investigation if the deceased was not in a custodial institution, and thus SCRA analysis would not have been conducted. There were minor contributions towards the cohort of individuals who died as a result of an accident, alcohol-related issues, a house fire, homicide, or a road traffic collision.

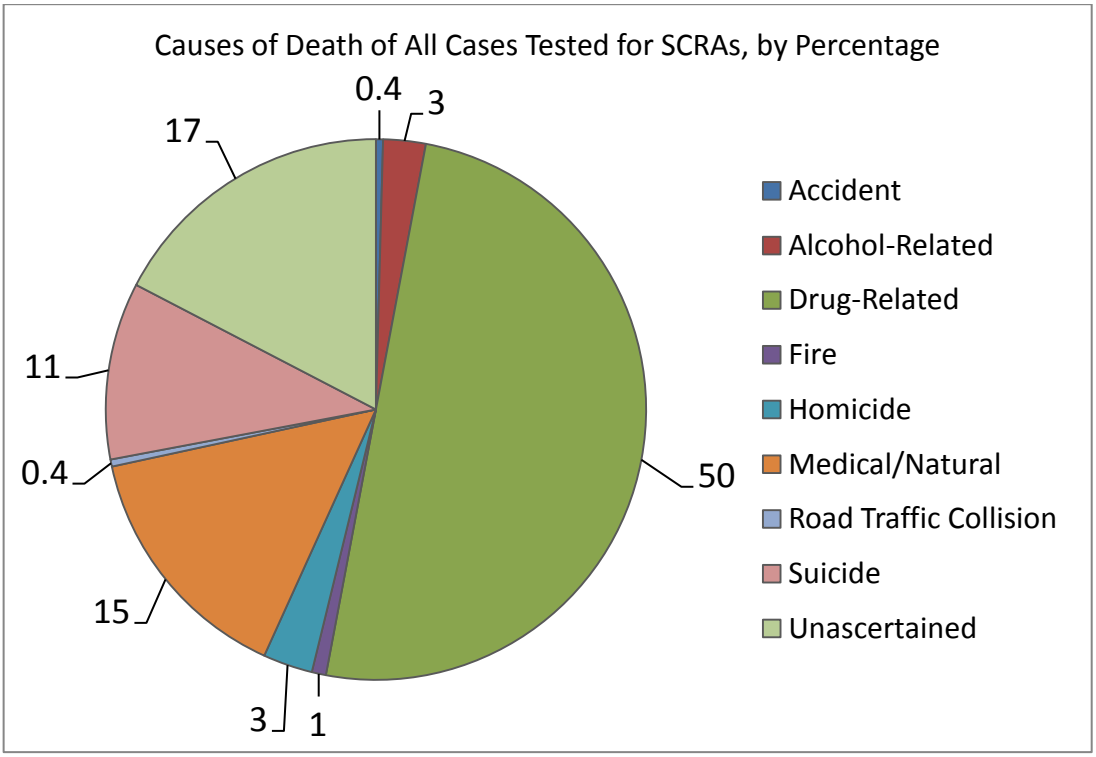


Figure 45 – Pie chart showing the causes of death for all cases tested for Synthetic Cannabinoid Receptor Agonists, by percentage. The cause of death for 4 cases were undisclosed and so are not included in this chart.

5.3.4. Conclusions

The application of the methods developed for the detection and quantitation of SCRAs in blood and urine to PM casework has shown that SCRAs are being used by a variety of people in the Scottish population. The numbers of cases where SCRAs have been detected are considerably lower than those for the likes of opioids, benzodiazepines and cocaine, however. From the categories and circumstances of deaths in SCRA-positive cases, it is apparent that the types of cases where SCRAs are detected tend to be in abusers of other recreational drugs, and individuals within, or recently released from, prison.

The cases where SCRAs have been detected are generally quite complex from a toxicological view point, with little interpretation of toxicity possible. The demographics of positive cases are similar to those found elsewhere in the literature, and indeed to those of Scottish drug users in general.

Due to the novelty of SCRAs and the lack of understanding regarding what constitutes a ‘toxic’ concentration, there is currently little value in reporting

concentrations in PM cases. As has been the case with other newly emerged drugs, such as etizolam and gabapentin, it is thought that as pathologists become more familiar with SCRAs and concentrations found in different case types that they will consider the concentrations to a higher degree. It is, therefore, useful to continue to quantitatively report concentrations in order to build up more information on concentrations considered important by pathologists in determination of causes of death.

This work has provided evidence that SCRAs are being used in a prison environment, but care must be taken not to over-interpret this point, as individuals with recent incarceration were preferentially tested for SCRAs. Likewise, the apparent correlation with SCRA use and suicide by hanging is perhaps something that could be investigated further, but may be another complication of the high number of prison cases (as 4 out of 5 hanging cases were in prison).

As discussed previously, the potential effect of the time between sampling and analysis, and compound instability should be considered. Similarly, the panel of drugs tested did not cover all SCRAs. Overall, however, important information on the nature of cases where SCRA use was detected has been obtained by this work.

5.4. Individuals Admitted to or Liberated From a Scottish Prison Service Facility

5.4.1. Introduction

For the month of November every year all individuals entering and leaving Scottish Prison Service (SPS) facilities are tested for drugs of abuse in urine using a point of care testing device, in a scheme called Annual Addictions Prevalence Testing (AAPT). This scheme covers the most commonly used drugs of abuse including cannabis, opiates, cocaine and amphetamines, however it does not include NPS such as SCRAs. It has been reported in the mainstream media that SCRAs in particular are commonly used in prisons in the UK (103, 149, 150). It is thought this may be due to their legality prior to the Psychoactive Substances Act 2016 (PSA), the absence of point-of-care tests sensitive and specific enough to detect their presence, and their reported effects on the perception of time (namely to speed up the passing of time, leading to their nickname 'bird killers')(111). Whether reports of widespread use of SCRAs in prisons are justified, however, remains unclear as there is a lack of prevalence studies in this area.

It was the aim of this project to assess the scale of use of SCRAs in individuals being admitted to or liberated from SPS facilities in November 2015, by way of additional testing on the AAPT samples.

NHS Ethical Approval was granted from the NHS West of Scotland REC under reference WS/15/0207. Conditions of this approval included the informed consent of participants, and in order to achieve this information and consent forms were provided to participants during sample collection (see Appendix F, Section 9.6). These had undergone review from the REC.

5.4.2. Method

5.4.2.1. Sample Collection

In order to conduct the AAPT, NHS staff collected urine from individuals undergoing the admission to or liberation from SPS. After testing for the standard panel, the individual was asked to sign a consent form and the remainder of the original urine sample was labeled as 'admission' or 'liberation' and sent by courier to FMS. On receipt at FMS, the samples were

labeled with a unique identifier which denoted the prison from which it was received and were placed in a freezer (≤-18 °C) to await analysis.

5.4.2.2. Sample Analysis

Method 1.1 was applied to these samples, comprising the analytes listed in Table 25, and the extraction and hydrolysis protocols and MP gradient detailed in Table 40.

For initial screening tests, two calibrators, at 0.5 and 10 ng/mL, were extracted along with the samples. For confirmation of screen-positive samples, a calibration was run for assessment of concentration. Table 45 shows details of the preparation of standards for screening and confirmation methods.

Table 45 – Preparation of calibrators and QC for the confirmation of Synthetic Cannabinoid Receptor Agonist screen-positive samples in the Scottish Prison Service cohort.

CAL	Final Concentration in urine (ng/mL)	Volume of 500 ng/mL Working Standard Solution (µL)	Volume of ACN (µL)
1	0.2	2	998
2	0.5	5	995
3	1.0	10	990
4	5.0	50	950
5	10.0	100	900
6	25.0	250	750
7	50.0	500	500
QC	4.2	42	958

5.4.3. Results and Discussion

Between the 1st and 30th November 2015, 725 samples were collected from the 7 prisons included in the study. The number and type of samples collected, and the percentage of the 2015 AAPT scheme cohort these comprise, are detailed in Table 46, broken down by SPS facility. It should be noted that the AAPT is mandatory for individuals undergoing admission to or liberation from SPS facilities in the month of November every year, while inclusion in the additional NPS project was voluntary, with explicit consent obtained.

Table 46 – Details of the Scottish Prison Service facilities, type and number of samples, and proportion of the Annual Addictions Prevalence Testing sample received for this cohort.

Facility	No. Admission Samples	No. Liberation Samples	Total No. Samples	% of AAPT Samples – Admission	% of AAPT Samples – Liberation
HMP Addiewell	69	34	109*	97	103†
HMP Barlinnie	109	63	173*	47	49
HMP Cornton Vale	62	25	87	87	86
HMP Edinburgh	27	35	62	77	81
HMP Greenock	6	20	26	86	95
HMP Low Moss	19	40	60*	53	105†
HMP Perth	140	68	208	91	78
Total	432	285	725*	N/A	N/A

* Six samples from HMP Addiewell and one each from HMP Barlinnie and HMP Low Moss were not labeled as admission or liberation and could not be identified as either.

† Number of samples tested in NPS project is greater than those reported as collected during the APT.

Inclusion in this study was generally high, with >75% coverage of all AAPT participants for all facilities except HMP Barlinnie, and admission samples from HMP Low Moss. The number of samples received labeled as liberation from HMP Addiewell and HMP Low Moss were higher than those reported in the AAPT (151). This appears to be error in the recording or reporting of the SPS AAPT results, or mis-labeling of samples for this study by staff collecting the samples.

Reasons for the variation, and occasional reduction in participation could be reflective of variation in the techniques employed by staff in providing information on the study and obtaining consent for this. Alternatively, it could be indicative of a higher incidence of NPS use and/or distrust of SPS or research staff. As the lowest participation rate was observed at HMP Barlinnie, which collected the highest number of samples for the AAPT, it is possible that staff were simply very busy and did not have the time to discuss the NPS project with potential participants either fully or at all. Generally the mean recruitment of participants was good, at 77% for admission and 85% for liberation, however the voluntary element to participation means this cannot be classified as a prevalence study.

5.4.3.1. HMP Addiewell

HMP Addiewell, situated in West Lothian, was opened in 2008 and houses up to 700 low-, medium- and high-security male offenders (152). Table 47 shows the results of the samples received from this facility. All of the samples received were negative for SCRAs with the exception of 1 sample which was not labeled as admission or liberation.

Table 47 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Addiewell

Sample Type	No. negative	No. positive
Admission	69	0
Liberation	34	0
Unlabelled	5	1
TOTAL	108	1

5.4.3.2. HMP Barlinnie

HMP Barlinnie, on the outskirts of Glasgow, was opened in 1882 and has a capacity of 1019, although the average number in custody for 2013 – 2014 was 1305 (153, 154). HMP Barlinnie houses male offenders who are on remand or have been convicted and have sentences less than 4 years, as well as offenders serving life sentences who are approaching a potential release date (153). Of the samples received from this facility, 8 were found positive for SCRAs, all being admission samples.

Table 48 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Barlinnie

Sample Type	No. negative	No. positive
Admission	101	8
Liberation	63	0
Unlabelled	1	0
TOTAL	165	8

5.4.3.3. HMP Cornton Vale

Located in Stirling, HMP Cornton Vale is the only all-female prison in Scotland and, at the time of the study, accommodated the majority of the female offenders in Scotland. This prison has a design capacity of 119 but has been reported to house around 340 inmates (155, 156).

Of the samples received from HMP Cornton Vale, 7 admission samples were determined to be positive for SCRAs. All samples labeled as liberation were negative.

Table 49 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Cornton Vale

Sample Type	No. negative	No. positive
Admission	55	7
Liberation	25	0
TOTAL	80	7

5.4.3.4. HMP Edinburgh

HMP Edinburgh originally opened in 1924, but was entirely rebuilt in the late 1990s – early 2000s and now holds around 900 offenders of all types (157).

Only 1 positive sample was detected from the HMP Edinburgh cohort, which was labeled as an admission sample.

Table 50 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Edinburgh

Sample Type	No. negative	No. positive
Admission	26	1
Liberation	35	0
TOTAL	61	1

5.4.3.5. HMP Greenock

HMP Greenock opened in 1910 and houses all types of male offenders, as well as all types of female offenders since 2002 (158, 159). It currently has the capacity for around 250 inmates (158).

All the samples received from HMP Greenock were found negative for SCRAs.

Table 51 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Greenock

Sample Type	No. negative	No. positive
Admission	6	0
Liberation	20	0
TOTAL	26	0

5.4.3.6. HMP Low Moss

The renovated HMP Low Moss, on the outskirts of Glasgow, was opened in 2012, although there had been a prison on the site since 1968. It has a design capacity of 784 and houses all types of male offenders (160).

All the samples received from HMP Low Moss were found negative for SCRAs.

Table 52 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Low Moss

Sample Type	No. negative	No. positive
Admission	19	0
Liberation	40	0
Unlabelled	1	0
TOTAL	60	0

5.4.3.7. HMP Perth

Whilst HMP Perth was originally completed in 1812, the most recent renovations took place in 2007 (161). This prison accommodates an average of 678 male offenders of all types (161).

SCRAs were detected in 4 samples from HMP Perth – all labeled as admission samples.

Table 53 – Results of Synthetic Cannabinoid Receptor Agonist testing of samples from HMP Perth

Sample Type	No. negative	No. positive
Admission	136	4
Liberation	68	0
TOTAL	204	4

5.4.3.8. Overall Results

Out of the 725 samples received overall, 21 were positive – 2.9%. All of the positive samples were labeled as admission samples, except one from HMP Addiewell which was unlabeled. Therefore, the percentage of admission samples which were positive for SCRAs was 4.9%. These data could be perceived as encouraging, as it appears that SCRAs are being used before, and on admission to, prison, but that this use is decreasing to zero on liberation. However, it is important to consider the reason behind SCRA use and the motivation for abstinence on liberation from prison. It has been reported that SCRAs are used in prison for a number of reasons, predominantly related to their psychoactive effects and lack of detection. The benefits of the former are the alleviation of boredom, escapism and feeling of time passing faster (111). The latter relates to the lack of suitably sensitive and specific mandatory drug testing instrumentation. It could be possible, then, that the use of SCRAs is just not appealing to individuals on their way out of prison in the same way as it is to those on their way in. Similarly, individuals undergoing liberation from prison could fear that their being under the influence of SCRAs could prevent their release, and abstain for the short term.

Figure 46 shows the equivalent results for the DOA testing conducted by the SPS for the AAPT. It is important to remember that this testing is not voluntary so all individuals must participate. Bearing this in mind, it is clear that the prevalence of SCRAs within this group is significantly lower than for most 'traditional' drugs of abuse.

It is important to note that all of the drugs tested reduce from admission to liberation, with the exception of the opiate substitutes methadone and buprenorphine (and methamphetamines but the number is insignificant on admission). The reduction of SCRAs on liberation then fits in with the general trend of drug use in this context.

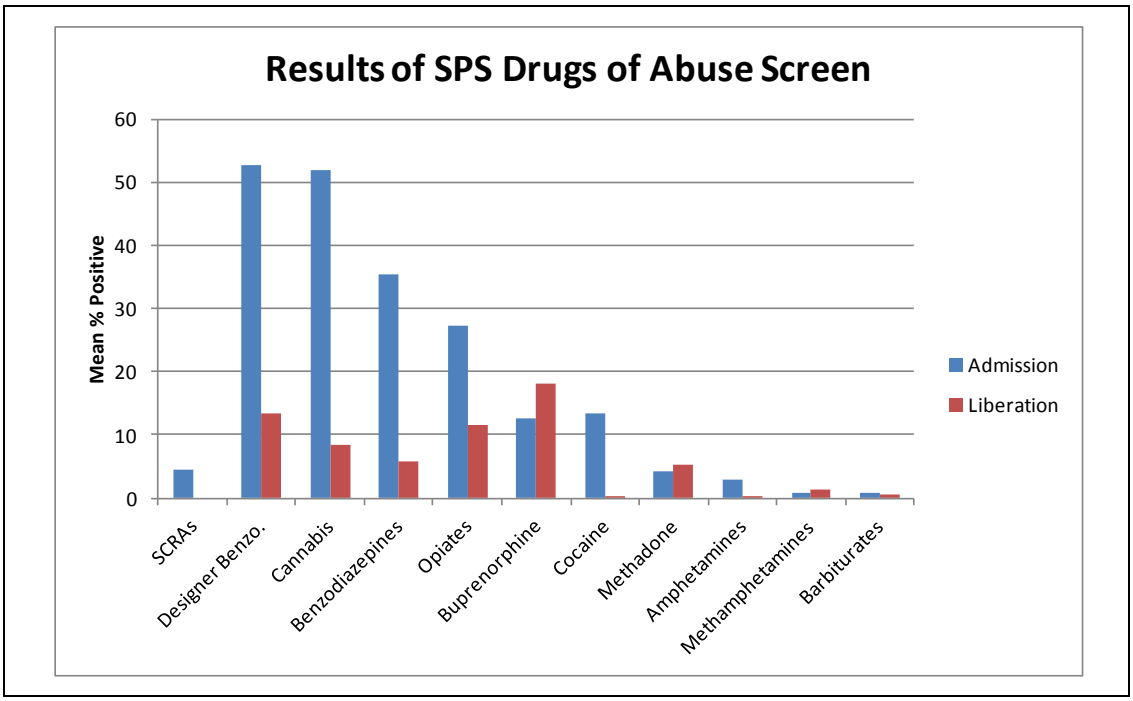


Figure 46 – Results from the Scottish Prison Service Annual Addictions Prevalence Testing for traditional drugs of abuse (151), showing relatively low positivity rate of Synthetic Cannabinoids Receptor Agonists.

The individual compounds and the number of samples in which these were detected are given in Table 54. This data shows that the AB-FUBINACA valine metabolite was the most commonly encountered compound with 15 samples positive. This compound is also a metabolite of MMB-FUBINACA, however MMB-FUBINACA was not reported in the UK until May 2016 so the presence of the metabolite is more likely to have resulted from the ingestion

of AB-FUBINACA (94, 162). While the rapidly changing nature of SCRA popularity must be considered, the relatively high detection rate of this compound in this cohort was also evident in the PM cohort (see Section 5.3).

Table 54 – Numbers of Synthetic Cannabinoid Receptor Agonists detected in Scottish Prison Service samples.

Compound	No. of Samples
AB-FUBINACA valine metabolite	15
AB-FUBINACA metabolite 2B	1
BB-22 3-carboxyindole	7
BB-22 or MDMB-CHMICA	1
AKB48 N5OH pentyl	6
AKB48 N-pentanoic acid metabolite	5
PB-22 N-pentanoic acid metabolite	3
AB-CHMINACA metabolite 1A	2
AB-CHMINACA metabolite 2	1

After the AB-FUBINACA valine metabolite, the most commonly encountered compound was the BB-22 3-carboxyindole metabolite. A response was observed in the BB-22/MDMB-CHMICA transition for one sample, and it was believed this was BB-22 as the BB-22 3-carboxyindole metabolite was also present in this sample. Research not available at the time of the study, but published at a later date stated that the BB-22 3-carboxyindole metabolite is also produced from the amide hydrolysis of MDMB-CHMICA (84). This metabolite, however, is present in minor quantities when MDMB-CHMICA is administered (<1% of the mean area ratio, and ranked number 30 of 31 metabolites in terms of prevalence in 10 samples). Findings in the PM cohort (see Section 5.3) where the MDMB-CHMICA and BB-22 are chromatographically resolved, and the MDMB-CHMICA *O*-desmethyl acid metabolite is included in the method, have indicated that the BB-22 3-carboxyindole metabolite is not detected where MDMB-CHMICA use is demonstrated (by presence of parent drug and/or major metabolite). It is, therefore, much more likely that the compound administered was BB-22 rather than MDMB-CHMICA in this case. The similarities between many

SCRAs and the likelihood of common metabolites in general should be borne in mind when reporting results where no parent drug is detected.

AKB48 metabolites (N5OH pentyl and N-pentanoic acid) were detected in 11 samples in total. Again, it is possible that these metabolites could arise from de-fluorination and further metabolism of 5F-AKB48, however the lack of the 5F-AKB48 N4OH pentyl – specific to 5F-AKB48 – strongly indicates this is not the case.

The PB-22 N-pentanoic acid metabolite was detected in 3 cases. This metabolite is common to PB-22 and its fluorinated analogue, 5F-PB-22 (80).

AB-CHMINACA metabolites 1A and 2 were detected in 2 and 1 sample(s) respectively. The sample in which AB-CHMINACA metabolite 2 was detected also contained metabolite 1A.

The potential instability of compounds should be borne in mind when considering the significance of the compounds detected, as rapid metabolism could lead to under-representation in these findings.

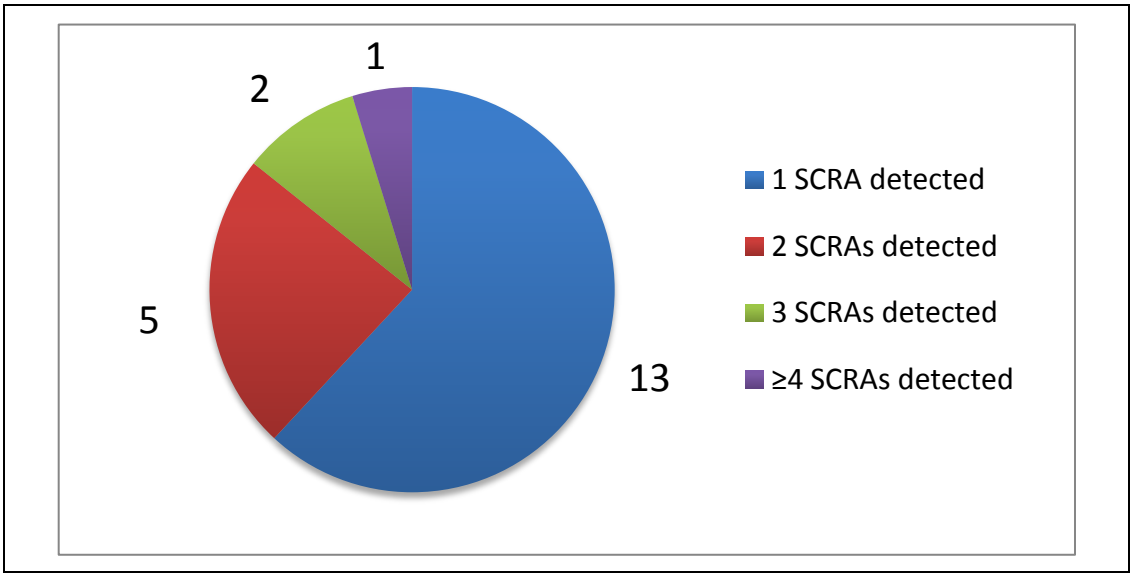


Figure 47 – Co-administration of Synthetic Cannabinoid Receptor Agonists as detected in Scottish Prison Service samples.

With regards to the number of compounds detected in positive samples, co-administration was observed, as demonstrated in Figure 47. The majority of positive cases (62%) only contained 1 detected compound, however 1

sample was found to contain 8 compounds, indicating the use of 5 distinct parent compounds.

The packaging for some SCRA products is often inaccurate with regards to type and number of compounds the product contains. For this reason it is not possible to know whether the incidence of co-administration of compounds was intentional.

After England and Wales, Scotland has the highest rate of imprisonment in Western Europe (104). Due to the lack of studies, it is not known what the prevalence of SCRA use is within custodial institutions in Scotland, or indeed the U.K. Surveys such as the Scottish Crime and Justice Survey or Crime Survey for England and Wales do not include individuals in prison so the incidences of drug use are not reflective of this population. The seizures of SCRAs in prisons in England and Wales has increased from <100 in 2010 to over 700 in 2014 (104). In a survey of 625 prisoners across multiple English and Welsh prisons, 33% reported ‘spice’ use in the last month, with prisoners estimating rates of between 40 – 90% of prisoners using SCRAs during group discussions (104). Of these individuals who had used in the last month, 46% admitted to using ‘almost daily’; which, if extrapolated, equates to almost 13000 individuals using SCRAs almost daily in prison. A rate of 4.9% of admission samples and 2.9% of all samples positive for SCRAs in this study seems low in comparison. However, it is important to consider the difference in the populations in terms of what point of their prison sentence they are currently at.

5.4.4. Conclusions

The work conducted in this section indicates that SCRAs were being used by individuals undergoing admission to SPS facilities in November 2015. Compared to more ‘traditional’ drugs of abuse, the proportion of people using SCRAs on admission to prison was low, and this number reduced to zero on liberation. This follows the trend seen in other drugs of abuse, with the exception of opioid substitutes. It is important to consider, however, that this work did not test the same individuals on admission and liberation.

While the study was relatively small in scale, and did not include all participants of the AAPT scheme, recruitment to this project was generally

high. As with all the work discussed, it is important to bear in mind that only a selection of SCRAs were included within the testing panel, and it is possible that the samples may have contained drugs that were not detected. Nonetheless, information on the scale of use and types of compounds used was gained, and the use of multiple compounds by the same individual was observed.

As mentioned previously, analysis of urine samples from individuals currently within their prison sentence would provide valuable information on the real scale of use of SCRAs in Scottish prisons. Similarly, a repeat of the work during another round of the AAPT would show what changes have occurred in SCRA trends since November 2015.

5.5. Individuals Undergoing Psychiatric Treatment from the NHS Greater Glasgow and Clyde Forensic Directorate

5.5.1. Introduction

There are approximately 220 patients under the care of the NHS Greater Glasgow and Clyde Forensic Directorate (NHS GGC FD) managed in medium secure, low secure and community settings. This includes the national medium secure learning disability service based at the Rowanbank clinic. The patient cohort has a range of diagnoses, although the majority (around 70%) have a primary diagnosis of schizophrenia. Many forensic patients have co-morbidity (76% of those in community setting), with either harmful use of or dependency on illicit substances and/or alcohol. The vast majority of patients are detained under a section of either the Mental Health (Care and Treatment) Scotland Act (2003) or the Criminal Procedure (Scotland) Act 1995. It is routinely a condition of the patient's suspension of detention or condition of discharge that they should not use illicit substances, alcohol or NPS.

Patients under the care of the forensic psychiatric services are regularly and randomly screened for drugs of abuse. This is done using an immunoassay screen and confirmation by either GC-MS (opiates and methadone), LC-MS/MS (amphetamines) or LC-TOF-MS (everything else). It is unknown if this cohort of patients are using NPS such as SCRAs, as they are not known to be routinely detected by current urine screening tests.

The aim of this study was therefore to estimate the prevalence of use of SCRAs in patients undergoing treatment by the NHS GGC FD; and to assess the ability of the current drug detection systems in place at the NHS GGC FD to detect NPS.

NHS Ethical Approval was granted from the NHS West of Scotland REC under reference 15/WS/0263. See Appendix G, Section 9.7, for ethical approval documentation.

5.5.2. Method

5.5.2.1. Sample Collection

Urine samples were collected from individuals under the treatment of the NHS GGC FD and were sent to a sub-contractor for testing for the standard panel of drugs of abuse. Patients were provided with an information sheet and asked to sign a form if they consented to their inclusion in this study. These forms were reviewed by the NHS REC and are provided within Appendix G, Section 9.7. The remainder of the sample was sent by courier to FMS, along with a corresponding copy of the drug testing results, and a list of their prescribed drugs. The samples and paperwork did not contain any information that would make them traceable to an individual by FMS, and were paired by a unique number noted on samples and paperwork. The results of the drug testing and the prescription information would likely make them identifiable to FD staff, however. Only one sample per individual was received at FMS; no individual was tested repeatedly.

On receipt at FMS, the samples were placed under freezer storage to await analysis.

5.5.2.2. Sample Analysis

Method 1.2 was applied to these samples, comprising the analytes listed in Table 25, and the extraction and hydrolysis protocols, and MP gradient detailed in Table 40.

5.5.3. Results and Discussion

Between 1st November 2015 and 30th November 2016, 95 urine samples were received by FMS. All samples received were found negative for the SCRAs contained within the analysis panel.

Due to the nature of SCRAs as high potency CB_1 agonists with high affinity, they have been linked to adverse psychological effects (14, 140, 163). It is therefore a positive finding that individuals experiencing poor psychological health were not found to be using these compounds.

It is, however, important to remember that these individuals were informed that they would be tested for drugs, albeit testing was conducted at random appointments. Similarly, while the panel is believed to include the SCRAs most likely to be encountered in this population, it is not exhaustive, particularly with regards to metabolites. The samples were frozen upon receipt at FMS, but as little is known regarding stability of SCRAs, it should be borne in mind that negative results could arise from the decay of the compounds included in the panel.

5.5.4. Conclusions

The results from this work indicate that individuals under the treatment of the NHS GGC FD do not use SCRAs, particularly the SCRAs included in method 1.2.

While this is positive from a treatment point of view, it is important to bear in mind that the individuals knew when they would be tested for drugs, and that the panel of SCRAs tested was not exhaustive.

One of the reasons proposed for SCRA use is to avoid detection in drugs tests, so it is apparent from analysis conducted that this is not the case for this cohort. Similarly, the drugs included in this panel were detected in other cohorts around the time of this study, so it is known that some of the compounds, at least, were available for use.

Due to the lack of cases positive for SCRAs in this cohort, it was not possible to determine whether the current systems in place for the detection of drugs of abuse, particularly NPS, were fit-for-purpose. It does appear, however, that the clinical mechanisms used for promoting and ensuring abstinence from drugs of abuse are working.

5.6. Individuals Under a Drug Treatment Order from the Glasgow Drugs Court

5.6.1. Introduction

The Glasgow Drug Court (GDC) is a special court within the Scottish judicial system, but run by the NHS, which applies treatment-based options and mandatory drug testing in place of custodial sentences to offenders with histories of drug addiction or misuse.

Individuals under the jurisdiction of the GDC attend to provide a urine sample for drug testing at a pre-arranged time. A dipstick test is employed, which covers the most commonly abused drug groups including benzodiazepines, opiates, cocaine and amphetamines, however does not detect SCRAs. Practitioners within the GDC had observed behaviour which they suspected was due to drug use, but the results of their analyses were negative, and consequently they suspected the undetected use of NPS.

Given the suspected scale of SCRA, particularly in the offending population, it was deemed necessary to assess the use of these drugs within individuals involved in the GDC system.

This work was considered service development, and as such, ethical approval from the NHS Research Ethics Committee was not required. Ethical approval was sought and granted from the UG MVLS REC under application number 200140101. See Appendix H, Section 9.8, for ethical approval documentation.

5.6.2. Method

5.6.2.1. Sample Collection

Samples were refrigerated after donation and initial on-site testing, then transferred to FMS on a monthly basis. On receipt at FMS, samples were given a unique identifier and placed in a freezer (≤ -18 °C) to await analysis.

Samples were accompanied by completed questionnaires about the individual's drug use (provided in Appendix I, Section 9.9). These were reviewed by the REC prior to the project beginning, and help was provided to the donor to complete these by NHS staff if required.

5.6.2.2. Sample Analysis

Method 1.2, detailed in Chapter 4 was applied to these samples, comprising the analytes listed in Table 25, and the extraction and hydrolysis protocols and MP gradient detailed in Table 40 .

5.6.3. Results and Discussion

Between 31st August 2015 and 19th February 2016, 73 samples were received with completed questionnaires.

There were no questionnaires that listed brand names or any other term considered to refer to SCRAs specifically. However, of the 73 questionnaires, 27 (37%) stated that 'cannabis', 'hash' or 'weed' was used with any frequency.

The results of the analysis were negative for the SCRAs and their metabolites tested in all samples with the exception of one. This sample was positive for the AKB48 N5OH pentyl and N-pentanoic acid metabolites, and MDMB-CHMICA *O*-desmethyl acid metabolite. The corresponding questionnaire stated that the participant smoked one draw of herbal cannabis every other day. Based on this response, it is unclear whether the individual was aware they were taking these drugs.

The prevalence of SCRAs in this cohort was therefore found to be 1.4%.

One of the reasons suggested for the perceived popularity of NPS is their use in avoiding positive drug tests. Based on this, it may be reasonable to suspect that individuals used to abusing cannabis might use SCRAs if they were subject to mandatory drug testing. The result of this, albeit small-scale, study is interesting as it suggests this not to be the case. It is possible that the treatment they are receiving from the NHS regarding their problematic drug use has contributed towards drug abstinence. This being said, the limiting factors noted previously in this work should be considered here as well, namely the panel of SCRA compounds being limited and the potential instability of the compounds. In addition to this, the fact that individuals were aware of when the drug testing would take place meant that they could abstain for the preceding period.

5.6.4. Conclusions

While the use of SCRAs was detected in this cohort, only one sample was positive, and it is unclear whether the use was intentional. As such, it is apparent that SCRA use is not a significant problem within individuals under the jurisdiction of the GDC.

As discussed for previous projects, it is important to bear in mind the incomplete panel of drugs tested, and the potential for compound instability, or extended time since sample collection, to cause false negative results. Having said that, it appears from this work that any unusual behaviour exhibited by individuals governed by the GDC was not due to the use of SCRAs. Nor do the individuals tested appear to be using SCRAs to avoid detection of more ‘traditional’ drugs of abuse.

6. Conclusions and Limitations

The use of SCRAs in the UK is an issue that is widely perceived as problematic, and is frequently highlighted by mainstream media. Evidence to support or refute these claims is challenging to produce as the trends of SCRA use are rapidly changing, and analytical detection in biological matrices is complex.

Through the work conducted and detailed here, a method was developed, optimised and validated for the detection and quantitation of SCRAs in blood and urine samples. The most likely to be encountered compounds were identified and the analytical method was deemed fit-for-purpose for detecting and quantifying these. Limits of detection generally ranged from 0.01 – 0.20 ng/mL in blood and urine; sufficient to see the low concentrations of SCRAs present after use. Accuracy and precision, within and between batches, were found to be acceptable for the compounds quantitatively validated, and linearity was established over the calibration range of interest, with $1/\chi$ weighting applied. The compounds were generally stable with ±20% of the t_0 injection when left under autosampler conditions for *ca.* 46 H, when compensated with the I.S. No interferences were observed in the analytical results where commonly encountered prescription and abused drugs were injected at a realistically encountered concentration.

As with all analytical techniques, certain limitations apply to this method. Significant matrix effects were observed for some compounds, predominantly in blood, which are likely to affect calculated concentrations in some PM samples. The sensitivity of the method as applied to blood was assessed based on the extraction from diluted packed red cells, rather than whole blood. As such it is unknown how the inclusion of plasma in whole blood would affect the LODs and LLOQs.

Additionally, the compounds included in the panel were chosen based on intelligence suggesting they were available to the Scottish population. The panel was not exhaustive and the stability of these compounds in biological samples is unknown. It is possible, then, that samples were positive for compounds not included in the panel, or concentrations had reduced below LODs, thus giving false negative results.

Intermediate methods were developed and validated to ensure they were fit for purpose in terms of sensitivity and selectivity.

These methods were then applied to 1177 samples collected from cohorts covering a variety of Scottish sub-populations: individuals presenting at an ED with suspected recreational drug toxicity; deceased individuals undergoing post-mortem examination; individuals undergoing admission to or liberation from SPS facilities; individuals under the care of the NHS GGC FD; and individuals under the jurisdiction of the GDC.

The results showed that the compounds selected for the method were being used within the Scottish population to varying degrees. No SCRAs were detected in the NHS GGC FD cohort (n=95), and only 1 sample was positive in the GDC cohort (n=73). This indicates that these groups of individuals are not using SCRAs in significant numbers to avoid detection of drug use, or for any other reason. Within the cohort of individuals presenting at the ED of GRI who were tested for SCRAs (n=34), 56% were found positive for one or more compound in one or more sample. This number was 11% in PM cases tested for SCRAs (n=250). Within samples collected from the SPS cohort, 5% of admission samples (n=432 in total) were found positive, and 3% overall (n=725 in total).

These results correlate with expectations of the low use of SCRAs relative to traditional drugs of abuse in Scottish sub-populations; indeed, SCRA use was found to be even lower than expected. Positive results were found in higher numbers in the ED and PM cohorts, as anticipated, due to analysis taking place on samples from participants suspected of using these compounds. Numbers of positive results in the FD and GDC cohorts were significantly lower than expected, particularly regarding the GDC cohort, where GDC staff had suspected SCRA use.

While qualitative results for SCRAs are sufficient for the purposes of this research in assessing scale and nature of use, particularly in urine samples, valuable quantitative information was gained in ED and PM cohorts.

The most commonly encountered compounds were the AB-FUBINACA/MMB-FUBINACA shared metabolite, MDMB-CHMICA and metabolite, 5F-MDMB-PINACA and metabolite, 5F-PB-22 and metabolites

and 5F-AKB48 and metabolites. These compounds are among those commonly reported in literature from the UK and also featured in WEDINOS results. These results indicated that a relatively limited number of SCRAs were being used. In that respect, the panels of compounds selected appear to have been suitable for the context within which this research was conducted. It was accepted, however, that the addition of novel compounds was reactive in nature and no attempt at anticipation of future trends in compounds was made as this was outside the scope of the work.

A number of limitations should be considered regarding these projects, however. Individuals from the NHS GGC FD and GDC cohorts knew they would be drugs tested and it is possible that they could abstain from use for the testing period. It has been suggested that SCRAs are used to avoid detection in populations undergoing drugs testing, though, and this is not apparently the case based on these results. Cases were only put forward for SCRA analysis when SCRA use was suspected for the ED and PM samples, not for every case, positively biasing the results. Consent was required from participants for the SPS and NHS GGC FD projects, and this could be withheld if participants had been using SCRAs. The projects discussed in this work cannot therefore be considered true prevalence studies.

Overall, the research described here provides invaluable information regarding the scale of use, specific compounds ingested and potential groups vulnerable to SCRA use in Scotland. The quantitative aspect of this work with regards to ED and PM cohorts can begin to address the lack of reference concentration ranges available for SCRA concentrations in living and deceased populations. This information can be used by practitioners of forensic toxicology, emergency medicine, and public policy to guide practice and employ techniques to detect SCRA use.

7. Further Work

The nature of drug use, particularly relating to SCRAs and NPS more generally, means that the compounds likely to be encountered in forensic casework will be constantly changing. For a method to continue to be fit-for-purpose it will need to be updated frequently as new compounds become available on the market. Continual quantitative revalidation is not very practical for a busy laboratory and it would be beneficial for a screening method, potentially on instrumentation such as quadrupole-time-of-flight (QTOF), to be developed. This would allow for quick identification of positive samples by comparison to a library of drugs, and then quantitation of compounds which appear to have some market longevity could be conducted by a method such as that developed in this work.

With regards to the method detailed in this thesis, further optimisation of the extraction protocol for blood, and/or the MP gradient might go some way to improve the variation and degree of ME observed for some compounds. Similarly, exploration of alternative I.S. may compensate for these ME.

An expansion of the cohorts tested would provide more data on the scale and nature of SCRA use in Scotland. For example, testing samples from individuals undergoing mandatory workplace drug testing would add evidence as to whether SCRAs are used in place of cannabis to avoid detection, or not. Likewise testing samples from individuals currently serving sentences in prison – rather than being admitted to or liberated from prisons – could add to this knowledge. A more in-depth examination of the role of SCRAs in suicide cases could be conducted to provide more context around the apparent association between SCRAs and hanging observed in the PM cohort.

In addition, given the time scale of the projects, repeating the work in, for example, the ED and SPS facilities would provide information on how the drug trends in these contexts have shifted since the original analysis.

8. References

1. White CM. The Pharmacologic and Clinical Effects of Illicit Synthetic Cannabinoids. J Clin Pharmacol. 2016; 57: 297-304

2. Howlett A, Barth F, Bonner T, Cabral G, Casellas P, Devane W, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological reviews. 2002;54(2):161-202.

3. Gurney S, Scott K, Kacinko S, Presley B, Logan B. Pharmacology, toxicology, and adverse effects of synthetic cannabinoid drugs. Forensic Sci Rev. 2014;26(1):53-78.

4. Pertwee R. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. British journal of pharmacology. 2008;153(2):199-215.

5. Huffman JW, Zengin G, Wu M-J, Lu J, Hynd G, Bushell K, et al. Structure–activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB1 and CB2 receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB2 receptor agonists. Bioorganic & Medicinal Chemistry. 2005;13(1):89-112.

6. Fine PG, Rosenfeld MJ. Cannabinoids for Neuropathic Pain. Current Pain and Headache Reports. 2014;18(10):451.

7. Murray RM, Quigley H, Quattrone D, Englund A, Di Forti M. Traditional marijuana, high-potency cannabis and synthetic cannabinoids: increasing risk for psychosis. World Psychiatry. 2016;15(3):195-204.

8. Banister SD, Moir M, Stuart J, Kevin RC, Wood KE, Longworth M, et al. Pharmacology of Indole and Indazole Synthetic Cannabinoid Designer Drugs AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA, and 5F-ADBICA. ACS Chem Neurosci. 2015;6(9):1546-1559.

9. Le Boisselier R, Alexandre J, Lelong-Boulouard V, Debruyne D. Focus on cannabinoids and synthetic cannabinoids. Clinical Pharmacology & Therapeutics. 2016; 101(2): 220-229

10. Russo EB. History of Cannabis and Its Preparations in Saga, Science, and Sobriquet. Chemistry & Biodiversity. 2007;4(8):1614-1648.

11. Brenneisen R. Chemistry and analysis of phytocannabinoids and other Cannabis constituents. In: ElSohly M.A. (ed) Marijuana and the Cannabinoids. Totowa, U.S. Humana Press; 2007. p. 17-49.

12. Mechoulam R, Gaoni Y. A Total Synthesis of dl-Δ1-Tetrahydrocannabinol, the Active Constituent of Hashish. Journal of the American Chemical Society. 1965;87(14):3273-3275.

13. Seely KA, Lapoint J, Moran JH, Fattore L. 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):234-243.

14. Castaneto MS, Gorelick DA, Desrosiers NA, Hartman RL, Pirard S, Huestis MA. Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications. Drug and alcohol dependence. 2014;144:12-41.

15. Di Forti M, Marconi A, Carra E, Fraietta S, Trotta A, Bonomo M, et al. Proportion of patients in south London with first-episode psychosis attributable to use of high potency cannabis: a case-control study. The Lancet Psychiatry. 2015;2(3):233-238.

16. Mechoulam R, Parker LA, Gallily R. Cannabidiol: An Overview of Some Pharmacological Aspects. The Journal of Clinical Pharmacology. 2002;42(S1):11S-9S.

17. Loeffler G, Delaney E, Hann M. International trends in spice use: Prevalence, motivation for use, relationship to other substances, and perception of use and safety for synthetic cannabinoids. Brain Research Bulletin. 2016;126(1):8-28

18. MIMS. Sativex | MIMS online: MIMS; 2017 [Available from: http://www.mims.co.uk/drugs/musculoskeletal-disorders/muscle-spasm/sativex [Accessed on 4th January 2017]

19. MHRA. MHRA statement on products containing Cannabidiol (CBD) - News stories - GOV.UK: Gov.UK; 2017 [Available from:

https://www.gov.uk/government/news/mhra-statement-on-products-containing-cannabidiol-cbd. [Accessed on 4th January 2017]

20. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, et al. 2-Arachidonoylgylcerol: a possible endogenous cannabinoid receptor ligand in brain. Biochemical and biophysical research communications. 1995;215(1):89-97.

21. Sedefov R, Gallegos A, King L, Lopez D, Auwärter V, Hughes B, et al. Understanding the 'Spice'phenomenon. Thematic papers, European Monitoring Centre for Drugs and Drug Addiction. 2009.

22. Mechoulam R, Hanuš Lr. A historical overview of chemical research on cannabinoids. Chemistry and Physics of Lipids. 2000;108(1–2):1-13.

23. Tait RJ, Caldicott D, Mountain D, Hill SL, Lenton S. A systematic review of adverse events arising from the use of synthetic cannabinoids and their associated treatment. Clin Toxicol (Phila). 2015;54:1-13.

24. Thomas BF, Gilliam AF, Burch DF, Roche MJ, Seltzman HH. Comparative Receptor Binding Analyses of Cannabinoid Agonists and Antagonists. Journal of Pharmacology and Experimental Therapeutics. 1998;285(1):285-92.

25. Seely KA, Prather PL, James LP, Moran JH. Marijuana-based drugs: innovative therapeutics or designer drugs of abuse? Molecular interventions. 2011;11(1):36.

26. EMCDDA. EMCDDA | Perspectives on drugs: synthetic cannabinoids in Europe: EMCDDA; 2017 [Available from: http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids. [Accessed on 5th January 2017]

27. UNODC. Synthetic Cannabinoids in Herbal Products. UNODC; 2011.

28. Gandhi AS, Zhu M, Pang S, Wohlfarth A, Scheidweiler KB, Liu H-f, et al. First characterization of AKB-48 metabolism, a novel synthetic cannabinoid, using human hepatocytes and high-resolution mass spectrometry. The AAPS journal. 2013;15(4):1091-1098.

29. Bilgrei OR. From “herbal highs” to the “heroin of cannabis”: Exploring the evolving discourse on synthetic cannabinoid use in a Norwegian Internet drug forum. International Journal of Drug Policy. 2016;29:1-8

30. Kneisel S, Auwärter V. Analysis of 30 synthetic cannabinoids in serum by liquid chromatography-electrospray ionization tandem mass spectrometry after liquid-liquid extraction. Journal of Mass Spectrometry. 2012;47(7):825-835.

31. Presley BC, Gurney SM, Scott KS, Kacinko SL, Logan BK. Metabolism and toxicological analysis of synthetic cannabinoids in biological fluids and tissues. Forensic Sci Rev. 2016;28(2):103-169.

32. Simmons J, Cookman L, Kang C, Skinner C. Three cases of “spice” exposure. Clinical Toxicology. 2011;49(5):431-433.

33. ACMD. Report on the Major Cannabinoid Agonists. London: UK Government; 2009. Available from: https://www.gov.uk/government/publications/acmd-report-on-the-major-cannabinoid-agonists [Accessed on 5th January 2017]

34. Shevyrin V, Melkozerov V, Nevero A, Eltsov O, Shafran Y, Morzherin Y, et al. Identification and analytical characteristics of synthetic cannabinoids with an indazole-3-carboxamide structure bearing a N-1-methoxycarbonylalkyl group. Analytical and Bioanalytical Chemistry. 2015:1-15.

35. The Misuse of Drugs Act 1971 (Amendment) Order 2009, (2009).

36. ACMD. Further Consideration of the Synthetic Cannabinoids. London; 2012. Available from: https://www.gov.uk/government/publications/acmd-further-consideration-of-the-synthetic-cannabinoids [Accessed on 15th January 2017]

37. ACMD. Third Generation' Synthetic Cannabinoids. London; 2014. Available from: https://www.gov.uk/publications/third-generation-synthetic-cannabinoids [Accessed on 16th June 2016]

38. EMCDDA. European Drug Report 2016. Lisbon; 2016.

39. EMCDDA. European Drug Report 2017. Lisbon; 2018.

40. EMCDDA. European Drug Report 2018. Lisbon; 2019.

41. EMCDDA. Infographic: new psychoactive substances (NPS) overview - substances reported to the EU Early Warning System since 1997 2019 Available from: http://www.emcdda.europa.eu/media-library/infographic-new-psychoactive-substances-nps-overview-%E2%80%94%C2%A0substances-reported-eu-early-warning-system-1997_en. [Accessed on 22nd February 2020]

42. Psychoactive Substances Act (2016).

43. CAST. Psychoactive Substances Act 2016 Forensic Strategy. London: Home Office; 2016.

44. Hansard. Synthetic Cannabinoids: Reclassification - Hansard: UK Parliament; 2018 Available from: https://hansard.parliament.uk/commons/2018-11-06/debates/3ACB4F7B-DF0F-4E38-9B41-962C5A3C05F4/SyntheticCannabinoidsReclassification. [Accessed 27th February 2019]

45. Alatise S. UK to reconsider classification of synthetic drug spice. Financial Times. 2019 10 January 2019.

46. Jeffrey J, McClue C, Jamieson L. Trading Standards Scotland Operation Alexander Tackling NPS 2016.

47. WEDINOS. Welsh Emerging Drugs & Identification of Novel Substances Project: Public Health Wales; 2015 Available from: http://www.wedinos.org/. [Accessed on 2nd December 2015]

48. EUChemicals. Buy Synthetic Cannabinoids | EuChemicals - Research Chemicals UK 2017 Available from: http://euchemicals.com/cannabinoids.html. [Accessed on 21st January 2017]

49. IceHeadShop. Herbal Incense 2017 Available from: https://www.iceheadshop.co.uk/research-chemicals/herbal-incense.html. [Accessed on 21st January 2017]

50. RCNET. Cannabinoids 2017 Available from: https://rcnetchemicals.com/synthetic-cannabinoids.html. [Accessed on 21st January 2017]

51. Winstock AR, Barratt MJ. The 12-month prevalence and nature of adverse experiences resulting in emergency medical presentations

associated with the use of synthetic cannabinoid products. Human Psychopharmacology: Clinical and Experimental. 2013;28(4):390-393.

52. EMCDDA. EDND | Substances 2019 Available from: https://ednd.emcdda.europa.eu/html.cfm/index7246EN.html [Accessed on 21st January 2017]

53. WEDINOS. PHILTRE Issue 1. Public Health Wales; 2013.

54. WEDINOS. PHILTRE Issue 2. Public Health Wales; 2014.

55. WEDINOS. PHILTRE Issue 3. Public Health Wales; 2014.

56. WEDINOS. PHILTRE Issue 4. Public Health Wales; 2014.

57. WEDINOS. PHILTRE Issue 5. Public Health Wales; 2015.

58. WEDINOS. PHILTRE Issue 6. Public Health Wales; 2015.

59. WEDINOS. PHILTRE Issue 7. Public Health Wales; 2015.

60. WEDINOS. PHILTRE Issue 8. Public Health Wales; 2016.

61. WEDINOS. PHILTRE Issue 9. Public Health Wales; 2016.

62. WEDINOS. PHILTRE Issue 10. Public Health Wales; 2016.

63. WEDINOS. PHILTRE Issue 11. Public Health Wales; 2017.

64. WEDINOS. PHILTRE Issue 12. Public Health Wales; 2017.

65. WEDINOS. PHILTRE Issue 13. Public Health Wales; 2017.

66. WEDINOS. PHILTRE Issue 14. Public Health Wales; 2018.

67. Cayman Chemicals. Synthetic Cannabinoids Flipbook 2017 Available from: https://www.caymanchem.com/forensics/flipbook. [Accessed on 31st January 2017]

68. Tai S, Fantegrossi WE. Pharmacological and Toxicological Effects of Synthetic Cannabinoids and Their Metabolites. Current Top Behav Neurosci. 2017;32:249-262

69. Bluelight. Synthetic Cannabinoids Thread Available from: http://www.bluelight.org/vb/threads/572411-Synthetic-Cannabinoids-Thread. [Accessed on 1st February 2017]

70. Forum ULH. Synthetic Cannabis / Herbal Incense Forum 2017 Available from: http://www.legalhighsforum.com/forumdisplay.php?40-

Synthetic-Cannabis-Herbal-Incense-Forum/page2&s=65481dec21c2c2ef18d4bb0387d15c4c. [Accessed on 1st February 2017]

71. Hermanns-Clausen M, Kneisel S, Szabo B, Auwarter V. Acute toxicity due to the confirmed consumption of synthetic cannabinoids: clinical and laboratory findings. Addiction. 2013;108(3):534-544.

72. Seywright A, Torrance HJ, Wylie FM, McKeown DA, Lowe DJ, Stevenson R. Analysis and clinical findings of cases positive for the novel synthetic cannabinoid receptor agonist MDMB-CHMICA. Clinical Toxicology. 2016;54(8):632-637.

73. Kneisel S, Teske J, Auwärter V. Analysis of synthetic cannabinoids in abstinence control: long drug detection windows in serum and implications for practitioners. Drug Test Anal. 2014;6(1-2):135-136.

74. Castaneto MS, Wohlfarth A, Desrosiers NA, Hartman RL, Gorelick DA, Huestis MA. Synthetic cannabinoids pharmacokinetics and detection methods in biological matrices. Drug Metabolism Reviews. 2015;47(2):124-174

75. Banister SD, Wilkinson SM, Longworth M, Stuart J, Apetz N, English K, et al. The synthesis and pharmacological evaluation of adamantane-derived indoles: cannabimimetic drugs of abuse. ACS Chem Neurosci. 2013;4(7):1081-1092.

76. Banister SD, Stuart J, Kevin RC, Edington A, Longworth M, Wilkinson SM, et al. Effects of Bioisosteric Fluorine in Synthetic Cannabinoid Designer Drugs JWH-018, AM-2201, UR-144, XLR-11, PB-22, 5F-PB-22, APICA, and STS-135. ACS Chemical Neuroscience. 2015;6(8):1445-1458.

77. Banister SD, Longworth M, Kevin R, Sachdev S, Santiago M, Stuart J, et al. Pharmacology of Valinate and tert-Leucinate Synthetic Cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and Their Analogues. ACS Chemical Neuroscience. 2016;7(9):1241-1254.

78. Canazza I, Ossato A, Trapella C, Fantinati A, De Luca MA, Margiani G, et al. Effect of the novel synthetic cannabinoids AKB48 and 5F-AKB48 on “tetrad”, sensorimotor, neurological and neurochemical responses in mice. In

vitro and in vivo pharmacological studies. Psychopharmacology. 2016;233(21):3685-3709.

79. De Luca MA, Castelli MP, Loi B, Porcu A, Martorelli M, Miliano C, et al. Native CB1 receptor affinity, intrinsic activity and accumbens shell dopamine stimulant properties of third generation SPICE/K2 cannabinoids: BB-22, 5F-PB-22, 5F-AKB-48 and STS-135. Neuropharmacology. 2016;105:630-638.

80. Wohlfarth A, Gandhi AS, Pang S, Zhu M, Scheidweiler KB, Huestis MA. Metabolism of synthetic cannabinoids PB-22 and its 5-fluoro analog, 5F-PB-22, by human hepatocyte incubation and high-resolution mass spectrometry. Analytical and bioanalytical chemistry. 2014;406(6):1763-1780.

81. Sobolevsky T, Prasolov I, Rodchenkov G. Study on the phase I metabolism of novel synthetic cannabinoids, APICA and its fluorinated analogue. Drug Testing and Analysis. 2015;7(2):131-142.

82. Grigoryev A, Kavanagh P, Pechnikov A. Human urinary metabolite pattern of a new synthetic cannabimimetic, methyl 2-(1-(cyclohexylmethyl)-1H-indole-3-carboxamido)-3,3-dimethylbutanoate. Forensic Toxicology. 2016;34(2):1-13.

83. Erratico C, Negreira N, Norouzizadeh H, Covaci A, Neels H, Maudens K, et al. In vitro and in vivo human metabolism of the synthetic cannabinoid AB-CHMINACA. Drug Testing and Analysis. 2015;7:866-876

84. Franz F, Angerer V, Moosmann B, Auwärter V. Phase I metabolism of the highly potent synthetic cannabinoid MDMB-CHMICA and detection in human urine samples. Drug Testing and Analysis. 2016;9(5):744-753

85. Chimalakonda KC, Seely KA, Bratton SM, Brents LK, Moran CL, Endres GW, et al. Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: identification of novel cannabinoid receptor ligands. Drug Metabolism and Disposition. 2012;40(11):2174-2184.

86. Carlier J, Diao X, Sempio C, Huestis MA. Identification of New Synthetic Cannabinoid ADB-CHMINACA (MAB-CHMINACA) Metabolites in Human Hepatocytes. The AAPS Journal. 2017;19(2):568-577.

87. Andersson M, Diao X, Wohlfarth A, Scheidweiler KB, Huestis MAJRCiMS. Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. 2016;30(8):1067-1078.

88. Vikingsson S, Josefsson M, Gréen H. Identification of AKB-48 and 5F-AKB-48 Metabolites in Authentic Human Urine Samples Using Human Liver Microsomes and Time of Flight Mass Spectrometry. Journal of analytical toxicology. 2015;39(6):426-435

89. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yamagishi I, et al. Postmortem distribution of MAB-CHMINACA in body fluids and solid tissues of a human cadaver. Forensic Toxicology. 2015;33(2):380-387.

90. Peterson BL, Couper FJ. Concentrations of AB-CHMINACA and AB-PINACA and Driving Behavior in Suspected Impaired Driving Cases. Journal of Analytical Toxicology. 2015;39(8):642-647.

91. Karinen R, Tuv SS, Oiestad EL, Vindenes V. Concentrations of APINACA, 5F-APINACA, UR-144 and its degradant product in blood samples from six impaired drivers compared to previous reported concentrations of other synthetic cannabinoids. Forensic Sci Int. 2015;246:98-103.

92. Kronstrand R, Roman M, Andersson M, Eklund A. Toxicological Findings of Synthetic Cannabinoids in Recreational Users. Journal of Analytical Toxicology. 2013;37(8):534-541.

93. Behonick G, Shanks KG, Firchau DJ, Mathur G, Lynch CF, Nashelsky M, et al. Four Postmortem Case Reports with Quantitative Detection of the Synthetic Cannabinoid, 5F-PB-22. Journal of Analytical Toxicology. 2014;38(8):559-562.

94. Castaneto MS, Wohlfarth A, Pang S, Zhu M, Scheidweiler KB, Kronstrand R, et al. Identification of AB-FUBINACA metabolites in human hepatocytes and urine using high-resolution mass spectrometry. Forensic Toxicology. 2015;33(2):295-310.

95. Scheidweiler KB, Jarvis MJ, Huestis MA. Nontargeted SWATH acquisition for identifying 47 synthetic cannabinoid metabolites in human

urine by liquid chromatography-high-resolution tandem mass spectrometry. Analytical and bioanalytical chemistry. 2014;407(3):883-897.

96. Hutchison RD, Ford BM, Franks LN, Wilson CD, Yarbrough AL, Fujiwara R, et al. Atypical Pharmacodynamic Properties and Metabolic Profile of the Abused Synthetic Cannabinoid AB-PINACA: Potential Contribution to Pronounced Adverse Effects Relative to Δ(9)-THC. Frontiers in Pharmacology. 2018;9:1084.

97. Gamage TF, Farquhar CE, McKinnie RJ, Kevin RC, McGregor IS, Trudell ML, et al. Synthetic Cannabinoid Hydroxypentyl Metabolites Retain Efficacy at Human Cannabinoid Receptors. Journal of Pharmacology and Experimental Therapeutics. 2019;368(3):414-422.

98. Fantegrossi WE, Moran JH, Radominska-Pandya A, Prather PL. Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: mechanism underlying greater toxicity? Life sciences. 2014;97(1):45-54.

99. Black C, Setterfield L, Murray L. Scottish Schools Adolescent Lifestyle and Substance Use Survey (SALSUS). Scottish Government; 2015 Available from: https://www2.gov.scot/Topics/Research/by-topic/health-community-care/social-research/SALSUS [Accessed 12th March 2019]

100. Crawford C, Clare T, Sharpe C, Wright C. United Kingdom Drug Situation - Focal Point Annual Report 2017. 2017 Available from: https://www.gov.uk/government/publications/united-kingdom-drug-situation-focal-point-annual-report [Accessed 12th March 2019]

101. MacLeod K, Pickering L, Gannon M, Greenwood S, Liddel D, Smith A, et al. Understanding the patterns of use, motives, and harms of New Psychoactive Substances in Scotland Scottish Government; 2016. Available from: https://www.gov.scot/publications/understanding-patterns-of-use-motives-harms-new-psychoactive-substances-scotland/ [Accessed 12th March 2019]

102. Flatley J. Drug Misuse: Findings From the 2017-2018 Crime Survey England and Wales. Home Office; 2018. Available from: https://www.gov.uk/government/statistics/drug-misuse-findings-from-the-2017-to-2018-csew [Accessed 12th March 2019]

103. Prime R, Ranns H, Pearce M, Engelen S, Roberts P. Changing Patterns of Substance Misuse in Adult Prisons and Service Responses. London; 2015.

104. Johnson M. Spice: The Bird Killer - What Prisoners Think About The Use Of Spice And Other Legal Highs In Prison. User Voice; 2016.

105. UK Focal Point. Drug Situation Annual Report 2017. Available from: https://www.gov.uk/government/publications/united-kingdom-drug-situation-focal-point-annual report [Accessed on 14th March 2019]

106. Winstock A, Lynskey M, Borschmann R, Waldron J. Risk of emergency medical treatment following consumption of cannabis or synthetic cannabinoids in a large global sample. J Psychopharmacol. 2015;29(6):698-703.

107. NEPTUNE. NEPTUNE Novel Psychoactive Treatment: UK Network 2019 Available from: http://neptune-clinical-guidance.co.uk/. [Accessed on 14th March 2019]

108. Abdulrahim D, Bowden-Jones O. Harms of Synthetic Cannabinoid Receptor Agonists (SCRAs) and Their Management. London: Novel Psychoactive Treatment UK Network (NEPTUNE); 2016.

109. Cooper ZD. Adverse effects of synthetic cannabinoids: management of acute toxicity and withdrawal. Current psychiatry reports. 2016;18(5):52.

110. Zimmermann US, Winkelmann PR, Pilhatsch M, Nees JA, Spanagel R, Schulz K. Withdrawal phenomena and dependence syndrome after the consumption of "spice gold". Deutsches Ärzteblatt International. 2009;106(27):464.

111. Ralphs R, Williams L, Askew R, Norton A. Adding Spice to the Porridge: The development of a synthetic cannabinoid market in an English prison. International Journal of Drug Policy.2017;40:57-69

112. Rubino T, Patrini G, Parenti M, Massi P, Parolaro D. Chronic treatment with a synthetic cannabinoid CP-55,940 alters G-protein expression in the rat central nervous system. Molecular Brain Research. 1997;44(2):191-197.

113. Macfarlane V, Christie G. Synthetic cannabinoid withdrawal: A new demand on detoxification services. Drug and Alcohol Review. 2015;34(2):147-153.

114. NRS. Drug Related Deaths in Scotland 2018. Edinburgh; 2019.

115. Huppertz LM, Kneisel S, Auwärter V, Kempf J. A comprehensive library-based, automated screening procedure for 46 synthetic cannabinoids in serum employing liquid chromatography-quadrupole ion trap mass spectrometry with high-temperature electrospray ionization. Journal of Mass Spectrometry. 2014;49(2):117-127.

116. Knittel JL, Holler JM, Chmiel JD, Vorce SP, Magluilo J, Jr., Levine B, et al. Analysis of Parent Synthetic Cannabinoids in Blood and Urinary Metabolites by Liquid Chromatography Tandem Mass Spectrometry. J Anal Toxicol. 2016;40(3):173-186

117. Ambroziak K, Adamowicz P. Simple screening procedure for 72 synthetic cannabinoids in whole blood by liquid chromatography–tandem mass spectrometry. Forensic Toxicology. 2018;36(2):280-290.

118. Hess C, Murach J, Krueger L, Scharrenbroch L, Unger M, Madea B, et al. Simultaneous detection of 93 synthetic cannabinoids by liquid chromatography-tandem mass spectrometry and retrospective application to real forensic samples. Drug Testing and Analysis. 2016;9(5):721-733

119. Kacinko SL, Xu A, Homan JW, McMullin MM, Warrington DM, Logan BK. Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Identification and Quantification of JWH-018, JWH-073, JWH-019, and JWH-250 in Human Whole Blood. Journal of Analytical Toxicology. 2011;35(7):386-393.

120. Borg D, Tverdovsky A, Stripp R. A Fast and Comprehensive Analysis of 32 Synthetic Cannabinoids Using Agilent Triple Quadrupole LC–MS-MS. Journal of Analytical Toxicology. 2017;41(1):6-16.

121. Jang M, Shin I, Kim J, Yang W. Simultaneous quantification of 37 synthetic cannabinoid metabolites in human urine by liquid chromatography-tandem mass spectrometry. Forensic Toxicology.2015;33(2):221-234

122. Gaunitz F, Kieliba T, Thevis M, Mercer-Chalmers-Bender K. Solid-phase extraction-liquid chromatography-tandem mass spectrometry method for the qualitative analysis of 61 synthetic cannabinoid metabolites in urine. Drug Testing and Analysis.2019 [Accepted Manuscript]

123. Staeheli SN, Veloso VP, Bovens M, Bissig C, Kraemer T, Poetzsch M. LC-MS/MS Screening Method Using Information-Dependent Acquisition of Enhanced Product Ion Mass Spectra for Synthetic Cannabinoids Including Metabolites in Urine. Drug testing and analysis. 2019. [Accepted Manuscript]

124. Hutter M, Kneisel S, Auwärter V, Neukamm MA. Determination of 22 synthetic cannabinoids in human hair by liquid chromatography–tandem mass spectrometry. Journal of Chromatography B. 2012;903:95-101.

125. Franz F, Jechle H, Angerer V, Pegoro M, Auwarter V, Neukamm MA. Synthetic cannabinoids in hair - Pragmatic approach for method updates, compound prevalences and concentration ranges in authentic hair samples. Anal Chim Acta. 2018;1006:61-73.

126. Kim J, Park Y, Park M, Kim E, Yang W, Baeck S, et al. Simultaneous determination of five naphthoylindole-based synthetic cannabinoids and metabolites and their deposition in human and rat hair. Journal of Pharmaceutical and Biomedical Analysis. 2015;102:162-175.

127. Kneisel S, Auwärter V, Kempf J. Analysis of 30 synthetic cannabinoids in oral fluid using liquid chromatography-electrospray ionization tandem mass spectrometry. Drug Testing and Analysis. 2013;5(8):657-669.

128. Christie R. EU Early Warning System Alert: Subject: 2 deaths and 3 non-fatal intoxications in Germany associated with MDMB-CHMICA (methyl-2-(1-(cyclohexylmethyl)-1 H-indol-3-ylcarbonylamino)-3,3-dimethylbutanoate). EMCDDA; 2015.

129. Hospitalisation From Synthetic Cannabinoid MMB-CHMINACA [press release]. Wales: NHS Wales 2015.

130. Simpson K. Drug Trend Bulletin Issue 2. Police Scotland; 2015.

131. Aldlgan A. Chromatographic Analysis and Survey Studies to Evaluate the Emerging Drugs of Synthetic Cannabinoids in Scotland and Saudi Arabia. Glasgow: University of Glasgow; 2016.

132. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS. Analytical Chemistry. 2003;75(13):3019-3030.

133. Mills B, Yepes A, Nugent K. Synthetic Cannabinoids. Am J Med Sci. 2015;350(1):59-62.

134. Abouchedid R, Hudson S, Thurtle N, Yamamoto T, Ho JH, Bailey G, et al. Analytical confirmation of synthetic cannabinoids in a cohort of 179 presentations with acute recreational drug toxicity to an Emergency Department in London, UK in the first half of 2015. Clinical Toxicology. 2017:1-8.

135. Rowley E, Benson D, Tiffee A, Hockensmith A, Zeng H, Jones GN, et al. Clinical and financial implications of emergency department visits for synthetic marijuana. The American Journal of Emergency Medicine. 2017;35(10):1506-1509

136. O'shea M, Singh ME, McGregor IS, Mallet PE. Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. Journal of psychopharmacology. 2004;18(4):502-508.

137. Hall W, Degenhardt L. Adverse health effects of non-medical cannabis use. The Lancet.374(9698):1383-91.

138. Frej M, Frej J. Glasgow Coma Scale 2014 Available from: http://www.glasgowcomascale.org/. [Accessed on 10th April 2017]

139. Persson HE, Sjöberg GK, Haines JA, de Garbino JP. Poisoning severity score. Grading of acute poisoning. Journal of Toxicology: Clinical Toxicology. 1998;36(3):205-213.

140. van Amsterdam J, Brunt T, van den Brink W. The adverse health effects of synthetic cannabinoids with emphasis on psychosis-like effects. Journal of psychopharmacology. 2015;29(3):254-63.

141. Bäckberg M, Tworek L, Beck O, Helander A. Analytically confirmed intoxications involving MDMB-CHMICA from the STRIDA Project. Journal of medical toxicology. 2017;13(1):52-60

142. Franz F, Haschimi B, King LA, Auwärter V. Extraordinary long detection window of a synthetic cannabinoid metabolite in human urine – Potential impact on therapeutic decisions. Drug Testing and Analysis.2020;12(3):391-396

143. Baselt R. Disposition of Toxic Drugs and Chemicals in Man. 11th ed. Seal Beach, California: Biomedical Publications; 2017.

144. UNODC. World Drug Report 2019 - Booklet 5 Cannabis and Hallucinogens. Vienna; 2019.

145. Shanks KG, Behonick GS. Death after use of the synthetic cannabinoid 5F-AMB. Forensic science international. 2016;262:21-24.

146. Shanks KG, Winston D, Heidingsfelder J, Behonick G. Case reports of synthetic cannabinoid XLR-11 associated fatalities. Forensic science international. 2015;252:6-9.

147. Shanks KG, Clark W, Behonick G. Death Associated With the Use of the Synthetic Cannabinoid ADB-FUBINACA. Journal of analytical toxicology. 2016;40(3):236-239

148. Westin AA, Frost J, Brede WR, Gundersen POM, Einvik S, Aarset H, et al. Sudden Cardiac Death Following Use of the Synthetic Cannabinoid MDMB-CHMICA. Journal of Analytical Toxicology. 2015;40(1):86-87

149. The Economist. At Her Majesty's pleasure. The Economist. 2015 Available from: https://economist.com/britain/2015/07/30/at-her-majestys-pleasure [Accessed on 10th November 2015]

150. BBC News. Legal highs: Call to investigate link to prison deaths - BBC News. 2015. Available from: https://www.bbc.co.uk/news/uk-england-34385206 [Accessed on 2nd October 2015]

151. SPS. Addiction Prevalence Testing for Performance Measurement Purposes 2015-16 (November 2015). 2016.

152. SPS. HMP Addiewell 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/Addiewell/HMP-Addiewell.aspx. [Accessed on 8th October 2018]

153. SPS. HMP Barlinnie 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/Barlinnie/HMP-Barlinnie.aspx. [Accessed on 8th October 2018]

154. Wikipedia. HM Prison Barlinnie - Wikipedia 2018 Available from: https://en.wikipedia.org/wiki/HM_Prison_Barlinnie. [Accessed on 8th October 2018]

155. SPS. HMP YOI Cornton Vale 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/CorntonVale/HMP-YOI-Cornton-Vale.aspx. [Accessed on 8th October 2018]

156. BBC NEWS | Scotland | Inmate mothers to keep children 2018 [updated 15th August 2004. Available from: http://news.bbc.co.uk/1/hi/scotland/3566700.stm. [Accessed on 8th October 2018]

157. SPS. HMP Edinburgh 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/Edinburgh/HMP-Edinburgh.aspx. [Accessed on 8th October 2018]

158. SPS. HMP Greenock 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/Greenock/HMP-Greenock.aspx. [Accessed on 8th October 2018]

159. Wikipedia. HM Prison Greenock - Wikipedia 2018 Available from: https://en.wikipedia.org/wiki/HM_Prison_Greenock. [Accessed on 8th October 2018]

160. SPS. HMP Low Moss 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/LowMoss/HMP-Low-Moss.aspx. [Accessed on 8th October 2018]

161. SPS. HMP Perth 2018 Available from: http://www.sps.gov.uk/Corporate/Prisons/Perth/HMP-Perth.aspx. [Accessed on 8th October 2018]

162. EMCDDA. EDND | Substances 2019 Available from: https://ednd.emcdda.europa.eu.html.cfm/index7246EN.html [Accessed on 12th March 2019]

163. Aguilar DD, Giuffrida A, Lodge DJ. Adolescent synthetic cannabinoid exposure produces enduring changes in dopamine neuron activity in a rodent model of schizophrenia susceptibility. Int J Neuropsychopharmacol. 2018:21(4):393-403

9. Appendices

9.1. Appendix A – Mobile Phase Gradients Tested in Section 4.3.3.2

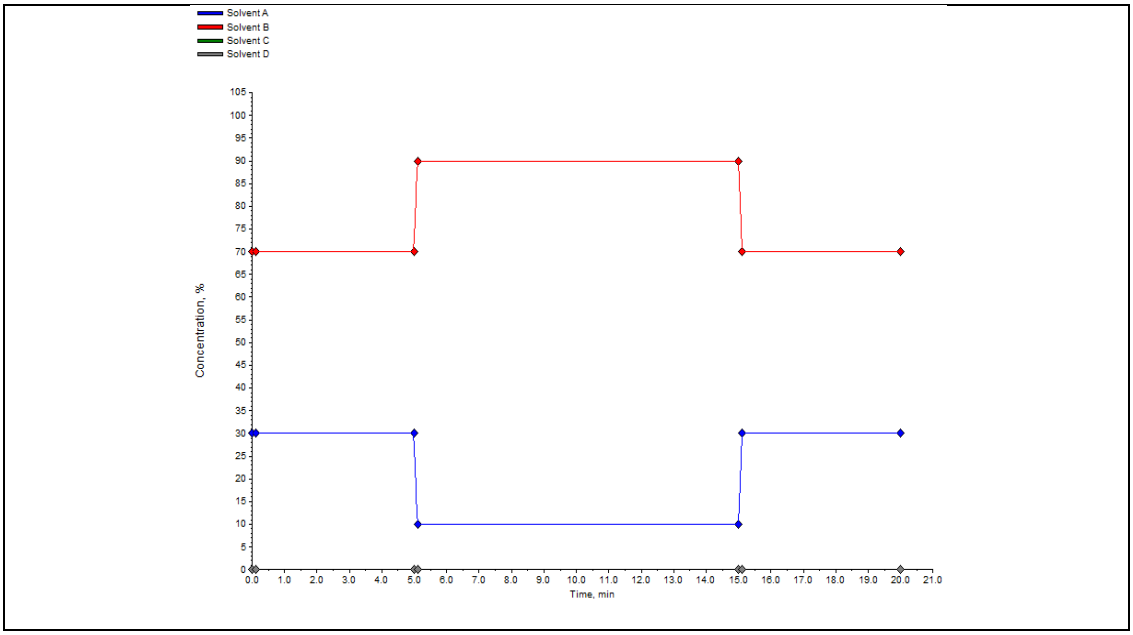


Figure 48 – Graphical representation of MP Gradient System A

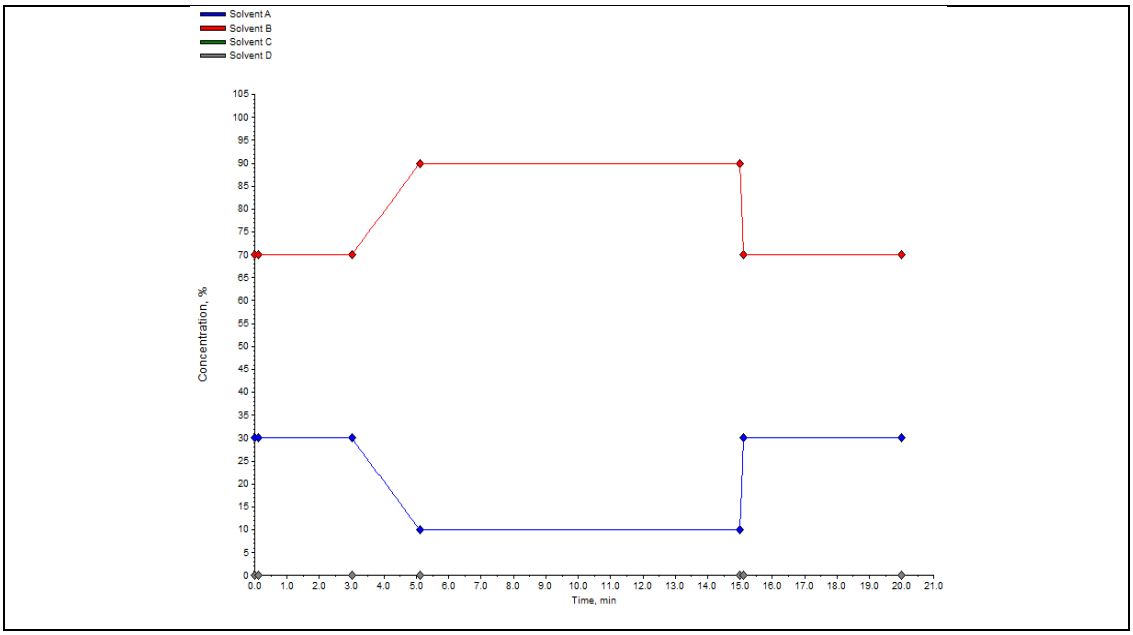


Figure 49 – Graphical representation of MP Gradient System B

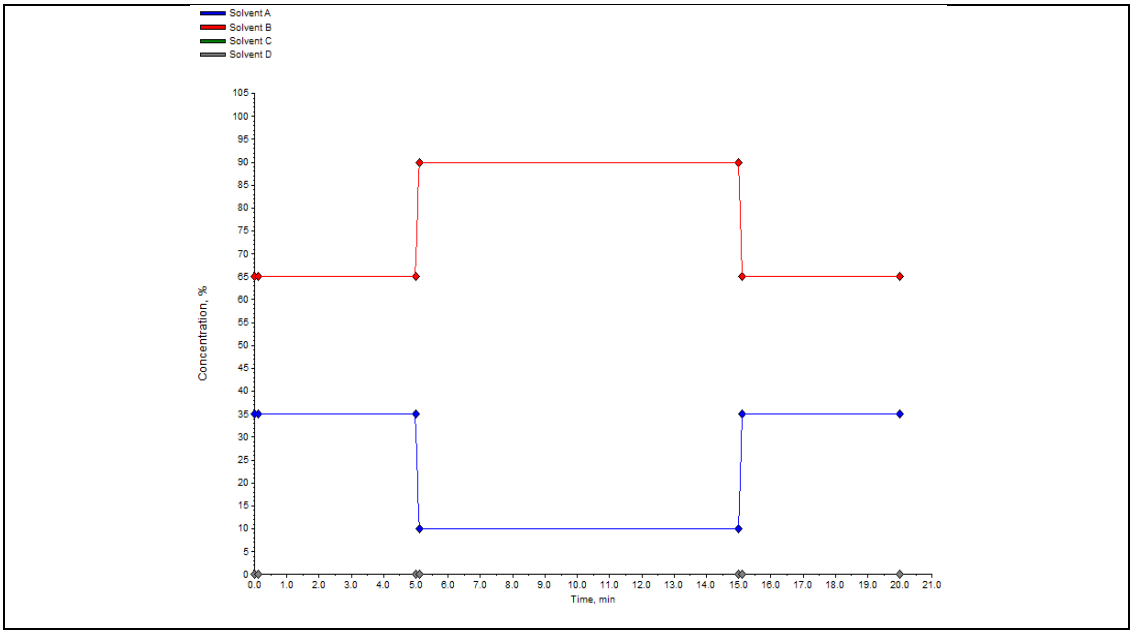


Figure 50 – Graphical representation of MP Gradient System C

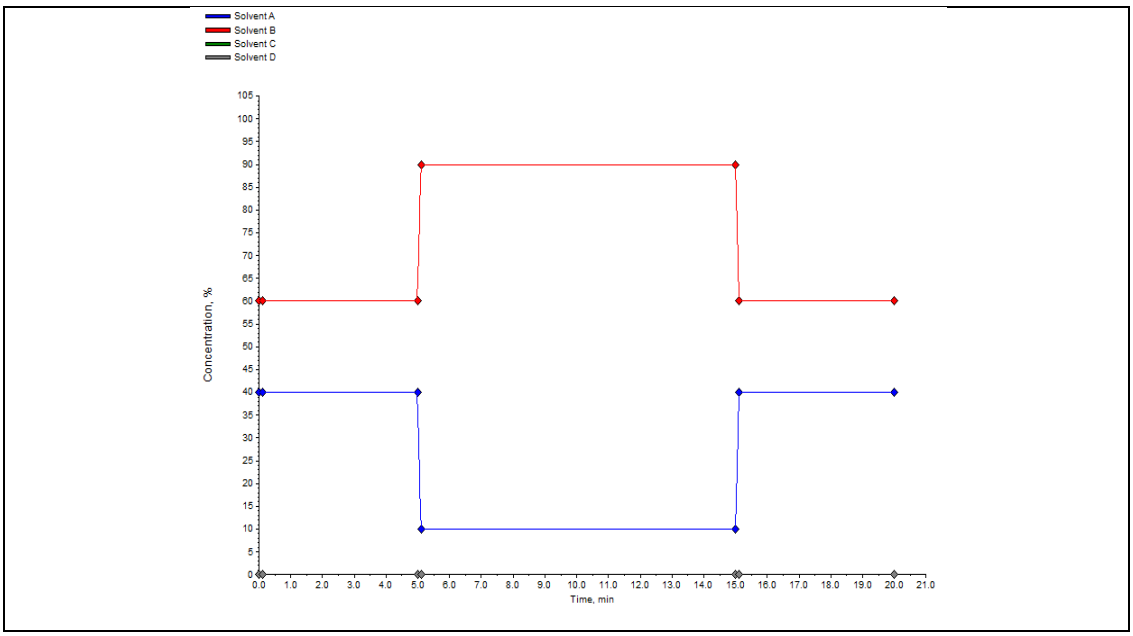


Figure 51 – Graphical representation of MP Gradient System D

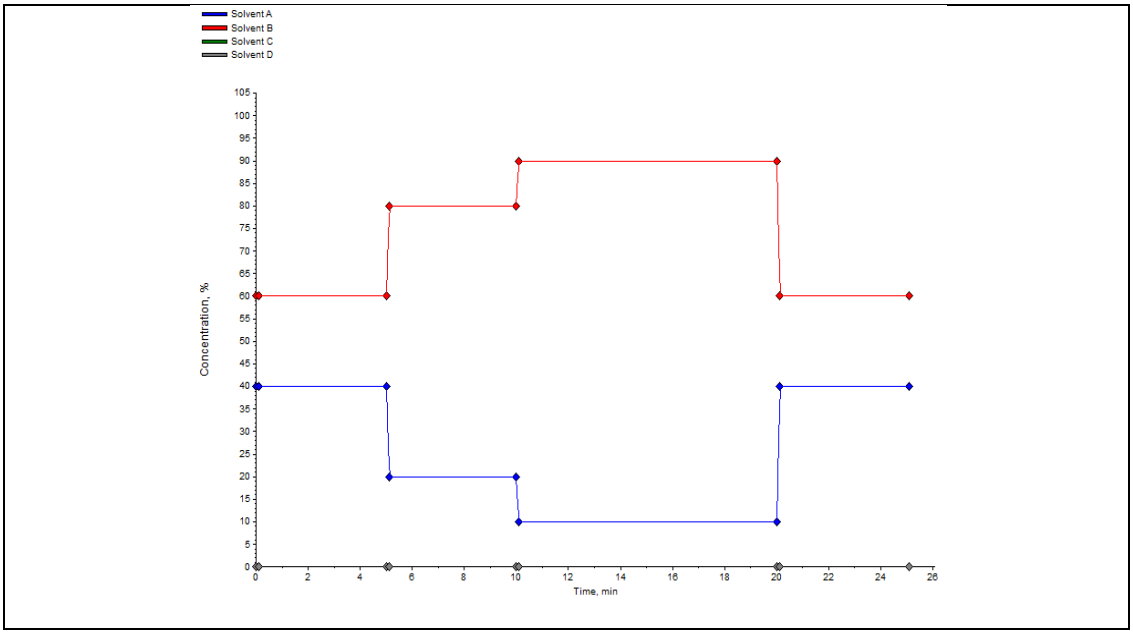


Figure 52 – Graphical representation of MP Gradient System E

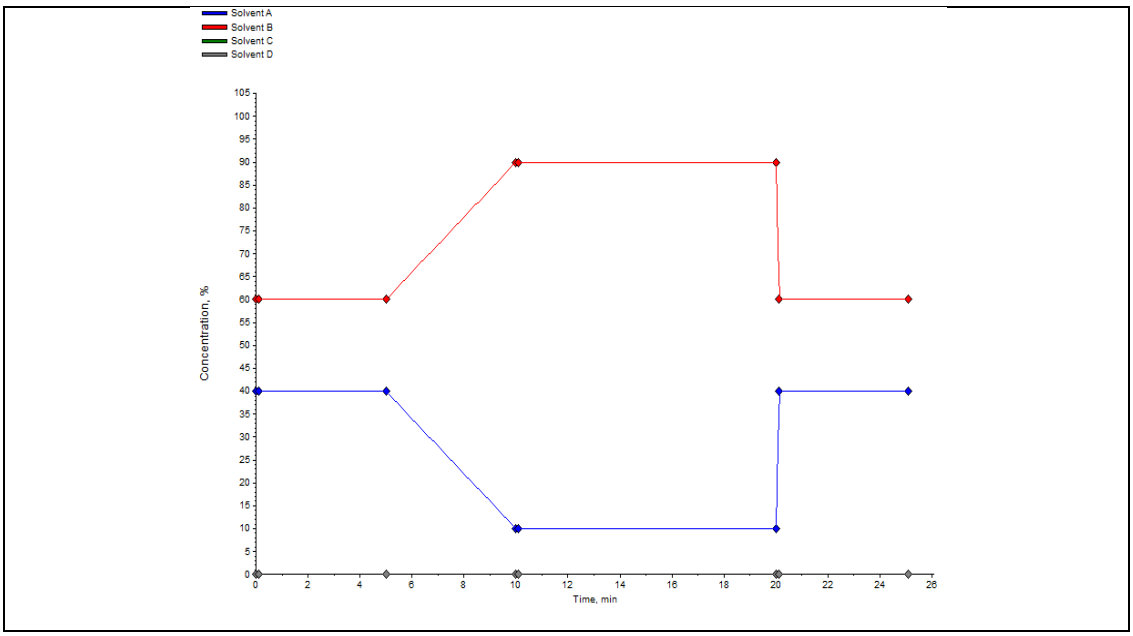


Figure 53 – Graphical representation of MP Gradient System F

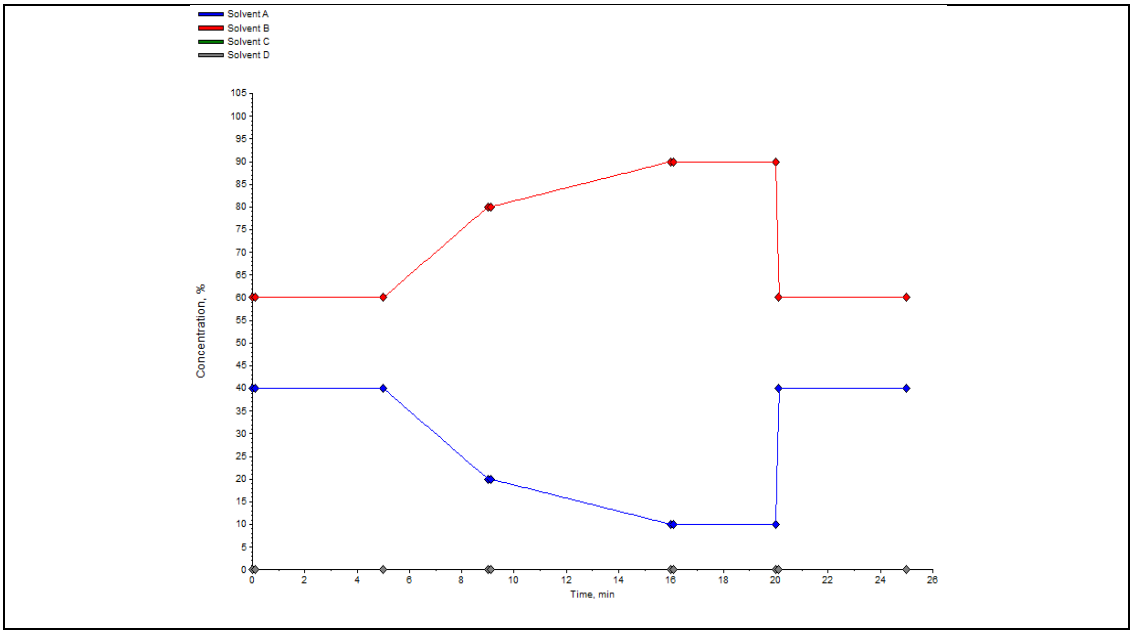


Figure 54 – Graphical representation of MP Gradient System G

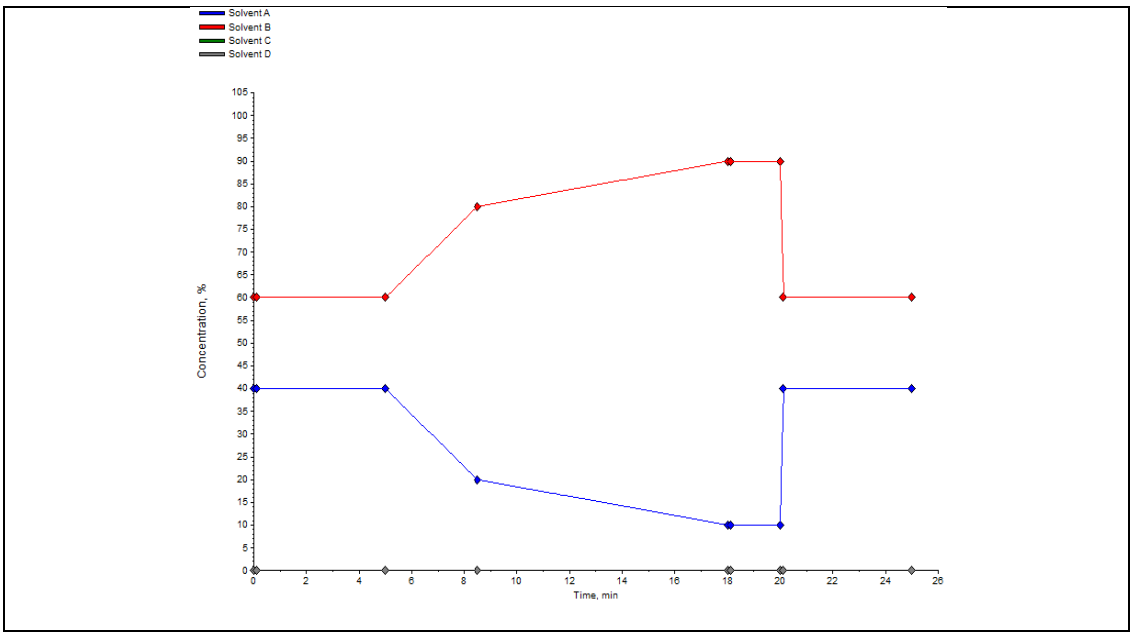


Figure 55 – Graphical representation of MP Gradient System H

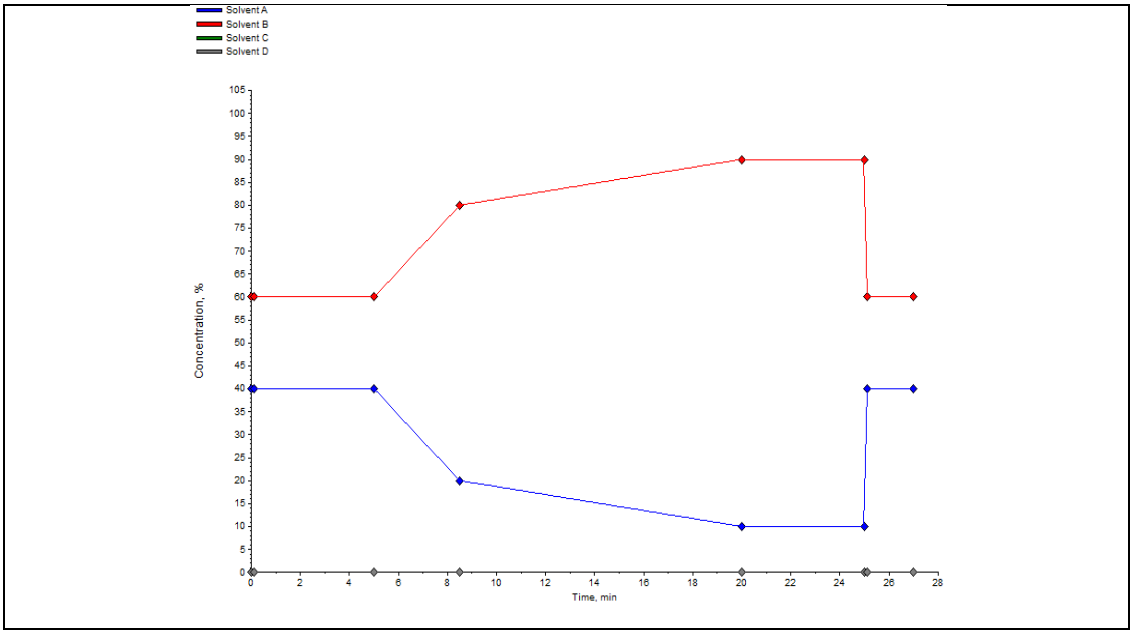


Figure 56 – Graphical representation of MP Gradient System I

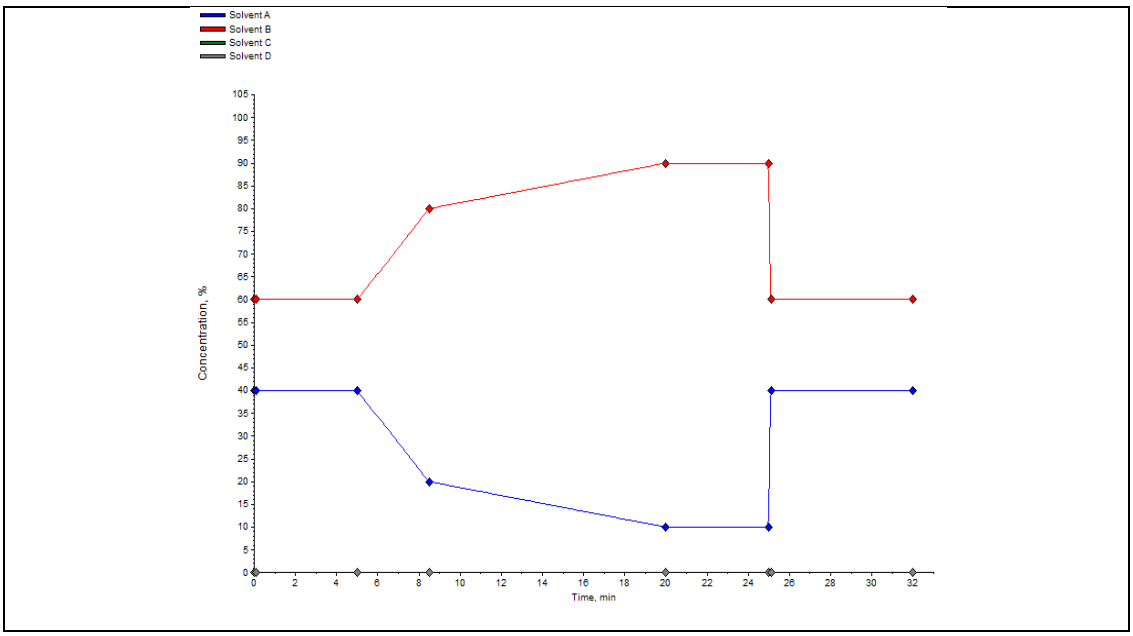


Figure 57 – Graphical representation of MP Gradient System J

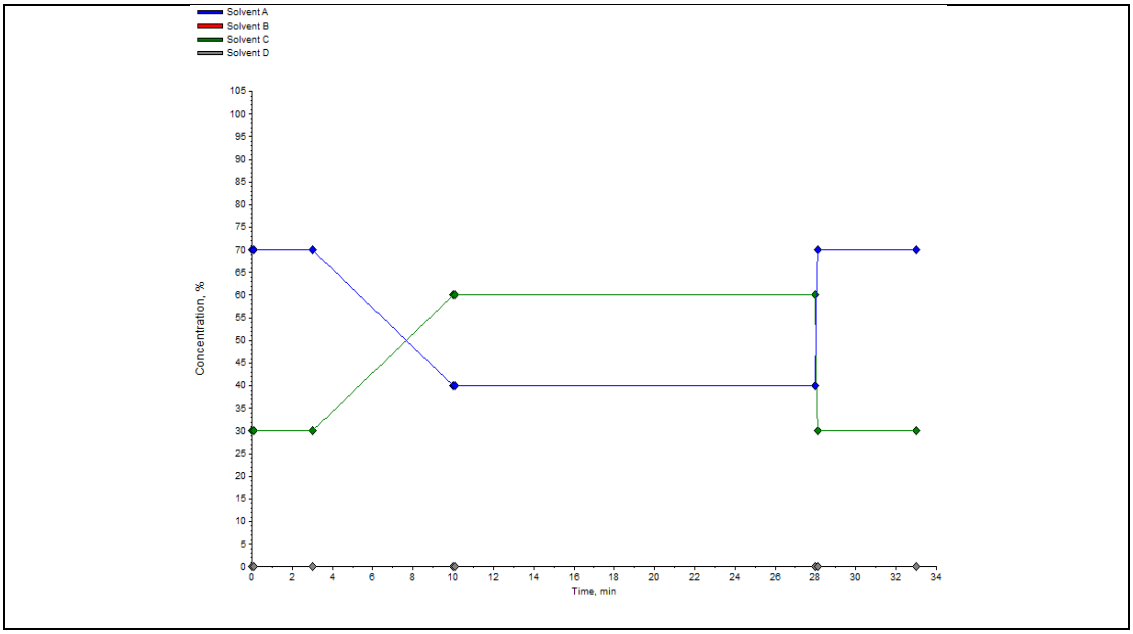


Figure 58 – Graphical representation of MP Gradient System K

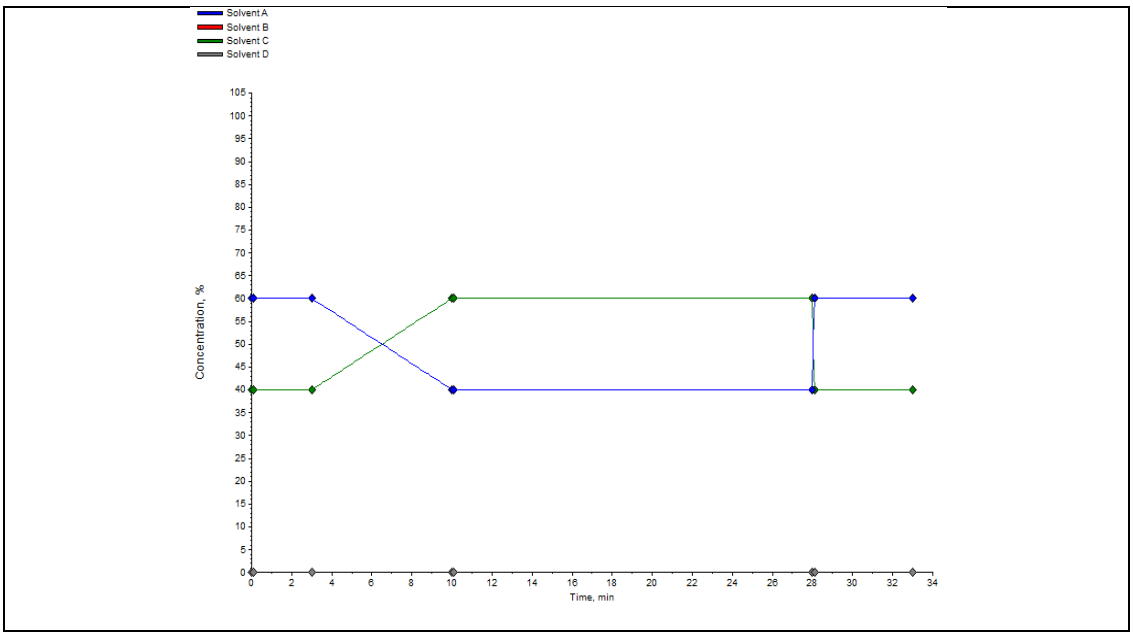


Figure 59 – Graphical representation of MP Gradient System L

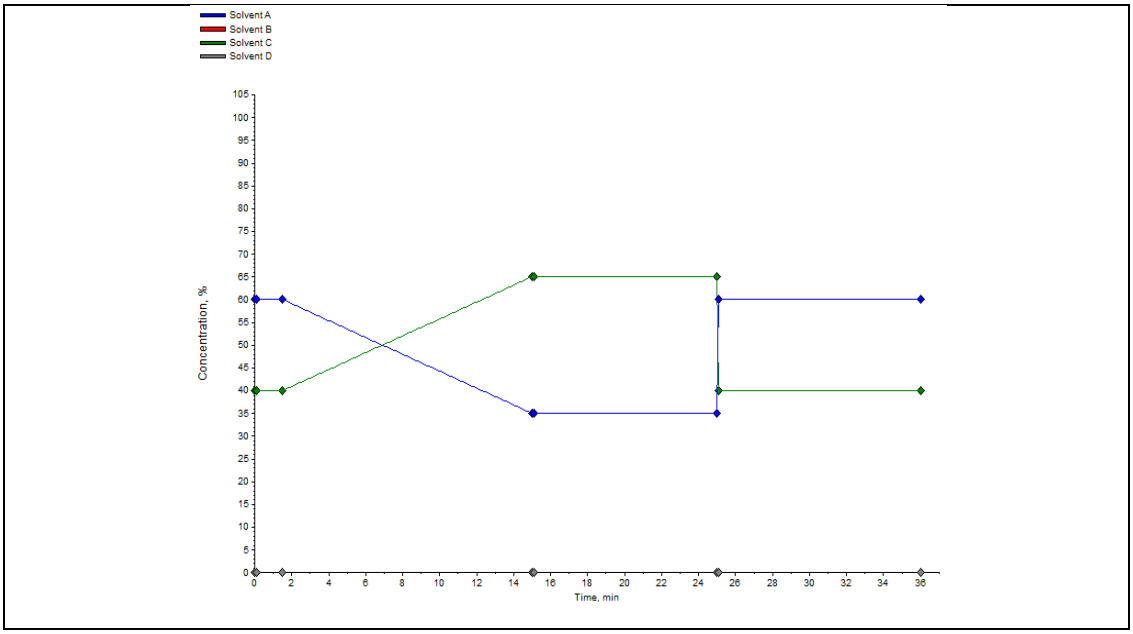


Figure 60 – Graphical representation of MP Gradient System M

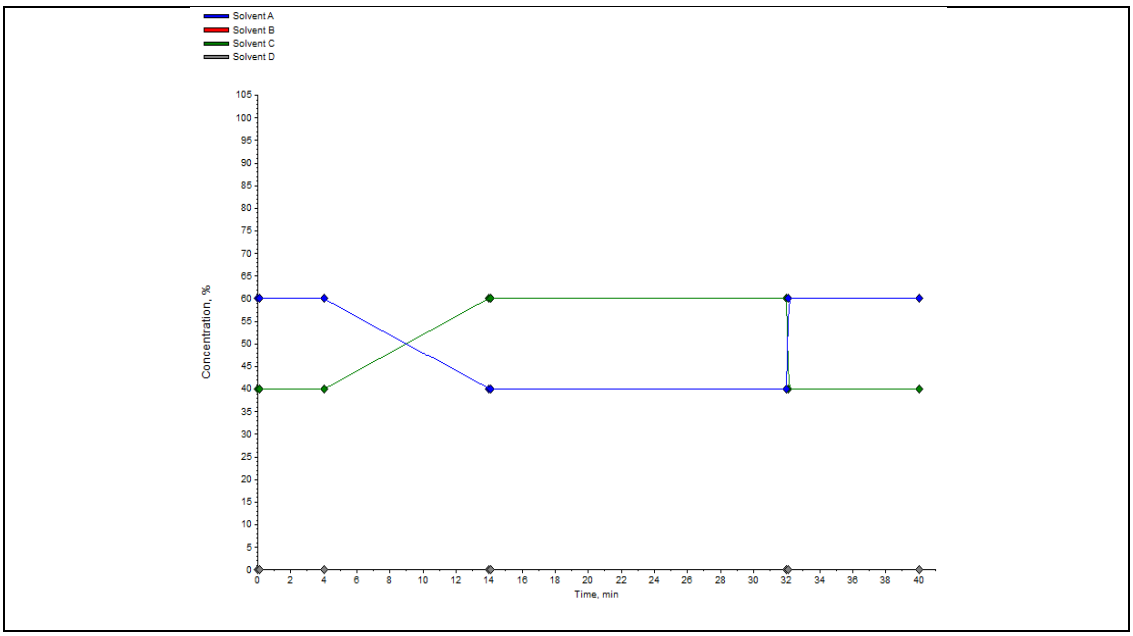


Figure 61 – Graphical representation of MP Gradient System N

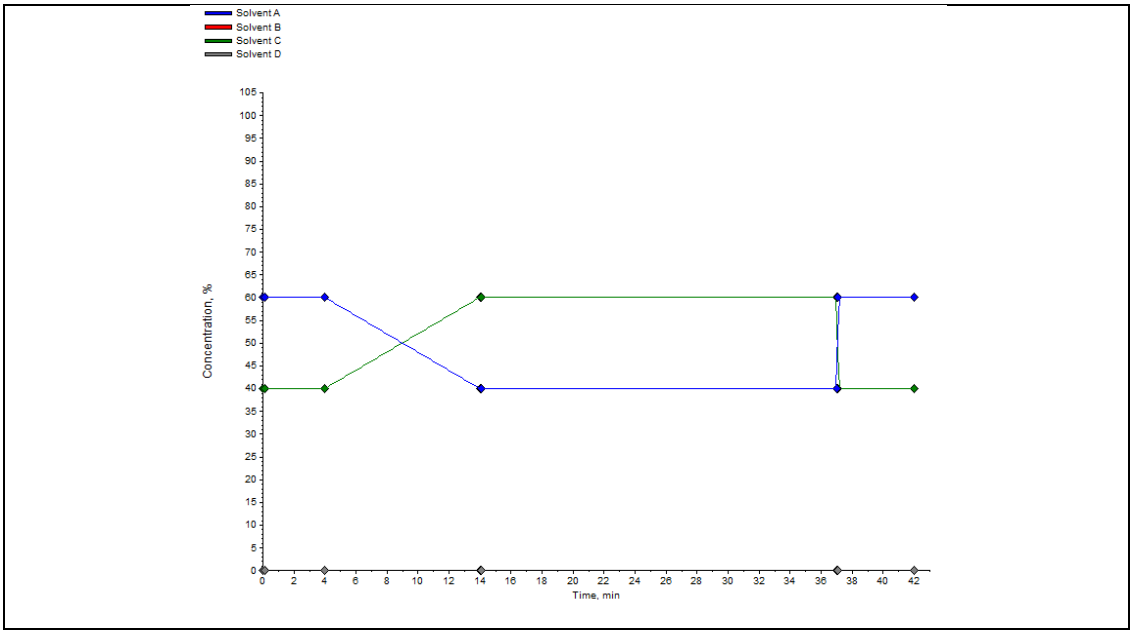


Figure 62 – Graphical representation of MP Gradient System O

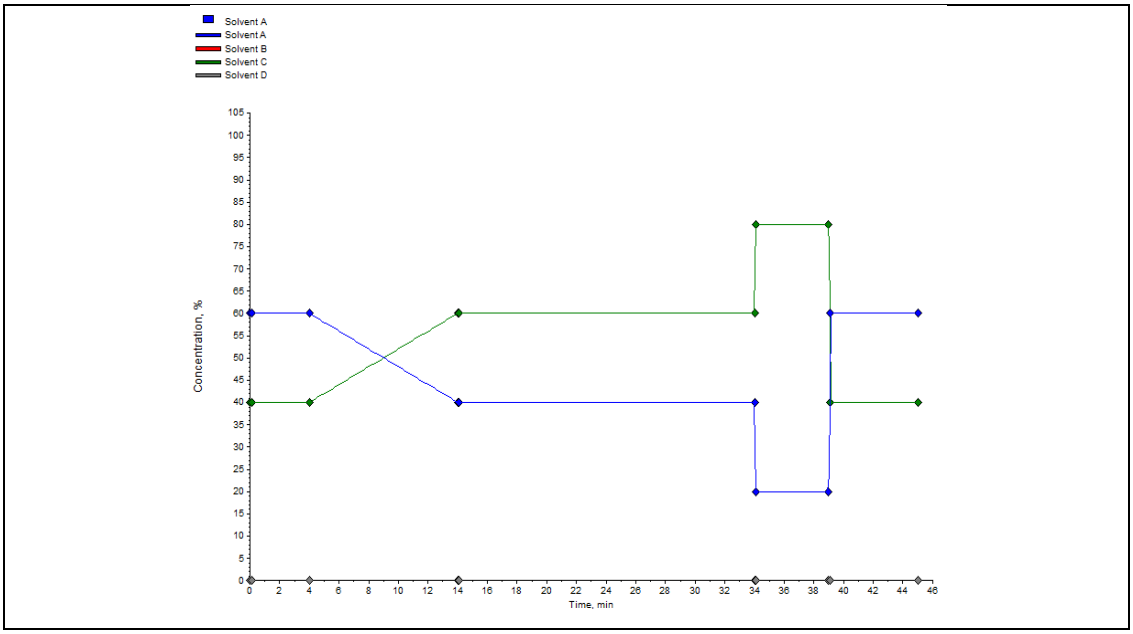


Figure 63 – Graphical representation of MP Gradient System P

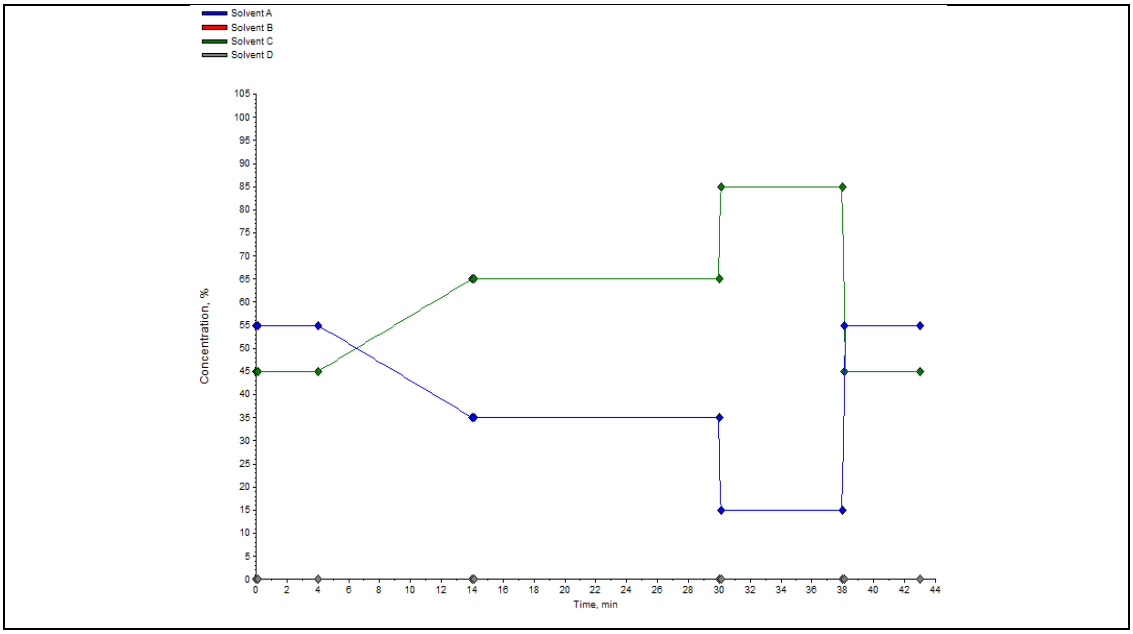


Figure 64 – Graphical representation of MP Gradient System Q

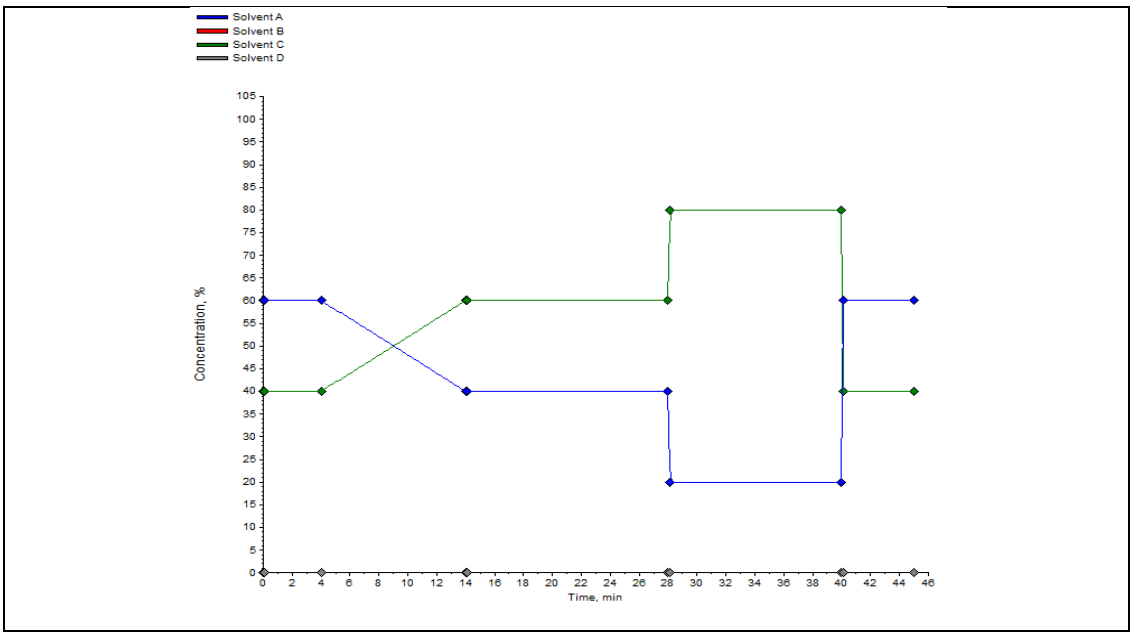


Figure 65 – Graphical representation of MP Gradient System R

9.2. Appendix B – Letter of Comfort from MVLS REC regarding the comparison of Prison 'A' and 'B' samples

University of Glasgow

Dear Dr Seywright

MVLS College Ethics Committee

Project: Development and validation of an analytical method for detecting drugs in blood and urine

The College Ethics Committee has reviewed your application. Based on the materials you submitted, we decided that the project does not require formal review from the Committee.

The project as described would be seen as service development and evaluation. We note that the HRA and Ministry of Justice are also of this view. It is acceptable to describe the process and results of a service evaluation in a PhD thesis and to share the learning with the wider scientific community through presentation at meetings or publications. However, the primary intention with this work is to develop an assay for local use. If sharing your work with others, I would be careful not to describe it as 'research'.

You can use this letter as evidence that your project was assessed by MVLS ethics – sometimes called a 'letter of comfort'. Your project may need other approvals before it can commence.

I wish you well in your project.

Yours sincerely

Terry Quinn
FESC, MD, FRCP, BSc (hons), MBChB (hons)
Senior Lecturer / Honorary Consultant

College of Medicine, Veterinary & Life Sciences
Institute of Cardiovascular and Medical Sciences
New Lister Building, Glasgow Royal Infirmary
Glasgow
terry.quinn@glasgow.gla.ac.uk
Tel – 0141 201 8519

The University of Glasgow, charity number SC004401

9.3. Appendix C – Details of additional analyses in Emergency Department cases

Table 55 – Details of Additional Analyses

Analysis	Analytes Included
Alcohol	Quantitates ethanol and acetone; qualitatively identifies acetaldehyde, methanol and isopropanol
Basic Drugs	A general screening method which qualitatively identifies many basic drugs and quantitates commonly used drugs.
Drugs of Abuse Screen	Presumptively identifies amphetamine, benzodiazepines, buprenorphine, cannabinoids, cocaine and related compounds, methadone, methamphetamines, and opiates
Cannabinoids	Quantitates Δ9-THC, 11-Nor-Δ9-THC-carboxylic acid
Benzodiazepines 1	Quantitates diazepam, desmethyldiazepam, temazepam, oxazepam, chlordiazepoxide, lorazepam, nitrazepam, 7-aminoflunitrazepam, etizolam, phenazepam, diclazepam, delorazepam, lormetazepam, flubromazepam and pyrazolam
Benzodiazepines 2	As Benzodiazepines 1, plus deschloroetizolam, nifoxipam, meclonazepam, clonazolam and flubromazolam
Opiates	Quantitates morphine, 6-monoacetyl-morphine (6-MAM), codeine and dihydrocodeine

9.4. Appendix D – Analysis and Clinical Findings of Cases Positive for the Novel Synthetic Cannabinoid Receptor Agonist MDMB-CHMICA

Permission to reproduce this article has been sought and granted by Taylor and Francis, publishers of Clinical Toxicology. This article is availble in full here: https://www.tandfonline.com/doi/full/10.1080/15563650.2016.1186805

Analysis and Clinical Findings of Cases Positive for the Novel Synthetic Cannabinoid Receptor Agonist MDMB-CHMICA

Alice Seywright[a], Hazel J. Torrance[a], Fiona M. Wylie[a], Denise A. McKeown[a], David J. Lowe[b,c], Richard Stevenson[b]

[a]Forensic Medicine and Science, University of Glasgow, Glasgow, UK; [b]Glasgow Royal Infirmary, Glasgow, UK; [c]Academic Unit of Anaesthesia, Pain and Critical Care Medicine, School of Medicine, University of Glasgow, Glasgow, UK

Corresponding author: Alice Seywright, +441413304574, alice.seywright@glasgow.ac.uk, Forensic Medicine and Science, Joseph Black Building, University Place, Glasgow, G12 8QQ, UK.

Keywords: MMB-CHMINACA, Blood analysis, NPS, Legal highs

Article written in UK English

Abstract

Context: MDMB-CHMICA is a synthetic cannabinoid receptor agonist which has caused concern due to its presence in cases of adverse reaction and death. *Method:* 43 cases of suspected synthetic cannabinoid ingestion were identified from patients presenting at an Emergency Department and from post-mortem casework. These were subjected to liquid-liquid extraction using tertiary-butyl methyl ether and quantitatively analysed by Electospray Ionisation Liquid Chromatography – tandem Mass Spectrometry. For positive samples, case and clinical details were sought and interrogated. *Results:* 11 samples were found positive for MDMB-CHMICA. Concentrations found ranged from <1 – 22 ng/mL (mean: 6 ng/mL, median: 3 ng/mL). The age range was 15 – 44 years (mean: 26 years, median: 21 years), with the majority (82%) of positive results found in males. Clinical presentations included hypothermia, hypoglycaemia, syncope, recurrent vomiting, altered mental state and serotonin toxicity, with corresponding concentrations of MDMB-CHMICA as low as <1 ng/mL. Duration of hospitalisation ranged from 3 – 24 hours (mean: 12 hours, median: 8 hours). *Discussion:* The concentration range presented in this case series is indicative of MDMB-CHMICA having a high potency, as is known to be the case for other synthetic cannabinoid receptor agonists. The age range and gender representation were consistent with that reported for users of other drugs of this type. The clinical presentations observed were typical of synthetic cannabinoid receptor agonists and show the difficulties in identifying reactions potentially associated with drugs of this type. *Conclusion:* The range of MDMB-CHMICA concentrations in Emergency Department presentations (n=9) and post-mortem cases (n=2) was reported. No correlation between the concentration of this drug and clinical presentation or cause of death was reported in

this sample. However, the potential for harm associated with low concentrations of MDMB-CHMICA and the symptoms of toxicity being non-specific was highlighted.

Introduction

MDMB-CHMICA (methyl-2-(1-(cyclohexylmethyl)-1 H-indole-3-ylcarbonylamino)-3,3-dimethyl butanoate, Figure 1), is a novel indole-based compound in the 'synthetic cannabinoid receptor agonist' (SCRA) drug group. It has previously been erroneously referred to as MMB-CHMINACA. Based on SCRA nomenclature, however, 'CHMINACA' indicates an indazole – rather than indole – structure, and 'MMB-' indicates an isopropyl - rather than tertiary butyl - group.

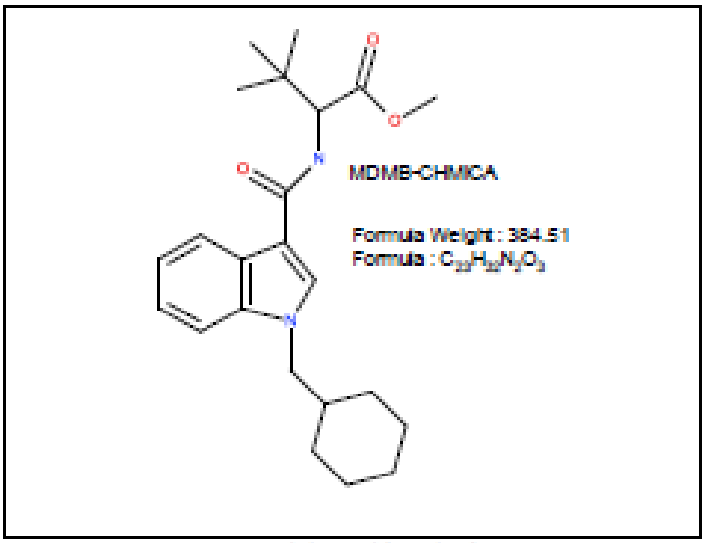


Figure 1 – Structure and chemical formula of MDMB-CHMICA

The potential dangers of MDMB-CHMICA were first highlighted by the European Monitoring Centre for Drug and Drug Addiction Early Warning System (EMCDDA EWS) in December 2014 when seven non-fatal intoxications were linked to the drug in Austria[1]. Shortly after these adverse reactions, ten intoxications – four proving fatal – were reported in Sweden[1]. A third alert was issued in April 2015 reporting two deaths and three non-fatal intoxications linked to the drug in Germany[2]. Adverse reactions reported in the latter included seizures, severe motor impairment, and persistent vomiting; recorded causes of death included probable methadone intoxication and suffocation on aspirated gastric contents related to ethanol intoxication. In addition to this, details of seizures of the drug were given, including 40 kg en route from China to Spain seized in Luxembourg[2]. As of the beginning of May 2016, MDMB-CHMICA is not controlled in the U.K., but has been confirmed as an ingredient in several products including 'AK47 Loaded', 'Manga Hot', 'Cloud 9-second

generation-Mad Hatters-incense', 'Black Diamond', 'AKB48-F', 'PB-22-F', 'Sky High' and 'Sweet Leaf Obliteration' in Europe[2, 3].

There is currently a paucity of data regarding the pharmacology and toxicology of MDMB-CHMICA, but case reports describe vomiting, seizures, and psychological distress as possible sequlae from ingestion. A case report from Norway implicates an ante-mortem serum concentration of 1.4 ng/mL MDMB-CHMICA as the probable cause of death, although mirtazapine (5.3 ng/mL), Δ^9-tetrahydrocannabinol (Δ^9-THC, 1.5 ng/mL), and cetirizine (not quantified) were also present[4].

The first reports of adverse reactions to MDMB-CHMICA in the U.K. were reported in the summer of 2015 with one in North Wales and one in Glasgow[5, 6]. In response to this the authors developed and validated an LC-MS/MS method, with a simple liquid-liquid extraction for the quantification of MDMB-CHMICA in whole blood.

Materials and Methods

Materials

MDMB-CHMICA was purchased from Chiron (Trondheim, Norway) and JWH-200-d_5 was purchased from LGC Standards (Teddington, U.K.). Phosphate buffer (pH 6, 0.1M) was prepared in-house from disodium hydrogen orthophosphate anhydrous and sodium dihydrogen orthophosphate dihydrate from Fisher Scientific (Loughborough, U.K.) and deionised water produced from a Purite (Thame, U.K.) deionised water system. *Tertiary* methyl butyl ether (*t*BME) and ammonium acetate were purchased from Sigma Aldrich (Gillingham, U.K.). Methanol and acetonitrile, both HPLC grade, and formic acid were obtained from VWR (Lutterworth, Leicestershire, U.K.). Blood products were purchased from the Scottish National Blood Transfusion Service (SNBTS) based at Gartnavel Hospital (Glasgow, U.K.).

Methods

Sample Collection and Data Analysis

The laboratory at Forensic Medicine and Science (FMS) provides post-mortem (PM) toxicology services for the cities of Glasgow, Edinburgh and Dundee and their surrounding regions, as well as an ongoing research project analysing Emergency Department (ED) admission samples for Novel Psychoactive Substances (NPS). Between the 1st of September and the 9th of December 2015, 43 cases were submitted for MDMB-CHMICA analysis to the laboratory, comprising 17 PM submissions and 26 cases from the ED of Glasgow Royal

Infirmary. The decision to conduct MDMB-CHMICA analysis on a sample was made on case circumstances suggestive of SCRA use. It is acknowledged that in some circumstances case details may have been misleading or lacking leading to incorrect inclusion or exclusion of cases.

Unpreserved blood samples were obtained either by collection at autopsy for PM cases, or during the clinical management of ED patients. The NHS Greater Glasgow and Clyde Research and Development Committee advised ethical approval was not required for this service development study. With regards to the PM cases, the medical histories of the deceased and basic PM findings were reviewed. Prior to toxicological analysis, only limited presenting symptoms and potential substances ingested were known for ED samples.

On receipt at Forensic Medicine and Science, the specimens were stored between 2 – 8 °C prior to analysis. These were submitted for MDMB-CHMICA analysis if the case circumstances suggested that SCRAs may have been used prior to death or hospital treatment. The additional analyses conducted were dependant on case circumstances and available sample volume, and included alcohol and the most prevalent prescription and recreational drugs.

On completion of toxicological analysis, each case was reviewed in terms of gender and age of individual, other toxicological findings, drug product ingested (if noted) and any other relevant circumstantial information. Where the case was a PM investigation, the assigned cause of death was also noted.

Toxicological Analysis

MDMB-CHMICA was detected and quantified using an Agilent 1260 Infinity Liquid Chromatography (LC) system coupled with tandem Mass Spectrometry (MS; ABSciex 3200 QTRAP® instrument). A calibration range of 1 – 100 ng/mL MDMB-CHMICA was employed. Extraction of the analyte plus internal standard (I.S., JWH-200-d_5 at 25 ng/mL) was undertaken through a liquid-liquid extraction process, by adding *t*BME (2 mL) to 100 µL blood sample plus 2 mL pH 6.0, 0.1 M phosphate buffer. Chromatographic separation took place on a Phenomenex Gemini C18 column (150 mm x 2.0 mm, 5 µm) fitted with a guard column of the same packing material, held at 40 °C, and using a mobile phase flow rate of 300 µL/min. The mobile phase was run isocratically and was composed of 0.1% formic acid and 2 mM ammonium acetate in 20% deionised H_2O and 80% methanol. MS detection was conducted using positive Electrospray Ionisation (ESI) with multi-reaction monitoring (MRM). The transitions monitored for MDMB-CHMICA were m/z 385 → 240 for the quantification transition and m/z 385 → 144 and 116 (qualifier transitions), with m/z 390 → 155 for the I.S.

The method was validated according to the following criteria: linearity with 1/x weighting was assessed by analysing calibrators of 1, 5, 10, 25, 50 and 100 ng/mL, evaluating the linear model and correlation co-efficient. Selectivity was determined by the absence of a MDMB-CHMICA peak in MDMB-CHMICA-free samples. The lower limit of quantification (LLOQ) was defined as the lowest calibrator, ensuring a signal-to-noise ratio of at least 10:1. The limit of detection (LOD) was designated as the lowest concentration affording a signal-to-noise ratio of 3:1. Inter- and intra-day accuracy and precision were determined at 10 and 42 ng/mL, with accuracy defined by the ratio of the mean actual concentration of triplicate standards to their expected concentration multiplied by 100, and the %CV of the triplicate results taken as the precision. Process efficiency was determined at 50 ng/mL by comparison of the mean peak area of calibrators extracted from blank blood in triplicate to those of an unextracted solution of equivalent concentration. Matrix effects were assessed at 50 ng/mL using the Matuszewski method[7] and 6 distinct sources of blank blood.

Results

Toxicological Analysis

Linearity was assessed over the calibration range 1 – 100 ng/mL and established (R^2 >0.99) using 1/x weighted regression. The LLOQ was assigned as the lowest calibrator (1 ng/mL), with LOD determined to be 0.5 ng/mL. Mean inter-day accuracy was found to be 102 and 104% at 10 and 42 ng/mL respectively; and the mean precision at the same concentrations was ≤8.3% and ≤5.3% respectively. Mean intra-day precision was found to be ≤4.7% at 10 ng/mL and ≤3.7% at 42 ng/mL; and the mean accuracy was calculated as 96% and 108% for 10 ng/mL and 42 ng/mL respectively. Process efficiency was calculated as 90% at 50 ng/mL. Slight ion enhancement was observed when the matrix effects were assessed, the most significant of these being an extracted peak area at 116 % of the unextracted equivalent.

The ion transitions employed in MDMB-CHMICA analysis were found to be identical to those used for another SCRA, BB-22 (1-(cyclohexylmethyl)-1H-indole-3-carboxylic acid 8-quinolinyl ester), which is similar structurally and shares the precursor ion m/z 385. In addition to this, these drugs were not resolved chromatographically on the MP system in use. In order to distinguish between the two drugs, the ratio of the quantitation transition (m/z 385 → 240) to the qualifier transition (m/z 385 → 116) was calculated and this value differed sufficiently to allow distinction. However, it is important to bear in mind the resemblance of these substances in terms of analytical behaviour.

Analysis of Case Samples

Between the 1st of September and the 9th of December 2015, 43 blood samples were submitted for MDMB-CHMICA analysis, comprising 17 PM cases and 26 ED cases, with a total of 11 found to be positive for the drug. Concentrations ranged from <1 to 22 ng/mL, with a mean of 6 ng/mL and median of 3 ng/mL. These cases are detailed in Table 1 and Table 2 for ED and PM cases respectively. With regards to the cases negative for this analyte, generally these were found positive for other drugs which would account for the observed symptoms. This, however, falls outside the scope of this study.

The age range was 15 – 44 years (mean: 26, median: 21). The gender split was 82% males to 18% females.

The most prevalent substance found in combination with MDMB-CHMICA in the samples tested was alcohol, which was present in 60% of MDMB-CHMICA-positive cases at concentrations from 79 to 237 mg/dL (mean: 163, median: 178). It should be noted that alcohol analysis was not conducted in case 10, as it was not requested by the pathologist. The main active component of cannabis, Δ^9-THC, or its metabolite, 11-nor-Δ^9-THC-COOH, was also present in 27% of cases.

The predominant clinical features on presentation at ED were syncope, present in 67% of ED cases, and recurrent vomiting, present in 33% of ED cases. With the exception of cases 1, 5 and 8 body temperatures were indicative of hypothermia with mean and median body temperatures of 35.1 °C and 34.8 °C respectively (range: 33.0 – 38.5 °C). Blood glucose concentrations, where noted, ranged from 3.3 – 8.5 mmol/L (mean and median both 5.5) with 75% within the range indicative of fasting. Heart rates ranged from 54 – 150 bpm (mean: 112, median: 120) with sinus tachycardia being the most common presentation (78%). Systolic blood pressure (SBP) ranged from 100 – 148 (mean: 118, median: 116), with the diastolic equivalent (DBP) ranging from 57 – 91 (mean: 71, median: 65). Glasgow Coma Scores (GCS) ranged from 4/15 – 15/15 with the mean and median being 12/15 and 14/15 respectively. Duration of hospitalisation varied, ranging between 3 – 24 hours (mean: 12 hours, median: 8 hours).

The cause of death noted for case number 10 was suicidal hanging; the individual had a history of poor mental health.

With regards to case 11, the cause of death was recorded as complications of chronic alcohol abuse and acute alcohol toxicity.

Discussion

The age range and gender representation is in-keeping with other studies where 'young' males or males in their early 20s have been the most common demographic to be encountered by medical staff treating symptoms of synthetic cannabis use[8, 9]. Of note is the consumption of the drug by an individual under 16 (case 4); there is a lack of understanding as to how MDMB-CHMICA use may affect development either physiologically or mentally.

It is not possible to compare the positivity rate between PM samples and ED admissions due to the non-standardised inclusion criteria for MDMB-CHMICA analysis. The effects of the interval between death and PM, PM sampling and analysis, and the degraded nature of PM blood on MDMB-CHMICA concentrations is unknown, and these complications were unlikely to be present to the same extent in the ED cases. An additional concern is the limited information available regarding potential metabolites of MDMB-CHMICA and no potential metabolites were included in the analytical method presented here.

The presence of alcohol in 60% of cases is unsurprising given its prevalence in Scottish society. Case numbers 6, 7 and 11 exhibit notably high alcohol concentrations; the symptoms of which may themselves require medical intervention. Literature has suggested that users of SCRAs are likely to have been or be current cannabis users, and SCRAs may be co-administered with cannabis by being smoked in the same 'joint'[10] The presence of cannabinoids or SCRAs in cases 2, 3, 6, 8 and 9 is indicative of these substances having been ingested within a similar timescale to or concurrently with MDMB-CHMICA.

With regards to cases 1, 8 and 9, no benzodiazepines were prescribed or used in the treatment of these individuals. The presence of diazepam and/or desmethyldiazepam was due to illicit consumption.

The drug products named, where provided, have largely been found to contain MDMB-CHMICA[8]. However it is not uncommon for drug product packaging to be misleading or incorrect in terms of ingredients, so this information should be treated with caution.

While little is known currently about the potential short- and long-term psychological effects of SCRAs, there is evidence of new psychotic phenomena exhibiting after SCRA use in individuals already undergoing treatment for psychiatric disorders[8, 11]. Known effects of SCRA use are hallucinations, psychosis, anxiety and panic attacks,

and it has been suggested that these may be due to disturbed dopaminergic neurotransmission; also a hypothesis for the aetiology of schizophrenia[12].

Recent media reports have focussed on the apparent prevalence of use of "legal highs" - specifically SCRAs - within prisons, and the Prison and Probation Ombudsman for England and Wales has recently issued a report into fatal cases of "legal high" use[14-15]. Some of these incidents included the use of SCRAs, although no deaths mentioned in this report suggest SCRAs intoxication as a direct cause of death, and the specific drug is not mentioned. The types of behaviours associated with SCRA use, some of which were observed in these cases, would be a troubling occurrence in prison settings, both in terms of safety of the individual under the influence of the drug and those managing the situation. Indeed, the U.K. Government intends to make possession of MDMB-CHMICA and other drugs covered by the Psychoactive Substances Bill an offence whilst in a custodial institution; possession of such a drug out in any other setting will not be prohibited[16].

A recent study analysed the urine of ED patients for Novel Psychoactive Substances (NPS), but acknowledged a limitation that SCRA drugs were not included within the scope of testing[17].

The observation of hypothermia was believed to be reflective of the drug's action, as all cases occurred during the summer months, when the ambient temperature was around 12 °C, and the patients were brought to hospital relatively quickly after ingestion of the drug.

With regards to case 2, the relatively high concentration of MDMB-CHMICA, along with other SCRAs, appeared to have contributed to the recurrent hypoglycaemia, necessitating an intravenous infusion of dextrose, with additional boluses. This pharmacological action is supported, as the patient was found to be smoking the product within the hospital ward during the episodes of profound hypoglycaemia whilst on a dextrose infusion. The overall majority of ED cases (75%) exhibited blood glucose concentrations indicative of fasting. Whilst this could be a symptom of generally poor self-care among drug users, it is an interesting finding as previous studies have linked the ingestion of synthetic cannabinoids to hyperglycaemia [18,19].

Heart rates were generally tachycardic (78%) while BP were generally unremarkable. Case number 1 exhibited a heart rate of 130 bpm and was hypertensive. Case 5 also exhibited hypertension, with a BP of 148/91. Case 7 was the only other case outside of the normal BP range, at a pre-hypertensive level of 134/76.

It was of concern that very low concentrations of MDMB-CHMICA were capable of inducing acute toxicity manifesting as a dissociative state (case 9) and clinical features of serotonin toxicity (case 7), suggestive of high

potency. Additional drugs or alcohol present in these cases, idiosyncratic reactions, and/or the presence of active MDMB-CHMICA metabolites must be acknowledged as potential contributing factors to adverse effects. It is not surprising, therefore, that overall the concentrations of MDMB-CHMICA observed do not appear to have any correlation to blood glucose concentration, GCS score, heart rate, blood pressure, or duration of hospitalisation in this case series. However, there does seem to be evidence of the drug causing a decrease in body temperature, which may be of interest in further studies.

With regards to treatment of individuals presenting at the ED, this was administered on a symptomatic basis and guided by local policy. Necessary treatment included the administration of antibiotics due to aspirated vomitus (case 1), intravenous dextrose administration (case 2), urinary catheterisation (case 7), sedation and the administration of cooled fluids (both case 5).

Neither of the PM cases in this case series had causes of death attributable to MDMB-CHMICA toxicity. It is expected that further elucidation of MDMB-CHMICA pharmacology will increase understanding of the potential for this drug to contribute to mortality.

Limitations

The authors acknowledge the small scale of the case series, which was determined by the number of cases submitted for MDMB-CHMICA analysis and the resulting positive cases. The number of cases submitted for analysis was determined by case information, which was limited in some cases and may have been inaccurate due to the illicit nature of the drug and mental state of the individual undergoing emergency treatment. It is possible that the inclusion of metabolites of MDMB-CHMICA would have increased the window of detection for the drug, and additional positive cases may have resulted. The metabolism of this drug is, however, not understood and no reference standards for potential metabolites are commercially available. This is therefore acknowledged as a limitation of this study.

Conclusion

We have presented a series of cases positive for MDMB-CHMICA and the relevant clinical findings, confirming that this drug was used within the Scottish population. Whilst no correlation of MDMB-CHMICA concentration to clinical effects can be confirmed from this limited case series, it was noted that adverse

symptoms were present in a case (case 3) exhibiting an MDMB-CHMICA concentration of <1 ng/mL where no other drugs or alcohol were detected.

Acknowledgements

The authors would like to thank the staff of the Emergency Department at Glasgow Royal Infirmary for sample collection, and Claire Parks and Lauren O'Connor for their work to complete the additional toxicological analyses on these samples.

The authors are also very grateful to the Scottish Government for the financial contribution provided towards the funding for some of this work.

Declarations of Interest

The authors report no declarations of interest.

References

1. EMCDDA. EMCDDA–Europol 2014 Annual Report on the implementation of Council Decision 2005/387/JHA Lisbon: EMCDDA, 2015.
2. Christie R. EU Early Warning System Alert: 2 deaths and 3 non-fatal intoxications in Germany associated with MDMB-CHMICA (methyl-2-(1-(cyclohexylmethyl)-1 H-indol-3-ylcarbonylamino)-3,3-dimethylbutanoate). Luxembourg: EMCDDA, 2015.
3. Public Health Wales. Welsh Emerging Drugs & Identification of Novel Substances Project 2014 [online] Available at: http://www.wedinos.org/db/samples. Accessed 02 October 2015.
4. Westin AA, Frost J, Brede WR, Gundersen POM, Einvik S, Aarset H, et al. Sudden Cardiac Death Following Use of the Synthetic Cannabinoid MDMB-CHMICA. Journal of Analytical Toxicology. 2015;40:86-7.
5. Hospitalisation From Synthetic Cannabinoid MMB-CHMINACA [press release]. Wales: NHS Wales 2015.
6. Simpson K. Drug Trend Bulletin Issue 2. Police Scotland, 2015.
7. Matuszewski B, Constanzer M, Chavez-Eng C. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Analytical Chemistry. 2003;75:3019-30.
8. Tait RJ, Caldicott D, Mountain D, Hill SL, Lenton S. A systematic review of adverse events arising from the use of synthetic cannabinoids and their associated treatment. Clinical Toxicology. 2015;54:1-13.
9. Mills B, Yepes A, Nugent K. Synthetic Cannabinoids. Am J Med Sci. 2015;350:59-62.

10. Winstock A, Lynskey M, Borschmann R, Waldron J. Risk of emergency medical treatment following consumption of cannabis or synthetic cannabinoids in a large global sample. Journal of Psychopharmacology. 2015;29:698-703.
11. Celofiga A, Koprivsek J, Klavz J. Use of synthetic cannabinoids in patients with psychotic disorders: case series. J Dual Diagn. 2014;10:168-73.
12. Kronstrand R, Roman M, Andersson M, Eklund A. Toxicological Findings of Synthetic Cannabinoids in Recreational Users. Journal of Analytical Toxicology. 2013;37:534-41.
13. BBC News. Legal highs: Call to investigate link to prison deaths [online] Available at: http://www.bbc.co.uk/news/uk-england-34385206. Accessed 02 October 2015.
14. The Economist. At Her Majesty's pleasure [online] Available at: http://www.economist.com/news/britain/21660148-legal-highs-are-rife-jails-new-laws-wont-help-much-her-majestys-pleasure. Accessed 05 August 2015.
15. Prison and Probation Ombudsman. Learning Lessons Bulletin Fatal Incident Investigations Number 9 2015.
16. Psychoactive Substances Bill, (2015) [online] Available at http://www.publications.parliament.uk/pa/bills/cbill/2015-2016/0088/cbill_2015-20160088_en_1.htm. Accessed 15 January 2016.
17. Lowe, D.J., Torrance, H.J., Ireland, A.J., Bloeck, F. and Stevenson, R. SODAS: Surveillance of Drugs of Abuse Study. European Journal of Emergency Medicine. 2015;15:16.
18. Seely, K.A., Prather, P.L., James, L.P., Moran, J.H. Marijuana-based drugs: innovative therapeutics or designer drugs of abuse? Molecular interventions. 2011;11(1):36.
19. Hess, C, Stockhausen, S, Kernbach-Wighton, G, Madea, B. Death due to diabetic ketoacidosis: Induction by the consumption of synthetic cannabinoids? Forensic Science International. 2015;257:e6-11.

Table 1 – Details of ED cases positive for MDMB-CHMICA

Case	Gender	Age (years)	MDMB-CHMICA Conc. (ng/mL)	Ethanol Conc. (mg/dL)	Other Substances Present (mg/L)	Estimated time from use to sampling	Circumstances/Clinical Presentation
1	F	20	5	130	Diazepam (0.075)	1 hour	Ingestion of 'Sweet Leaf' product. GCS[§] 4/15. Temp. 37.5 °C. Glucose 7.1 mmol/L. Heart rate 150 bpm sinus tachycardia, BP[‖] 130/90. Persistent vomiting, syncope, respiratory acidosis (Venous blood gases: [H^+] 67[#], [La^-] 1.9**, [HCO_3^-] 25[††], BE -4.7[‡‡]). 8 hours hospitalisation.
2	M	41	22	ND	5F-AKB48 (present), 5F-PB-22 (present), 5F-PB-22 3-carboxyindole metabolite (present)	1 hour	Ingestion of 'Black Mamba' product. GCS[§] 15/15. Temp. 33 °C. Glucose 3.3 mmol/L. Heart rate 40 bpm sinus bradycardia, BP[‖] 110/60. Syncope, recurrent hypoglycaemia (12 hours), background of alcoholism, routine bloods normal. 24 hours hospitalisation.
3	M	25	<5*	80	5F-AKB48 (present), 11-nor-Δ9-THC-COOH‡ (4 ng/mL)	>3 hours	Ingestion of 'Sweet Leaf' and 'Saint Row' products. GCS[§] 15/15. Temp. 35.2 °C. Glucose 3.9 mmol/L. Heart rate 94 bpm sinus tachycardia, BP[‖] 102/65. Syncope, vomiting, routine bloods normal. 3 hours hospitalisation.
4	M	15	<2*	ND	ND	1 hour	Ingestion of 'Red Exodus'. GCS[§] 13/15. Temp. 36 °C. Glucose 4.3 mmol/L. Heart rate 54 bpm sinus bradycardia, BP[‖] 100/57. Persistent vomiting, syncope, routine bloods normal. 22 hours hospitalisation.
5	M	21	<1	ND	ND	>24 hours	Admitted to ED with acute behavioural disturbance and drug-induced psychosis. Spontaneous urinating/defecating, thought disorder, aggression. Temp. 38.5 °C. Heart rate 150 bpm, BP[‖] 148/91. Routine bloods normal. 2 hours hospitalisation.
6	F	16	<1	225	11-nor-Δ^9-THC-COOH[‡] (9 ng/mL)	1 hour	Ingestion of 'Red Exodus'. GCS[§] 14/15. Temp. 33.9 °C. Glucose 5.6 mmol/L. Heart rate 130 bpm sinus tachycardia, BP[‖] 118/74. Combative, acute behavioural disturbance, routine bloods normal. 3 hours hospitalisation.
7	M	18	2	229	ND	40 min.	Ingestion of 'Damnation'. GCS[§] 7/15. Temp. 34.8 °C. Glucose 5.9 mmol/L. Heart rate 120 sinus tachycardia, BP[‖] 134/76. Serotonin toxicity (clonus, hyperreflexia), acute behavioural disturbance, mild metabolic acidosis (Venous blood gases: [H^+] 54[#], [La^-] 3.2**, [HCO_3^-] 23[††], BE -4.8[‡‡]). 19 hours hospitalisation.
8	M	24	1	ND	Δ^9-THC[¶] (5 ng/mL), 11-nor-Δ^9-THC-COOH[‡] (35 ng/mL), diazepam (0.2), desmethyldiazepam (0.13), morphine (<0.05)	4 hours	Ingestion of 'Obliteration'. GCS[§] 14/15. Temp. 37.2 °C .Glucose 5.3 mmol/L. Heart rate 108 sinus tachycardia, BP[‖] 116/61. Syncope, routine bloods normal. 17 hours hospitalisation.
9	M	20	4	79	Diazepam (0.28), desmethyldiazepam (0.34), 11-nor-Δ^9-THC-COOH[‡] (23 ng/mL)	2 hours	Ingestion of 'K2'. GCS[§] 12/15. Temp. 34.2 °C. Glucose 8.5 mmol/L. Heart rate 130 sinus tachycardia, BP[‖] 100/63. Syncope, dissociative state, confused. 7 hours hospitalisation.

ND Non-Detected; NA Not analysed
* LOD amended due to limited sample volume [†] β-hydroxybutyrate [‡] 11-Nor-Δ^9-Tetrahydrocannabinol carboxylic acid [¶] Δ^9-Tetrahydrocannabinol [§] Glasgow Coma Scale [‖] Blood Pressure
[#] Hydrogen ions (nmol/L) ** Lactate (mmol/L) [††] Bicarbonate (mmol/L) [‡‡] Base Excess (mmol/L)

Table 2 - Details of PM cases positive for MDMB-CHMICA

Case	Gender	Age (years)	MDMB-CHMICA Conc. (ng/mL)	Ethanol Conc. (mg/dL)	Other Substances Present (mg/L)	Circumstances/Clinical Presentation
10	M	44	1	NA	Amitriptyline (0.13)	Found dead by hanging.
11	M	38	<1	237	Acetone (<100), BHB[†] (249)	History of alcoholism: found dead at home.

NA Not analysed [†] β-hydroxybutyrate

9.5. Appendix E – Research ethics approval documentation – Post-Mortem casework

29th July 2019

MVLS College Ethics Committee

Project Title: Investigation into Novel Psychoactive Substance (NPS) in post-mortem samples in Scotland

Project No: 200180192

Dear Dr Torrance,

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project.

- Project end date: End March 2020
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: (http://www.gla.ac.uk/media/media_227599_en.pdf)
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely,

Jesse Dawson
MD, BSc (Hons), FRCP, FESO
Professor of Stroke Medicine
Consultant Physician
Clinical Lead Scottish Stroke Research Network / NRS Stroke Research Champion
Chair MVLS Research Ethics Committee

Institute of Cardiovascular and Medical Sciences
College of Medical, Veterinary & Life Sciences
University of Glasgow
Room M0.05
Office Block
Queen Elizabeth University Hospital
Glasgow
G51 4TF

jesse.dawson@glasgow.ac.uk

9.6. Appendix F – Research ethics approval documentation – Scottish Prison Service

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 3
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhsggc.org.uk

Miss Alice Turnbull
Forensic Medicine and Science
Joseph Black Building
University of Glasgow
University Place
Glasgow
G12 8QQ

Date 9th October 2015
Your Ref
Our Ref
Direct line 0141 211 2123
Fax 0141 211 1847
E-mail WOSREC3@ggc.scot.nhs.uk

Dear Miss Turnbull

Study title:	**Prevalence of Drugs of Abuse including Novel Psychoactive Substances in Prisoners at Scottish Prison Service Facilities**
REC reference:	**15/WS/0207**
IRAS project ID:	**190131**

Thank you for responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC. A list of the Sub-Committee members is attached.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mrs Liz Jamieson, wosrec3@ggc.scot.nhs.uk.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor Insurance or Indemnity (non NHS Sponsors only) [Insurance or Indemnity Letter]		12 August 2015
IRAS Checklist XML [Checklist_08102015]		08 October 2015
Other [Confirmation To Seek Consent]		08 October 2015
Participant consent form [Participant Consent Form]	1	09 September 2015
Participant information sheet (PIS) [Participant Information Sheet]	2	05 October 2015
REC Application Form [REC_Form_11092015]		11 September 2015
Research protocol or project proposal [Research Proposal]	2	05 October 2015
Summary CV for Chief Investigator (CI) [CV AT]		
Summary CV for supervisor (student research) [CV HJT]		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/WS/0207	Please quote this number on all correspondence

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

Yours sincerely

Liz Jamieson REC Manager
On behalf of Dr Adam Burnel, Chair

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Dr Debra Stuart, University of Glasgow*

West of Scotland REC 3

Sub-Committee of the REC meeting held between 29th September and 09 October 2015

Committee Members involved in the review:

Name	*Profession*	*Present*	*Notes*
Dr Adam Burnel	Consultant Psychiatrist - Chair	Yes	
Mr Eoin MacGillivray	Retired Dentist - Vice Chair	Yes	
Mrs Rosie Rutherford	Volunteer - Lay Plus Member and Alternate Vice Chair	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Liz Jamieson	REC Manager

University of Glasgow
Forensic Medicine and Science

Participant Information Sheet

Prevalence of Drugs of Abuse, Including Novel Psychoactive Substances, in Prisoners at Scottish Prison Service (SPS) Facilities

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

The purpose of this study is to determine how common the use of Novel Psychoactive Substances (NPS) or 'legal highs' is in a prison population, and what kind of substances these are. Whilst many of these drugs are still legal in the U.K., some of them can be very harmful and information about how common these are in prisons is important so that prison staff can be trained to manage bad reactions to such drugs.

Why have I been chosen?

You have been chosen as you are currently undergoing admission to or liberation from an SPS facility.

Do I have to take part?

Taking part is completely up to you. No legal action will result from you participating in this study, whatever the result.

What will happen to me if I take part?

To take part in this study, you are asked to sign the consent form which will permit your urine sample to be tested for this research project. The urine sample will be anonymised with a unique identifier number which is not linked to the consent form. No individual can or will be identifiable at any time. The results of the test will have no effect on your legal status or custodial sentence.

University of Glasgow
Forensic Medicine and Science

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. No individual results will be communicated to third parties. Your anonymised results along with other participants will be published in peer-reviewed journals and presented at international conferences.

What will happen to the results of the research study?

Any publications resulting from this study will not contain any identifying information and will deal with the combined results from all participants.

Who is organising and funding the research?

This study is organised and funded by Forensic Medicine and Science at the University of Glasgow in conjunction with the Scottish Prison Service.

Who has reviewed the study?

This study has been reviewed by an independent group, the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow, and by the Research Ethics Forum at the NHS.

11. Contact for Further Information

For further information concerning this study please contact Alice Tumbull at Alice.Tumbull@glasgow.ac.uk

Thank you very much for participating in this study.

If you have any concerns regarding the conduct of this research project, you can contact the College of MVLS Ethics team, email: mvls-ethics-admin@glasgow.ac.uk

University of Glasgow
Forensic Medicine and Science

CONSENT FORM

Prevalence of Drugs of Abuse, Including Novel Psychoactive Substances, in Prisoners at Scottish Prison Service (SPS) Facilities

Name of Researcher: Alice Turnbull

Please initial box

I confirm that I have read and understand the information sheet dated_____ for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and my legal rights will not be affected by participation in this study. ☐

I agree that my urine sample can be included in this study. ☐

I agree to my urine being tested for drugs of abuse including NPS. ☐

Name of participant	Date	Signature
Name of Person taking consent (Different from researcher)	Date	Signature

(Consent Form to be retained by SPS)

Ver. 1 09/09/15

9.7. Appendix G – Research ethics approval documentation – Forensic Directorate

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde

West of Scotland REC 3
Ground Floor – The Tennent Institute
Western Infirmary
38 Church Street
Glasgow G11 6NT
www.nhsggc.org.uk

Dr Richard Stevenson
Consultant
NHS Greater Glasgow and Clyde
Emergency Department
Glasgow Royal Infirmary
Glasgow
G4 0SF

Date 1st December 2015
Your Ref
Our Ref
Direct line 0141 211 2123
Fax 0141 211 1847
E-mail WOSREC3@ggc.scot.nhs.uk

Dear Dr Stevenson

Study title:	**Extended Urine Toxicology Screening of Forensic Psychiatric Patients**
REC reference:	**15/WS/0263**
IRAS project ID:	**187536**

Thank you for your email dated 30th November 2015. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 27 November 2015

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Other [Email confirming additional conditions]		30 November 2015

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering letter on headed paper [Covering letter]	1	15 October 2015
Other [Email confirming additional conditions]		30 November 2015
REC Application Form [REC_Form_26102015]		26 October 2015
Research protocol or project proposal [Protocol]	1.2	23 October 2015
Summary CV for Chief Investigator (CI) [RichardStevensonResarchCV]	1	

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices

at all participating sites.

15/WS/0263	Please quote this number on all correspondence

Yours sincerely

Liz Jamieson
REC Manager

Copy to: Dr Michael Barber, NHS Greater Glasgow and Clyde R&D

University of Glasgow
Forensic Medicine and Science

Participant Information Sheet

Prevalence of Novel Psychoactive Substances in Urine Samples Submitted for Toxicology Testing in Forensic Psychiatric Patients

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

The purpose of this study is to determine how effective the current NHS procedures are for testing urine samples for drugs of abuse, particularly novel psychoactive substances (NPS).

Why have I been chosen?

You have been chosen as you are currently a patient at an NHS Greater Glasgow and Clyde (GGC) Forensic Directorate facility.

Do I have to take part?

Taking part is completely up to you.

What will happen to me if I take part?

To take part in this study, you are asked to sign the consent form which will permit your urine sample to be tested for this research project. The urine sample will be anonymised and no individual involved in this study can or will identify you at any time.

University of Glasgow
Forensic Medicine and Science

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. No individual results will be communicated to third parties. Your anonymised results along with other participants will be published in peer-reviewed journals and presented at international conferences.

What will happen to the results of the research study?

Any publications resulting from this study will not contain any identifying information and will deal with the combined results from all participants.

Who is organising and funding the research?

This study is organised and funded by Forensic Medicine and Science at the University of Glasgow in conjunction with the NHS GGC.

Who has reviewed the study?

This study has been reviewed by an independent group, the College of Medical, Veterinary and Life Sciences Research Ethics Committee, University of Glasgow, and by the Research Ethics Forum at the NHS.

11. Contact for Further Information

For further information concerning this study please contact Alice Turnbull at ForensicMedicine.Office@glasgow.ac.uk

Thank you very much for participating in this study.

If you have any concerns regarding the conduct of this research project, you can contact the College of MVLS Ethics team, email: mvls-ethics-admin@glasgow.ac.uk

University of Glasgow
Forensic Medicine and Science

CONSENT FORM

Prevalence of Novel Psychoactive Substances in Urine Samples Submitted for Toxicology Testing in Forensic Psychiatric Patients

Name of Researcher: Alice Turnbull

Please initial box

I confirm that I have read and understand the information sheet dated_____ for the above study and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary . ☐

I agree that my urine sample can be included in this study. ☐

I agree to my urine being tested for drugs of abuse including NPS. ☐

Name of participant	Date	Signature
Name of Person taking consent (Different from researcher)	Date	Signature

(Consent Form to be retained by NHS)

9.8. Appendix H – Research ethics approval documentation – Glasgow Drugs Court

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

8th May 2015

Dear Lauren O'Connor, Hazel Torrance, Denise McKeown, Jo McManus, Lynn Macdonald

MVLS College Ethics Committee

Project Title: Detecting drugs of abuse including NPS in urine samples
Project No: 200140101

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: April 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

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

9.9. Appendix I – Questionnaire completed by Glasgow Drug Court cohort participants

University of Glasgow | Section of Forensic Medicine & Science

ID no.

Participant Questionnaire

Detecting drugs of abuse including NPS in Urine Samples

You have agreed to take part in the above research study. Please answer the following questions to the best of you ability. The more information you can give the better the results of the study will be.

All questionnaires are anonymised and will only be labelled with a number that will allow us to match them to the urine sample you have given permission for us to test. No identifiable information will be made available to any outside organisation.

Thank you for taking part.

Q1. What drug(s) (legal and illegal) have you taken in the last week?

1.
2.
3.
4.
5.
6.
7.

Others: _____

Q2. What did the drug(s) look like?

Tablet, powder, liquid, crystals, plant matter....

1.
2.
3.
4.
5.
6.
7.

Others: _____

Q3. When was the last time you used the drug(s)?

1.

2.

3.

4.

5.

6.

7.

Others:

Q4. How much, of each, did you use at last use?

1.

2.

3.

4.

5.

6.

7.

Others:

Q5. How did you take each drug(s)?
Injected, snorted, smoked, swallowed.....

1.

2.

3.

4.

5.

6.

7.

Others:

Q6. Where did you purchase the drug(s)?
Internet, Dealer, Headshop, Friend, Market stall, corner shop
Other?

1.

2.

3.

4.

5.

6.

7.

Others: _____

Q7. Do you use the drug(s) regularly?

1.

2.

3.

4.

5.

6.

7.

Others: _____