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Detection and quantitation of synthetic cannabinoids in whole blood, urine, and herbal products and its application to postmortem cases in Johannesburg, South Africa

by

Dale Pon

A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree:

MSc (Med) in Forensic Medicine

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Johannesburg, 2016

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not, necessarily, to be attributed to the NRF.

Declaration

I declare that this Dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



(Signature of candidate)

16th day of August 2016

Presentations arising from this study

Chrom SA 2015 Chromatography Postgraduate Student Seminar (oral presentation), 13 August 2015, University of the Witwatersrand, Johannesburg.

Faculty of Health Sciences - School of Clinical Medicine Research Day 2015 (poster presentation), 30 September 2015, University of the Witwatersrand, Johannesburg.

SASBCP (South African Society for Basic and Clinical Pharmacology) & TOXSA (Toxicological Society of South Africa) Congress 2015 (poster presentation), 31 August – 2 September 2015, University of the Witwatersrand, Johannesburg.

Abstract

A study was conducted by J.W. Huffman in 1994, to design new compounds with effects comparable to natural cannabinoids, for example THC. This resulted in the synthesis of JWH-018, which, along with C8 analogs of the cannabinoid CP 47,497 became the most common synthetic additives in several herbal blends known as 'Spice'. These herbal blends were originally sold online and in head shops (shops specialising in cannabis and tobacco paraphernalia) without age restriction or legal implications. In 2008 synthetic cannabinoids were identified in these mixtures and from early 2009, numerous countries began implementing legislation to monitor and control these drugs.

The aim of this project was to develop and validate a LC-MS method for the detection and quantitation of several synthetic cannabinoids (JWH-018, JWH-019, JWH-073, JWH-081, JWH-122 JWH-200, JWH-250, AM-2201, (±)-CP 47,497, (C8)-CP 47,497, HU-211) and selected metabolites (JWH-018 N-(4-hydroxypentyl) metabolite and JWH-073 N-(3-hydroxybutyl) metabolite) in whole blood and urine. Further aims were to apply it to postmortem cases at the Johannesburg Forensic Pathology Services Medicolegal Laboratory (FPS-MLL) to assess the prevalence of these synthetic cannabinoids amongst the local postmortem population; as well as to known positive powder and urine samples obtained from a horseracing laboratory In Australia.

Urine samples were extracted utilising an SPE method, while blood samples were extracted utilising an LLE method. LC-MS analysis was performed on a Thermo Fisher Q Exactive Orbitrap. Analytical parameters including: limit of detection (LOD), limit of quantitation

(LOQ), stability, matrix effects, selectivity, linearity, repeatability, accuracy, and recovery were assessed for each analyte.

None of the postmortem cases were found to contain any of the targeted analytes, although validated methods for urine and whole blood were developed based on existing routine screening methods. The sample population could be extended to living subjects such as those in drug rehabilitation centres or in hospitals to get a more accurate representation of the overall usage in South Africa.

Plagiarism declaration

I, **Dale Pon** (Student number: **318564**), am a student registered for the degree of **Master of Science in Medicine** in the academic year **2016**.

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Abbreviations and Nomenclature

11-OH-THC	11-Hydroxy-Δ^9-tetrahydrocannabinol.
ACN	Acetonitrile
CV	Coefficient of variation
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FPS-MLL	Forensic Pathology Services Medico-Legal Laboratory
GC-MS	Gas chromatography mass spectrometry
HAc	Acetic acid
HPLC	High performance liquid chromatography
LC-HRAM-MS	Liquid chromatography- high resolution accurate mass- mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MS	Mass spectrometry
NIMSS	National Injury Mortality Surveillance System
NMR	Nuclear magnetic resonance
QC	Quality control
SD	Standard deviation
S/N	Signal to noise
SPE	Solid phase extraction
THC	Δ^9-tetrahydrocannabinol
TLC	Thin layer chromatography
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography

Chapter 1: Literature Review

1.1. Cannabis - Introduction

Cannabis (also known as Marijuana, and more informally as 'weed', 'pot', 'grass', or 'herb') has long been used as a drug of abuse due to its psychoactive effects. There is much debate and controversy surrounding the potential legalisation of cannabis, due to some of its therapeutic properties in the treatment of glaucomas, the stimulation of appetite in AIDS patients as well as the suppression of nausea resulting from chemotherapy (Adams and Martin, 1996).

Cannabis (in the form of hemp) was used by the ancient Chinese and Greeks to make clothing and ropes, while the Romans used it in the construction of ships (Adams and Martin, 1996; Clarke and Merlin, 2013). It is also regarded as one of the oldest drugs in history where some research has indicated its use as far back as 2700 BC in China. It has also long been used as a medicine in a variety of countries, including China, India, the Middle East, South Africa and South America (Adams and Martin, 1996; Stafford, 2013).

First isolated in 1964 (Gaoni and Mechoulam, 1964), tetrahydrocannabinol is the main active constituent of cannabis. Cannabis is used for a variety of purposes; as a recreational drug, in religious/spiritual rites or even for medicinal applications (Touw, 1981). Cannabis forms part of a group of compounds known as cannabinoids (produced by plants of *Cannabis sativa* and *Cannabis indica*).

Cannabinoids are terpenophenolic compounds (i.e. a combination of a terpene, an unsaturated hydrocarbon group, C_6H_{10}; and a phenol, a benzene ring with a directly linked hydroxyl group, C_6H_5OH) which contain 21 carbons. There are three general types of cannabinoids; the phytocannabinoids (which occur in the cannabis plant), the endogenous cannabinoids (cannabinoids produced in the bodies of humans and animals), and synthetic cannabinoids (cannabinoids synthesised in a laboratory). In cannabis, the main active compound is the cannabinoid, Δ^9-tetrahydrocannabinol (THC) (Figure 1.1.); although there are several others with known biological activity, such as cannabinol (CBN), cannabidiol (CBD) (which is thought to have analgesic and anti-inflammatory activity, without the psychoactive effect of THC), cannabichromene (CBC), cannabigerol (CBG), tetrahydrocannabivarin and Δ^8-tetrahydrocannabinol (National Cancer Institute, 2014).

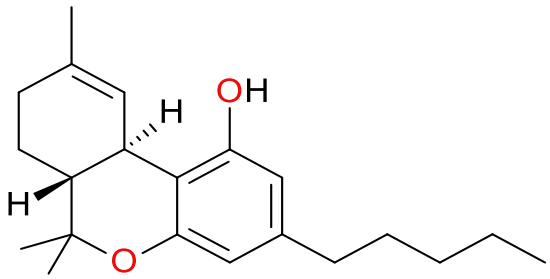


Figure 1.1. Δ9-Tetrahydrocannabinol

1.2. Cannabinoid Receptors

There are two kinds of cannabinoid receptors that have been found, named CB_1 and CB_2. Both of these receptors are G protein-coupled receptors, which are receptors that activate cellular responses by the activation of signal transduction pathways (Adams and Martin, 1996; Devane et al., 1988).

CB_1 receptors are distributed predominantly throughout the brain and are found in high density in the basal ganglia, the cerebellar molecular layer, the innermost layers of the

olfactory bulb, and parts of the hippocampal formation; while moderately distributed in the rest of the brain. Sparse amounts of CB_1 receptors are also found in the brain stem and spinal cord (Glass et al., 1997; Herkenham et al., 1991).

CB_2 receptors have been found predominantly in immune tissues such as the spleen, tonsils, and thymus, as well as in some peripheral blood mononucleated cells (PBMCs) (Galiegue et al., 1995). In 1992 anandamide, the first naturally occurring substance within the brain which binds to CB_1 receptors was discovered (Devane et al., 1992). Subsequently other naturally occurring substances which bind to CB_1 receptors have been discovered and these substances, along with the receptors are known as the 'endogenous cannabinoid system'. Cannabis tends to affect the limbic (the part of the brain that affects memory, cognition, and psychomotor performance) and mesolimbic (the part of the brain associated with feelings of reward) pathways as well as the areas of pain perception within the brain (Adams and Martin, 1996). This results in the commonly seen short-term effects of talkativeness; feeling of well-being; drowsiness; loss of inhibitions; decreased nausea; increased appetite; loss of coordination; bloodshot eyes; dryness of the eyes, mouth and throat as well as anxiety and paranoia. Furthermore, although research into the long-term effects of cannabis is limited, the most likely effects are increased risk of respiratory diseases associated with smoking, such as cancer; decreased memory and learning abilities; and decreased motivation towards studying, working and concentration (NCPIC (National Cannabis Prevention and Information Centre), 2008).

1.3. Synthetic Cannabinoids - Introduction

Synthetic cannabinoids can be classified according to their chemical structure, into six groups: classical cannabinoids (THC, other constituents of cannabis as well as their structurally related synthetic analogues); nonclassical cannabinoids (i.e. cyclohexylphenols and 3-arylcyclohexanols); hybrid cannabinoids (have structural features of both classical and nonclassical cannabinoids); aminoalkylindoles (which consist of the subclasses naphtoylindoles, phenylacetylindoles, naphthylmethylindoles and benzoylindoles); eicosanoids (endocannabinoids such as anandamide and their synthetic analogues); and others (such as diarylpyrazoles, naphtoylpyrroles, naphthylmethylindenes or derivatives of naphthalene-1-yl-(4-pentyloxynaphthalen-1-yl)methanone) (Howlett et al., 2002). Examples of some of the classes can be seen in Figure 1.2.

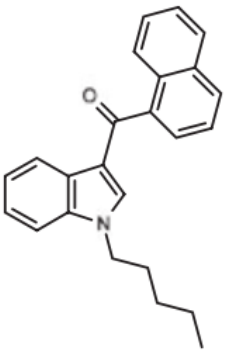
Research on synthetic cannabinoids has been on-going since the 1980s, with Pfizer developing CP 47,497 (Figure 1.3) (the "CP" indicating it is a cyclohexylphenol) as part of research to investigate which moieties of the molecule are the binding sites for analgesic activity. As a result it was discovered that CP 47,497 had an analgesic effect of similar potency to morphine (Melvin et al., 1984).

Aminoalkylindoles, specifically pravadoline (WIN 48,098) and WIN 55,212-2 (Figure 1.3), were initially developed to be potential analogues of nonsteroidal anti-inflammatory drugs (NSAIDs), but were found to exhibit antinociceptive (pain inhibiting) properties. These properties were eventually explained by their interaction with the cannabinoid receptors, even though they have no structural relationship to traditional cannabinoids (Bell et al., 1991; D'Ambra et al., 1992).

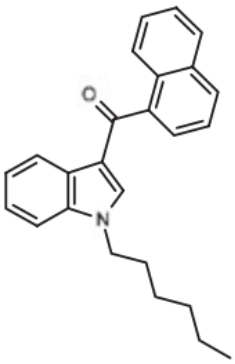
In an effort to design new indoles with effects comparable to natural cannabinoids such as THC, a study was conducted on cannabimimetic (which is defined by the United States Legislation in Section 1152 of the Synthetic Drug Abuse Prevention Act of 2012 as a "substance that is a cannabinoid receptor type 1 (CB_1 receptor) agonist as demonstrated by binding studies and functional assays" (US Congress, 2012)) indole structures which resulted in the synthesis of several naphthoylindole compounds (which are aminoalkylindoles), most notably JWH-018, but also JWH-007, JWH-015, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, and JWH-398 (Huffman et al., 1994).

1) Aminoalkylindoles

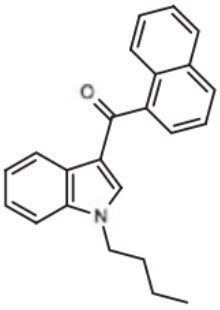
a) Naphthoylindoles



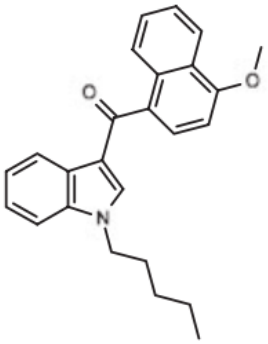
JWH-018



JWH-019

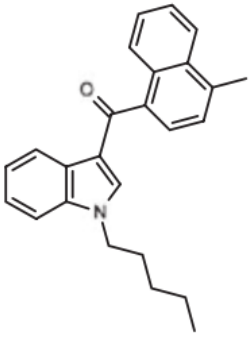


JWH-073

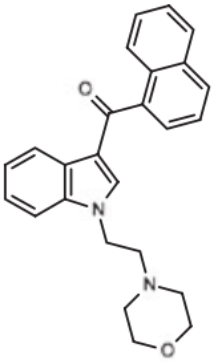


JWH-081

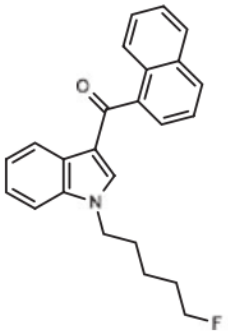
b) Phenylacetylindoles



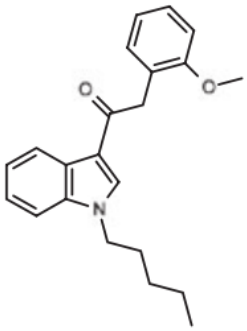
JWH-122



JWH-200

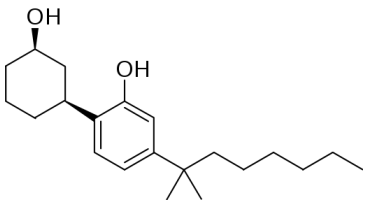


AM-2201

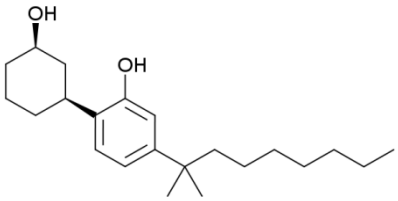


JWH-250

2) Cyclohexylphenols

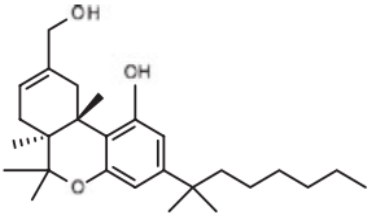


CP 47,497



CP 47,497 (C8)

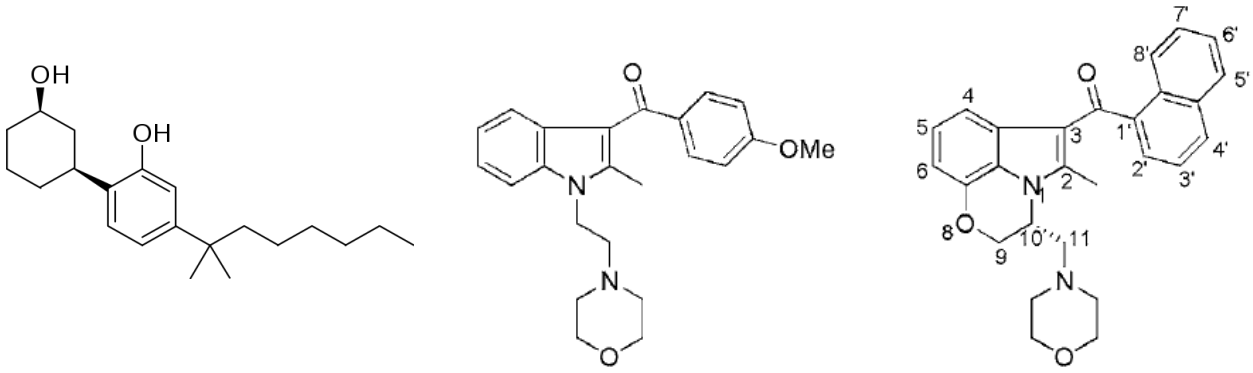
3) Classical cannabinoids



HU-211

Figure 1.2. Chemical structures of several synthetic cannabinoids.

Adapted from (United Nations Office on Drugs and Crime, 2011)



(Onavi, 2006)

Figure 1.3. Chemical Structures of CP 47,497 (left), Pravadoline (WIN 48,098, middle), and WIN 55,212-2 (right)

1.4. Synthetic Cannabinoids - From the laboratory to the street

JWH-018, along with C8 homologues of the non-classical cannabinoid CP 47,497 are the most common synthetic additives in a variety of herbal blends known as 'Spice' (Auwärter et al., 2009; Uchiyama et al., 2009). 'Spice' (and associated names such as K2, see Figure 1.4.) has been sold in numerous countries, including Switzerland, Austria, and Germany, since 2004 and is marketed as incense, although it is smoked by users (Auwärter et al., 2009). Its popularity can be attributed to the fact that commonly used drug tests at the time were unable to detect these drugs in urine and blood as well as its ease of availability, from head shops (shops selling predominantly cannabis-smoking accessories) and the internet. Furthermore, 'Spice' is sold without age restriction by vendors. These products are sold for anywhere between 5 USD and 15 USD per gram ("Buy K2 incense," 2016; Reed, 2010).



https://d14rmgtrwzf5a.cloudfront.net/sites/default/files/images/drugfactsspice2.jpg



https://upload.wikimedia.org/wikipedia/commons/8 82/USMC-100201-M-3762C-001.jpg

Figure 1.4. Examples of Spice (left) and K2 (right)

1.5. Synthetic Cannabinoids - Prevalence

Research performed in the USA in 2012 (Johnston et al., 2012) found the prevalence of synthetic cannabinoids use to be 8.8% for 15-16 year olds and 11.3% for 17-18 year olds. An online study conducted by Mixmag and Guardian on over 15 500 people in 2011 (Global Drug Survey, 2012) found that 14.2% of the UK respondents had experimented with synthetic cannabinoids (3.3% in the previous 12 months) while 14% of US respondents had tried it in the previous 12 months. Two separate studies performed in Australia (Barratt et al., 2013; Scott et al., 2012) found that a significant number of synthetic cannabinoid users were also regular cannabis users (Barratt et al. (2013) found that 96% of the synthetic cannabinoid users were also cannabis users).

Research undertaken in Japan on herbal samples acquired from the internet between June 2008 and June 2009 (Uchiyama et al., 2010) found six different synthetic cannabinoids present spread among the samples, namely cannabicyclohexanol (CP 47,497 dimethyloctyl monologue; (C8)-CP 47,497), the trans-Diastereoisomer of (C8)-CP 47,497, CP 47,497, Oleamide, JWH-018, and JWH-073. A study conducted in Germany on blood serum samples from forensic psychiatric clinics, and rehabilitation clinics, in cases of criminal investigation as well as critical care units (in the cases of severe intoxication) between August 2011 and January 2012 (Kneisel and Auwärter, 2012) found 11 different synthetic cannabinoids out of a total of 30 that were tested for. Another study performed in Germany on blood serum samples from hospitals, detoxification and therapy centres, forensic psychiatric centres and institutes of forensic medicine (Dresen et al., 2011) tested for 10 different synthetic cannabinoids and found 5 present in 56.4% of the subjects.

Very few postmortem studies have been investigated but one conducted by Shanks et al. (2012b) on cases received by their laboratory tested for the presence of JWH-018 and JWH-073 in whole blood and found a 40% positivity rate, although their sample size was small (n = 45).

1.6. Synthetic Cannabinoids - Pharmacology

The synthetic cannabinoids are usually administered by smoking since many of them are highly lipophilic and vaporise without decomposition under smoking conditions. This is thought to allow for the rapid onset of pharmacological effects. There have been some reports of oral consumption, but this is a less common manner of administration as the onset of any pharmacological action may be delayed due to initial first phase metabolism (United Nations Office on Drugs and Crime, 2011).

The pharmacokinetics of the synthetic cannabinoids is only partially known, but the metabolism has been investigated by a variety of studies, both *in vitro* and *in vivo*. Most of the compounds are metabolised extensively by multiple phase 1 reactions, including monohydroxylation, dihydroxylation, oxidation of the hydroxylated metabolites, and N-dealkylation (Hutter et al., 2012; Wintermeyer et al., 2010; Zhang et al., 2006). In urine, metabolites are found in the form of glucuronic acid conjugates (Sobolevsky et al., 2010). Research has also found that the synthetic cannabinoids JWH-018 and AM-2201 undergo cytochrome P450-mediated oxidation with CYP2C9 and CYP1A2 the major P450s involved (Chimalakonda et al., 2012).

There have been numerous case reports which have documented the psychopathological and neuropsychiatric effects of certain synthetic cannabinoids. The effects included: blurred

vision, tremors, agitation, anxiety, hallucinations, nausea, vomiting, psychosis, paranoia, tachycardia, hypokalemia, and hypertension (Forrester et al., 2011; Hermanns-Clausen et al., 2013a, 2013b; Papanti et al., 2013).

1.7. Synthetic Cannabinoids - Legislation

In 2008 synthetic cannabinoids were identified in the above studied herbal mixtures and subsequently, from early 2009 onwards, countries including Chile ("'Chile prohibits use of drug 'Spice," 2009); France ("'Decree of February 24, 2009 amending the Decree of 22 February 1990 establishing the list of substances classified as narcotics,'" 2009); Germany ("'Narcotics Law : fashionable drug Spice is prohibited by Emergency Ordinance,'" 2009); Ireland (Kennedy, 2010); New Zealand (Ryan, 2014); Poland (*"Report Health Committee of the Government's draft law amending the law on preventing drug addiction (form No. 1207).,"* 2009); Romania (*"GEO 6/2010 amending and supplementing Law no. 143/2000 on preventing and combating trafficking and illicit drug use and completing Law no. 339/2005 on the legal regime of plants, narcotic and psychotropic substances and preparations.,"* 2010); Russia (*"Resolution dated December 31, 2009 № 1186 On Amending Certain Resolutions of the Government of the Russian Federation on issues related to drug trafficking,"* 2010); South Korea ("'One days from the '5- Meo - mipteu and psychotropic drugs designation"," 2009); and Turkey (*"Decision Number : 2011/1310,"* 2011) have legislation in place, which either classifies several of the compounds as "medicinal preparations", "controlled substances" or outright illegal. Several US states have banned the sale of 'Spice' and related products, while in the UK a generic definition which includes structurally similar compounds and not just compounds present in herbal products has been developed (United Nations Office on Drugs and Crime, 2011). In December 2011, the Office

of National Drug Control Policy of the Executive Office of the President of the United States of America (Office of National Drug Control Policy of the Executive Office of the President, 2011) published a "Fact Sheet" in which it recognised synthetic marijuana as a "rapidly emerging threat" as well as describing the efforts taken by various states to monitor and control it.

1.8. Synthetic Cannabinoids - South Africa

A News24 article from late 2011 (Pienaar, 2011) reports the availability of a legal synthetic drug in South Africa with "a similar effect on the body to that of dagga". Upon further investigation the herbal blend was found to contain JWH-073, a synthetic cannabinoid. As mentioned previously, these drugs aren't detectable by routine screening tests, and GC-MS (gas chromatography mass spectrometry) was required to detect it. Current legislation in South Africa (i.e. the South African Medicines and Medical Devices Regulatory Authority Act (No. 132 of 1998) (*The South African Medicines and Medical Devices Regulatory Authority Act (No. 132 of 1998)*, 1998) as well as the Drugs and Drug Trafficking Act (No. 140 of 1992) (*Drugs and Drug Trafficking Act (No. 140 of 1992)*, 1992)) means that cannabis (also known locally as dagga) and its plant derivatives are considered banned substances.

A recent amendment (15 March 2012) to the Medicines and Related Substances Act, (No. 101 of 1965) (*Medicines and Related Substances Act (No. 101 of 1965)*, 1965) listed the following synthetic cannabinoids: JWH-018; JWH-073; JWH-200; CP 47,497; CP 47,497-C6; CP 47,497-C7; CP 47,497-C8; CP 47,497-C9; and HU-210 as Schedule 7 substances, which is the highest pharmaceutical scheduled classification, meaning that these substances are to be strictly controlled and monitored.

A PubMed search including the terms ‘South Africa’, ‘synthetic cannabinoid’, and ‘Spice’ yielded no results, therefore due to the limited information one may conclude that such research has not been conducted or published in South Africa. However, a quick internet search for “spice k2 South Africa” yields several classifieds type sites on which several companies claim to sell Spice and K2 locally, although they tend to offer bulk quantities.

1.9. Synthetic Cannabinoids Analysis – Literature Overview

Tables A1 and A2 in the Appendix summarise extraction methods from literature for the analysis of synthetic cannabinoids in urine and whole blood respectively conducted by various research cohorts.

Majority of the synthetic cannabinoid extraction methods make use of either liquid-liquid extraction (LLE) or solid-phase extraction (SPE). One group (Scheidweiler and Huestis, 2014) also utilised supported liquid extraction (SLE) (after an initial hydrolysis step) in which the same organic phases in LLE are used, but are coated onto an inert diatomaceous earth instead of being shaken together, to quantitate the synthetic cannabinoids in urine.

In 2009 Auwärter et al. initially attempted to use routine qualitative analytical methods such as GC-MS, multi-target LC-MS/MS (liquid chromatography tandem mass spectrometry), and immunological screening methods to characterise ‘Spice’ products purchased from the internet. These methods did not reveal any evidence of illicit drugs or known active pharmaceutical ingredients due to no standards being available with comparable structures. Following this these products were subsequently characterised by thin layer chromatography (TLC), UV-vis spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and electron ionisation (EI)-MS (Auwärter et al., 2009).

Around a similar time Uchiyama et al. (2009) in Japan also analysed and characterised herbal products purchased off the internet, by means of LC-MS, GC-MS, high-resolution MS, and NMR. From this information they elucidated the structure of one of the unknown compounds and determined that it was the same as a cannabinoid (CP 47,497) previously synthesised by Pfizer (Melvin et al., 1984).

Shortly afterwards (in 2012), a group of researchers at NMS Labs in the USA utilised TLC, GC-MS, HPLC (high performance liquid chromatography), and LC-TOF-MS (liquid chromatography- time of flight mass spectrometry) to identify and quantitate several synthetic cannabinoids in herbal incense blends (Logan et al., 2012).

After this initial period of identification and confirmation most researchers have developed and validated variants of LC-MS/MS methods to analyse synthetic cannabinoids in a variety of matrices. This has also been aided by synthetic cannabinoid standards becoming commercially available from companies such as LGC and Cerilliant. In 2010 a German group (Teske et al., 2010) developed an LC-MS/MS method to detect JWH-018 in blood serum. Following this, another German research group developed and validated LC-MS/MS methods to quantitate several synthetic cannabinoids in serum (Dresen et al., 2011; Kneisel and Auwärter, 2012), while an American research group developed and validated a similar method for the identification and quantitation of these drugs in whole blood (Kacinko et al., 2011). Some researchers in Italy developed a matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS) method for use with herbal blends, which they claim to be simpler, faster, and have a higher throughput than the usual GC-MS and LC-MS methods (Gottardo et al., 2012). With the recent development of UPLC (ultra-performance liquid chromatography), some methods have been developed for the analysis

of synthetic cannabinoids. One method made use of UPLC-TOF for the qualitative detection in non-biological specimens (Shanks et al., 2012a) while another utilised UPLC-MS/MS to determine concentrations as low as 0,01 ng/mL of two synthetic cannabinoids (JWH-018 and JWH-073) in whole blood (Shanks et al., 2012b).

Similar approaches have been taken to analyse the metabolites of synthetic cannabinoids. De Jager et al. (2012) developed an LC-MS/MS method for the identification and quantitation of the urinary metabolites of eight JWH-type cannabinoids. Wohlfarth et al. (2013), developed a qualitative LC-MS/MS method (with a traditional hydrolysis step) to identify several metabolites of selected synthetic cannabinoids as well as their parent compounds in urine. Due to the lack of a comprehensive quantitative urinary method for synthetic cannabinoids Scheidweiler and Huestis (2014) developed and validated an LC-MS/MS method for the quantitation (limit of detections (LOD) in the range of 0,1 – 0,5 ng/mL) of 20 synthetic cannabinoids and 21 metabolites, as well as the semi-quantitation of 12 additional metabolites in urine.

All of these methods have been developed as specialised targeted methods, and it is not known whether these were incorporated into general screening methods or not.

1.10. Conclusion

Over the past few years the rapid rise in synthetic cannabinoids use and availability around the world has prompted various countries to put legislation in place in order to monitor and control these substances (United Nations Office on Drugs and Crime, 2011). In addition, South Africa has promulgated legislation scheduling some of the first generation synthetic cannabinoids as mentioned above. Unfortunately no data are available regarding the

prevalence of synthetic cannabinoid use in South Africa, nor is it known whether South African forensic laboratories can identify them.

1.11. Aims and Objectives

The aim of this project is to develop a liquid chromatography coupled with mass spectrometry (LC-MS) method for the detection and quantitation of synthetic cannabinoids in whole blood and urine; and to apply this method to investigate the prevalence of synthetic cannabinoids in unnatural deaths in Johannesburg, South Africa by analysis of whole blood and urine from postmortem cases.

The objectives of this project are:

- To develop and validate a LC-MS method for the detection and quantitation of several synthetic cannabinoids (JWH-018, JWH-019, JWH-073, JWH-081, JWH-122 JWH-200, JWH-250, AM-2201, CP 47,497, (C8)-CP 47,497, HU-211) and selected metabolites (JWH-018 N-4OH pentyl metabolite and JWH-073 N-3OH butyl metabolite) in whole blood and urine.
- To investigate the prevalence of synthetic cannabinoid use amongst victims autopsied at the Forensic Pathology Services Medico-legal Laboratory (FPS-MLL), Johannesburg, South Africa.
- To determine the stability of the selected synthetic cannabinoids in whole blood and urine under normal storage conditions.

1.12. Expected Results

Synthetic cannabinoids are quite a new addition to the drug market and as such it was expected to feature in a small percentage of the sample population alongside the common drugs of abuse such as cannabis and heroin.

Chapter 2: Materials and Methodology

This study was a prospective, cross-sectional, descriptive study.

2.1. Materials

Spice Cannabinoid Mix 1 consisting of JWH-200, JWH-250, (±)-CP 47,497, (C8)-CP 47,497, and HU-211 (100 µg/mL in ACN (acetonitrile)); Spice Cannabinoid Mix 2 consisting of JWH-019, JWH-081, JWH-122, and AM-2201 (100 µg /mL in ACN); THC (1 mg/mL in MeOH), 11-OH-THC (11-Hydroxy-Δ^9-tetrahydrocannabinol) (100 µg/mL in MeOH), JWH-073 (100 µg/mL in ACN), JWH-018 N-(4-hydroxypentyl) metabolite (100 µg/mL in MeOH), and JWH-073 N-(3-hydroxybutyl) metabolite (100 µg/mL in MeOH) solutions were purchased from Cerilliant, Sigma-Aldrich. JWH-018 (10 mg/mL in MeOH) and JWH-200 (10 mg/mL in ACN) solutions were purchased from Cayman Chemical. 1-Naphthalenyl (1-pentyl-1H-indol-3-yl)-methanone-D_{11} (JWH-018-D_{11}) (100 µg/mL in MeOH) and (1-Butyl-1H-indol-3-yl)-1-naphthalenyl-methanone-D_9 (JWH-073-D_9) (100 µg/mL in MeOH) solutions were purchased from Chiron AS. Beta glucuronidase/arylsulphatase was purchased from Roche and di-potassium hydrogen orthophosphate, sodium carbonate, sodium dihydrogen orthophosphate, and sodium hydroxide were purchased from Associated Chemical Enterprises, ACE. Acetonitrile and methanol were purchased from Honeywell Burdick and Jackson; 1-chlorobutane was purchased from Minema; ethanol was purchased from Radchem; dichloromethane, ethyl acetate, ethyl ether, hexane, and isopropanol were purchased from RCI Labscan; methyl tert-butyl ether was purchased from Riedel-de Haën; and 1-chlorobutane, acetic acid, formic acid, and sodium acetate were purchased from Sigma-Aldrich. X Select CSH: C18 5 µm, 2.1 x 150 mm column used was purchased from

Waters. Beckman TJ-6, Beckman JS-6, and Beckman Coulter Allegra X-12R centrifuges were used and double deionised water from a Siemens LaboStar evoqua water purifier was used for this research.

2.1.1. Preparation of Calibrators and Quality Controls (QCs)

A combined synthetic cannabinoids spiking solution was prepared in methanol containing 1 µg/mL of each of the standards. The spiking solution was stored at -20°C. Calibrators and Quality Controls were prepared by spiking blank urine and whole blood from known drug-free volunteers at the following concentrations:

Urine:

Calibrators: 0,0225 ng/mL, 0,225 ng/mL, 1,125 ng/mL, 2,25 ng/mL, 3,375 ng/mL, 4,5 ng/mL, and 6,75 ng/mL.

QCs: High (4,5 ng/mL), Medium (1,125 ng/mL), and Low (0,1125 ng/mL).

Blood:

Calibrators: 0,0675 ng/mL, 0,675 ng/mL, 3,375 ng/mL, 6,75 ng/mL, 10,125 ng/mL, 13,5 ng/mL, and 20,25 ng/mL.

QCs: High (13,5 ng/mL), Medium (3,375 ng/mL), and Low (0,3375 ng/mL).

A combined internal standard solution was prepared in methanol containing 1-Naphthalenyl (1-pentyl-1H-indol-3-yl)-methanone-D_{11} (JWH-018-D_{11}) and (1-Butyl-1H-indol-3-yl)-1-naphthalenyl-methanone-D_9 (JWH-073-D_9) at 30 ng/mL.

2.2. Sample Collection

Samples were collected at the FPS-MLL Johannesburg situated at 25A Hospital Street, Braamfontein, 2001. Samples were obtained during routine medico-legal autopsies, where dissection and assessment of the body would already be required for the purposes of establishing with greater certainty the cause and circumstance of death as per the South African Inquests Act 58 of 1959, Section 2 i.e. "If the body of a person who has allegedly died from other than natural causes is available, it shall be examined by the district surgeon or any other medical practitioner, who may, if he deems it necessary for the purpose of ascertaining with greater certainty the cause of death, make or cause to be made an examination of any internal organ or any part or any of the contents of the body, or of any other substance or thing" (*Inquests Act 58 of 1959*, 1959)

The population comprised all cases of unnatural deaths (fulfilling the below acceptance criteria) received at the FPS-MLL Johannesburg, which services the Johannesburg metropolitan area, over a five month period. The following areas fall under the jurisdiction of Johannesburg: Alexandra, Booysens, Bramley, Brixton, Cleveland, Diepsloot, Douglasdale, Fairlands, Hillbrow, Honeydew, Jeppe, JHB Central, Langlaagte, Linden, Midrand, Moffatview, Mondeor, Norwood, Parkview, Randburg, Rosebank, Sandringham, Sandton, Sophiatown, and Yeoville.

Samples were analysed at the Laboratory of the National Horseracing Authority of Southern Africa situated at the Turffontein Racecourse.

Inclusion criteria:

- All decedents between the ages of 14 and 60 years.
- Decedents admitted to the FPS-MLL Johannesburg for medicolegal postmortem investigations.

Exclusion criteria:

- Cases which displayed signs of late decomposition, including putrefaction, adipocerous formation or mummification.
- Cases of decedents who were extensively burnt.
- Cases of decedents who had been previously hospitalised.
- Cases of decedents younger than 14 years of age or older than 60 years of age.
- Cases where autopsy was performed more than 72 hours after death as decomposition would already be present.

Urine (approximately 5 – 10 mL) (n=85) was obtained, where available by puncturing the bladder using a syringe and needle. Blood (approximately 5 mL) (n = 126) was obtained using a syringe and needle from an incision made at the inner femoral region, and if this was unsuccessful, it was obtained from the upper subclavian region.

Urine and whole blood samples were analysed as part of the validation batches described below.

Small quantities of seized powders (n = 3) and positive urine samples (n = 4) were donated by Racing Analytical Services Limited, Victoria, Australia and couriered to South Africa where they were analysed.

Ethics clearance was obtained from the University of the Witwatersrand Human Research Ethics Committee (HREC), Clearance Certificate Number: M130428 (Figure A4 in the Appendix).

2.3. Sample Storage

Urine samples were collected and split between plastic tubes (no preservative) and 25 mL glass containers with screw tops. Blood samples were collected and split between plastic blood tubes containing EDTA (ethylenediaminetetraacetic acid) and 25 mL glass containers with screw tops. Samples were stored at -20°C until analysis.

2.4. Extraction of Urine Samples

The extraction procedure for the extraction of drugs of abuse from urine was identical to that used routinely by the Laboratory of the National Horseracing Authority of Southern Africa, as described below.

3 mL of each urine sample, calibrator, and QC were transferred into separate 50 mL glass tubes, 3 mL of 2 M sodium acetate buffer (pH 5,5) and 20 µL of beta glucuronidase/ arylsulphatase solution was added to each sample. The samples were then hydrolysed overnight in a water bath at 37°C. This was followed by the addition of 160 µL of 5 M aqueous sodium hydroxide and 3 mL of 0,25 M phosphate buffer (pH 8) to keep a constant basic medium. After vortexing and leaving the samples to stand for 30 min to allow it to reach room temperature, 100 µL of internal standard was added and the samples were centrifuged for 20 minutes at approximately 3000 g. Samples were applied to C18 SPE cartridges conditioned with 2 mL MeOH followed by 2 mL double deionised water. The

columns were dried and eluted with 8 mL dichloromethane: ethanol (96:4), and the eluant dried under nitrogen gas at 40°C (5 - 10 psi), followed by reconstitution in 100 µL methanol, and submitted for LC-MS analysis.

Application to Real Samples from Australia

Small amounts (approximately 50 mg) of three different powders seized in Australia, confirmed to contain several synthetic cannabinoids and four positive workplace testing urine samples (different compounds than what was included in this study) from Australia were kindly donated by Paul Zahra of Racing Analytical Services Limited, Victoria, as positive control samples. The list of compounds included in the Australian method was also provided.

The powders were diluted to an estimated concentration of 5 ng/mL in MeOH, and then split into 2 portions, of which one was hydrolysed. Both the diluted and the hydrolysed samples were then analysed. The urine samples were treated as described in Section 2.4 and analysed as described in Section 2.7.

2.5. Extraction of Whole Blood Samples

2.5.1. Adaptation of Urine Extraction Method

An attempt was made to adapt the urine method described above for whole blood samples (without a hydrolysis step), which resulted in clogged SPE cartridges. A precipitation step was added in an attempt clean up the samples and remove proteins found in the blood. The SPE cartridges continued to get clogged due to the nature of the postmortem whole blood matrix, and subsequently this method was abandoned.

2.5.2. Evaluation of Sample Preparation Methods in Literature

A pooled blood sample was spiked with standard synthetic cannabinoid mixture at 30 ng/mL and used to evaluate four different extraction methods from literature.

A) LLE method developed by Kacinko et al., (2011):

In a clean glass tube, 1 mL of sample, 1 mL of sodium carbonate buffer (pH 9,0), 50 µL internal standard, and 15 mL of hexane: ethyl acetate (99:1) was added. The tube was thoroughly mixed, and then centrifuged for 15 minutes at approximately 3000 g. The tube was frozen in a dry ice bath and the organic layer transferred to a clean glass tube. The organic layer was evaporated to dryness under nitrogen gas, using a Turbovap drier at 40°C, reconstituted in 100 µL methanol, and then submitted for LC-MS analysis.

B) LLE method developed by Ammann et al., 2012.

In a clean glass tube, 1 mL of sample, 2 mL of sodium carbonate buffer (pH 9,0), 50 µL internal standard, and 10 mL of 1-chlorobutane: isopropanol (90:10) was added. The tube was thoroughly mixed, and then centrifuged for 15 minutes at approximately 3000 g. The tube was frozen in a dry ice bath and the organic layer transferred to a clean glass tube. The organic layer was evaporated to dryness under nitrogen gas, using a Turbovap drier at 40°C, reconstituted in 100 µL methanol, and then submitted for LC-MS analysis.

C) LLE method developed by Shanks et al., 2012 (b).

In a clean glass tube, 1 mL of sample, 2 mL of sodium carbonate buffer (pH 9,0), 50 µL internal standard, and 10 mL of ethyl ether was added. The tube was thoroughly mixed, and then centrifuged for 15 minutes at approximately 3000 g. The tube was frozen in a dry ice

bath and the organic layer transferred to a clean glass tube. The organic layer was evaporated to dryness under nitrogen gas, using a Turbovap drier at 40°C, reconstituted in 100 µL methanol, and then submitted for LC-MS analysis.

D) SPE method developed by Holm et al., 2013.

In a clean glass tube, 1 mL of sample was added, along with 50 µL internal standard. To this, 3 mL of acetonitrile: methanol (85:15) was added to precipitate the proteins. The tube was subsequently centrifuged for 15 minutes at approximately 3000 g, the organic layer transferred to a clean glass tube, and 4,5 mL 0,1 M acetic acid was added. This was added to a Strata-X Reversed Phase SPE cartridge which had been conditioned with 4 mL of acetonitrile: methanol (85:15) followed by 4 mL of 0,1 M acetic acid. Washing was performed with 3,5 mL of 0,1 M acetic acid followed by 3,5 mL of acetonitrile: 0,1 M acetic acid (70:30). The sample was eluted with two portions of 1.25 mL acetonitrile: acetic acid (98:2). The eluate was further dried under vacuum for 5 minutes, evaporated to dryness under nitrogen gas (using a Turbovap drier at 40°C), reconstituted in 100 µL methanol, and then submitted for LC-MS analysis.

The method by Ammann et al. (2012) was found to be the most suitable for sample preparation and was further evaluated before being used for all subsequent Whole Blood experiments, as can be seen in the Results chapter.

2.6. Liquid Chromatography-Mass Spectrometry (LC-MS) Background

For many years GC-MS was the gold standard for the systematic toxicological analysis of forensic samples, however the recent development of LC-MS from the early 1990s onwards

has enabled analysis of hydrophilic, thermolabile, and non-volatile analytes that weren't covered by GC-MS in a satisfactory manner (Peters, 2011; Sergi and Napoletano, 2012).

HPLC (the "LC" part of LC-MS) is a physical separation technique by which components of a mixture are separated based on their interactions with the mobile and stationary phases. In the current study reverse phase chromatography is being utilised which entails the use of a polar mobile phase (solvent) and a non-polar stationary phase (column). The above interaction of the components of the analyte with the mobile and stationary phase results in the various components being retained by the column for varying times (known as the retention time) depending on their polarity. In reverse phase chromatography non-polar components have higher retention times while polar compounds have lower retention times (Sirard, 2012).

Mass spectrometry is an analytical technique which identifies and quantifies molecules based on the mass to charge (m/z) ratio of charged gas-phase ions. The sample is introduced (usually after having gone through a chromatographic method), and subsequently converted into charged gas phase ions by ionisation which are then transferred to the mass analyser. The ionisation technique used in this study was electrospray ionisation (ESI), a technique in which a high voltage is applied to the liquid sample to create the aerosol. ESI is considered a soft-ionisation technique as very little fragmentation occurs. Some of the advantages of using ESI are its wide range of polarity, its applicability to thermally labile compounds, as well as ionisation of compounds with high molecular weights (Dooley, 2015).

2.7. LC-MS Method

The LC-MS settings used in this study were identical to those used routinely by the Laboratory of the National Horseracing Authority of Southern Africa where the laboratory work was conducted. This was done to assess how easily a new screening method could be integrated into already existing routine drug screening methods, so that the development of a completely new method would not be necessary.

The LC-MS system consisted of a Thermo Fisher Q Exactive Orbitrap mass spectrometer fitted with a heated electrospray (HESI-II) and an Agilent 1260 Infinity HPLC system with a XSelect CSH C18 column (5 µm, 2,1 x 150 mm). Gradient elution was performed using 5 mM ammonium acetate in 0,1% formic acid, water: acetonitrile (98:2) (Solvent A) and 5 mM ammonium acetate in 0,1% formic acid, water: acetonitrile (2:98) (Solvent B). The gradient started with 98% Solvent A at a flow rate of 0,3 mL/min and decreased over 20 minutes to 2% Solvent A, which was kept for 5 minutes. After this the flow rate was increased to 0,35 mL/min for 3 minutes. Starting conditions were restored over 7 minutes to allow the system to re-equilibrate. The temperature was kept at 40°C and the pressure remained between 80 and 150 psi.

2.7.1. Data Processing

Processing of data was done using Thermo Fisher ToxID (a sample of the generated report can be seen in Figure A3 in the Appendix). The ToxID software simply requires an Excel spread sheet comprising the compound name, elemental composition, polarity, and expected retention time; and it generates a PDF report with a trace of the extracted ions for each compound based on the accurate mass calculated from the monoisotopic mass to

charge ratio (m/z) (see Tables 3.1 and 3.4 for accurate masses and retention times of all the compounds).

For the study of the various whole blood extraction techniques the intensities of the chromatogram peaks were monitored and compared.

All processing was done with the allowable mass deviation set at 5 ppm (delta ppm = [(theoretical m/z – detected m/z)/theoretical m/z]) and retention time drift at 0,3 min. A signal to noise (S/N) ratio of ≥ 3 was required for confirmation. An explanation of the S/N ratio can be found below in 2.8.3.

2.8. Method Validation Criteria

Method validation was broadly based on the SANAS (South African National Accreditation System) TG 41-01 document (SANAS, 2008) which provides the recommended guidelines for both the verification and validation of forensic chemistry methods. These guidelines were used for the acceptance criteria.

Parameters that were investigated include selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, recovery, matrix effects, and stability. See Table A5 in the Appendix for the equations used.

Seven calibrators (two replicates per batch) were prepared (in pooled negative urine and whole blood from volunteers) and analysed on four different days for urine and three different days for whole blood. Three QCs (three replicates) were included in each batch.

2.8.1. Selectivity

Selectivity is the measure of the ability of the method to identify and quantitate the analytes in the presence of other substances, either endogenous or exogenous, in a sample matrix (Vessman et al., 2001).

To assess the selectivity of the method the following criteria were used to process the data: retention time variance ≤ 0,3 minutes and accurate mass deviation ≤ 5 ppm was used to check for co-eluting or closely eluting compounds within the same accurate mass range.

Blank samples from five individual non-drug users were also analysed with the first batch to check for co-eluting matrix peaks that could give rise to false positive results.

2.8.2. Linearity

The linearity of an analytical method is defined as its ability to produce results that are directly (or by mathematical transformations) proportional to the concentration of analytes in samples over a stipulated range (United Nations Office on Drugs and Crime, 2009).

Linearity was assessed using calibrators over the range: 0,0225 ng/mL to 6,75 ng/mL for urine, and 0,0675 ng/mL to 20,25 ng/mL for whole blood.

Deuterated 1-Naphthalenyl (1-pentyl-1H-indol-3-yl)-methanone-D_{11} (JWH-018-D_{11}) and (1-Butyl-1H-indol-3-yl)-1-naphthalenyl-methanone-D_9 (JWH-073-D_9) were used as internal standards to compensate for the loss of analyte during sample preparation.

The criteria for acceptance for linearity were an R^2 value ≥ 0,970 and a percentage difference of ≤ 25% for calibrators and QCs. In addition, no more than 2 points were excluded from

each set of standards. Failure to meet these criteria resulted in the validation being rejected for the specific compound. These criteria are based on those used by the Laboratory of the National Horseracing Authority of Southern Africa for substances below 50 ppb.

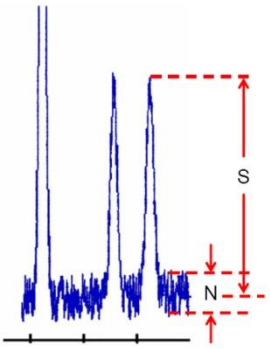
2.8.3. Limit of Detection and Quantitation

Limit of detection is defined as the smallest measured concentration from which it is possible to deduce the presence of an analyte with reasonable statistical certainty, and distinguish it from background noise. The minimum requirement is widely accepted to be an S/N ratio of 3. It is not regarded as a rugged or robust parameter as it is affected by minor changes in the analytical system (such as temperature, purity of reagents, matrix effects, and instrumental conditions) (United Nations Office on Drugs and Crime, 2009).

Limit of quantitation is defined as the smallest measured concentration from which it is possible to quantitate the analyte with an acceptable level of accuracy and precision (United Nations Office on Drugs and Crime, 2009). The minimum requirement is widely accepted to be a signal to noise ratio of 10, so that the signal is certain to be the analyte and not background noise or unusual fluctuation (Armbruster et al., 1994).

The aim of the project was not to determine the absolute LOD and LOQ for each compound. A calibration range was selected based on concentrations which would indicate recent use of synthetic cannabinoids prior to death.

The signal to noise ratio is illustrated in Figure 2.1 below.



http://www.sepscience.com/images/Fig1_HPLCSol124.jpg

Figure 2.1. Illustration of Signal to Noise Ratio

The LOD was determined as the lowest calibrator which exhibited an S/N ratio of greater than 3, while the LOQ was determined to be the lowest calibrator which exhibited an S/N ratio of greater than 10.

2.8.4. Accuracy and Precision

Accuracy is the measure of the difference, due to systematic method and laboratory error, between the expectation of the test result and the accepted reference value, i.e. it is the degree of closeness of the determined value to the known 'true' value (Food and Drug Administration, 2013; United Nations Office on Drugs and Crime, 2009).

Precision reflects the random errors which occur in the method by measuring the closeness of the analytical results obtained from a series of replicate measurements of the same measure under the stated conditions. There are two common sets of conditions by which precision is measured; repeatable and reproducible conditions. Repeatability conditions are those conditions which occur when an analyst analyses samples on the same day with the same instrument or materials in the same laboratory. Reproducible conditions are those that occur when one or several of the above is variable, e.g. different analysts, different days,

different instruments, or different laboratories (United Nations Office on Drugs and Crime, 2009).

Accuracy and precision were calculated using the three replicate results for the High QC on four days for urine and three days for whole blood. Accuracy represented as the difference of the QC High and the theoretical value, and precision as the standard deviation (SD) and coefficient of variance (CV) of the replicates. Acceptance criteria were values within ≤ 25% of the theoretical values, % RSD values ≤ 15%, and CV ≤ 10.

2.8.5. Matrix Effects

The matrix effect is defined as the interference (direct or indirect) of other substances in the sample on the response (Food and Drug Administration, 2013).

The matrix effect was not fully investigated in this study. Urine and whole blood samples were taken from five drug-free volunteers at the Forensic Pathology Services Medicolegal Laboratory, Johannesburg and from the Laboratory of the National Horseracing Authority of Southern Africa, Turffontein as blank samples. All calibrators and QCs were prepared in pooled urine or whole blood, respectively, to eliminate any matrix effects.

2.8.6. Stability

Stability relates to the resistance of the substances to chemical changes such as decomposition or disintegration. One of the purposes of performing stability studies is to assess the extent to which the analytes are stable during the entire analytical procedure as well as during storage before and after analysis (United Nations Office on Drugs and Crime, 2009).

In this study long term stability of urine and blood was assessed by storing spiked QCs for 3 months at 4°C (the temperature of most refrigerators) and at -20°C (the temperature of most freezers). In addition freeze-thaw stability of the samples was also assessed (i.e. after being frozen for 3 months the samples were thawed and frozen 3 times on 3 consecutive days, then analysed). Only QC High results (4,5 ng/mL in urine, and 13.5 ng/mL in blood) were used to assess stability.

For the statistical analyses means and standard deviations were determined.

For sample collection and storage refer to 2.3 and 2.4 above.

Chapter 3: Results

3.1. Urine Method Validation

The LC-MS method for synthetic cannabinoids in urine was based on the existing validated routine method used by the Laboratory of the National Horseracing Authority of Southern Africa, as described under Sections 2.4 and 2.7. Only the additional synthetic cannabinoids were validated. A table containing a summary of the validation results can be found below (Table 3.1).

Table 3.1: Urine Validation Results

Drugs	Ionisation Mode	Retention time (min)	Accurate mass (amu)	R^2 Value	No. of Calibrators	Concentration Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
JWH-018	Positive	19,47	342,18463	0,993	5	1,125 - 6,750	1,125	1,125
JWH-019	Positive	20,12	356,20023	0,993	5	1,125 - 6,750	1,125	1,125
JWH-073	Positive	18,69	328,16898	0,996	6	0,225 - 6,750	0,225	0,225
JWH-081	Positive	19,75	372,19507	0,997	6	0,225 - 6,750	0,225	0,225
JWH-122	Positive	20,23	356,20023	0,994	5	1,125 - 6,750	1,125	2,250
JWH-200	Positive	12,61	385,19031	0,994	5	1,125 - 6,750	1,125	2,250
JWH-250	Positive	18,45	336,19513	0,996	5	1,125 - 6,750	1,125	1,125
AM-2201	Positive	17,83	360,1752	0,992	5	1,125 - 6,750	1,125	2,250
(±)-CP 47,497	Negative	19,10	317,24854	*	*	*	*	*
(C8)-CP 47,497	Negative	19,99	331,26425	*	*	*	*	*
HU-211	Positive	20,33	387,28876	0,992	5	1,125 - 6,750	1,125	2,250
THC	Positive	20,97	315,23151	0,993	5	1,125 - 6,750	1,125	2,250
11-OH-THC	Positive	17,58	331,22623	0,991	3	3,375 - 6,750	3,375	3,375
JWH-018 N-4OH pentyl metabolite	Positive	15,41	358,17938	0,993	5	1,125 - 6,750	1,125	2,250
JWH-073 N-3OH butyl metabolite	Positive	15,24	344,16388	0,997	5	1,125 - 6,750	1,125	1,125

* indicates results that did not meet the validation criteria.

LOD = lowest calibrator which showed a S/N ratio ≥ 3.

LOQ = lowest calibrator which showed a S/N ratio ≥ 10.

3.1.1. Selectivity

No interfering compounds were found in the blank urine samples. All the extracted ion peaks fell within the retention time (0,3 min) and accurate mass (5 ppm) limits.

3.1.2. Linearity

All of the studied compounds except (±)-CP 47,497 and (C8)-CP 47,497, showed excellent linearity ($R^2 \geq 0{,}991$) as can be seen in Table 3.1.

3.1.3. Limit of Detection and Quantitation

Both the LODs and LOQs for the analytes in urine ranged from 0,225 ng/mL to 3,375 ng/mL (seen in Table 3.1).

3.1.4. Accuracy and Precision

A table summarising the accuracy and precision of the High QCs of the synthetic cannabinoids in urine can be seen below (Table 3.2) (the complete table can be found in the Appendix, Figure A8).

The accuracy of the analytes ranged from 95 - 109% in urine.

Table 3.2: Summary of the Accuracy and Precision of the High QCs (4,5 ng/mL) of the Synthetic Cannabinoids in Urine

Drugs	No. of QCs	Mean (ng/mL)	Accuracy %	SD	% RSD	CV
JWH-018	7	4,688	104	0,456	9,519	0,095
JWH-019	7	4,675	104	0,442	9,248	0,092
JWH-073	7	4,788	106	0,333	6,883	0,069
JWH-081	6	4,597	102	0,296	6,304	0,063
JWH-122	7	4,791	106	0,444	9,156	0,092
JWH-200	3	4,434	99	0,218	4,909	0,049
JWH-250	5	4,415	98	0,241	5,478	0,055
AM-2201	5	4,266	95	0,371	9,175	0,092
(±)-CP 47,497	*	*	*	*	*	*
(C8)-CP 47,497	*	*	*	*	*	*
HU-211	8	4,885	109	0,241	5,066	0,051
THC	8	4,573	102	0,365	7,873	0,079
11-OH-THC	5	4,430	98	0,434	9,629	0,096
JWH-018 N-4OH pentyl metabolite	5	4,610	102	0,408	8,933	0,089
JWH-073 N-3OH butyl metabolite	5	4,327	96	0,488	11,858	0,119

* indicates results that did not meet the validation criteria.

3.1.5. Stability

Table 3.3 below shows the long-term stabilities of the studied compounds.

Table 3.3: Long-term Stabilities (% Recovery) of the Synthetic Cannabinoids in Urine in Glass and Plastic Containers

Drugs	Medium	Temperatures		
		-20°C	4°C	Freeze-Thaw
JWH-018	Glass	78,71 ± 0,62	68,14 ± 7,71	78,71 ± 1,40
	Plastic	67,56 ± 74,25	40,20 ± 19,21	49,81 ± 13,82
JWH-019	Glass	67,64 ± 4,45	62,79 ± 9,48	73,35 ± 12,43
	Plastic	46,09 ± 49,89	25,38 ± 12,15	30,87 ± 8,85
JWH-073	Glass	75,90 ± 1,17	67,50 ± 5,57	78,38 ± 0,87
	Plastic	71,33 ± 77,49	49,90 ± 16,25	58,82 ± 9,40
JWH-081	Glass	57,56 ± 4,91	55,02 ± 3,93	66,69 ± 6,94
	Plastic	38,53 ± 39,04	30,66 ± 4,12	36,43 ± 2,29
JWH-122	Glass	72,00 ± 9,37	66,47 ± 5,88	90,09 ± 27,83
	Plastic	49,66 ± 52,66	29,09 ± 15,27	37,30 ± 14,31
JWH-200	Glass	75,01 ± 16,05	63,17 ± 20,54	75,12 ± 19,16
	Plastic	82,57 ± 89,30	64,42 ± 6,19	73,74 ± 16,05
JWH-250	Glass	79,04 ± 5,10	70,74 ± 3,45	69,50 ± 10,99
	Plastic	84,99 ± 92,47	61,52 ± 15,26	67,48 ± 13,81
AM-2201	Glass	85,80 ± 4,14	75,56 ± 4,21	73,23 ± 17,57
	Plastic	81,86 ± 89,98	65,33 ± 6,13	66,23 ± 2,42
(±)-CP 47,497	Glass	144,18 ± 7,32	91,95 ± 18,51	81,43 ± 39,25
	Plastic	193,73 ± 212,33	141,45 ± 38,06	129,66 ± 37,01
(C8)-CP 47,497	Glass	79,34 ± 38,74	57,52 ± 50,71	59,45 ± 50,17
	Plastic	157,98 ± 174,72	108,00 ± 43,42	99,39 ± 29,65
HU-211	Glass	118,35 ± 2,43	87,46 ± 10,41	117,80 ± 10,06
	Plastic	127,55 ± 139,58	97,00 ± 26,85	97,73 ± 31,11
THC	Glass	117,66 ± 18,32	65,08 ± 12,48	140,64 ± 57,48
	Plastic	155,00 ± 182,98	67,15 ± 32,73	88,86 ± 33,53
11-OH-THC	Glass	167,56 ± 19,22	100,17 ± 12,02	143,35 ± 42,18
	Plastic	237,87 ± 272,42	164,49 ± 24,43	162,44 ± 8,06
JWH-018 N-4OH pentyl metabolite	Glass	79,26 ± 12,04	77,36 ± 15,09	74,62 ± 20,94
	Plastic	99,04 ± 113,73	72,95 ± 6,28	75,20 ± 11,07
JWH-073 N-3OH butyl metabolite	Glass	90,22 ± 8,29	80,78 ± 4,26	73,29 ± 10,38
	Plastic	103,27 ± 111,35	86,03 ± 4,44	89,20 ± 2,26

n = 3

3.2. Blood Extraction Method

A comparison of the peak intensities of the drugs in the four extraction methods described in 2.5.2) can be seen in Figure 3.1. As can be seen, the extractions based on Kacinko et al. (2011) and Holm et al. (2013) had intensities that were noticeably higher than the other two methods, although the method based on Ammann et al. (2012) also resulted in consistently high intensities. It was decided that the method based on Ammann et al. (2012) would be further investigated, as it could easily be integrated with an existing screening method. An attempt was made to improve the extraction efficiency by substituting the isopropanol component with ethyl acetate and methyl tert-butyl ether respectively (as these are commonly used solvents in forensics). A comparison of the results of the optimisation can be seen in Figure 3.2. The 1-chorobutane with 10% isopropanol showed unmatched intensities, between 3 and 5 times higher than the rest, and was used for the subsequent validation.

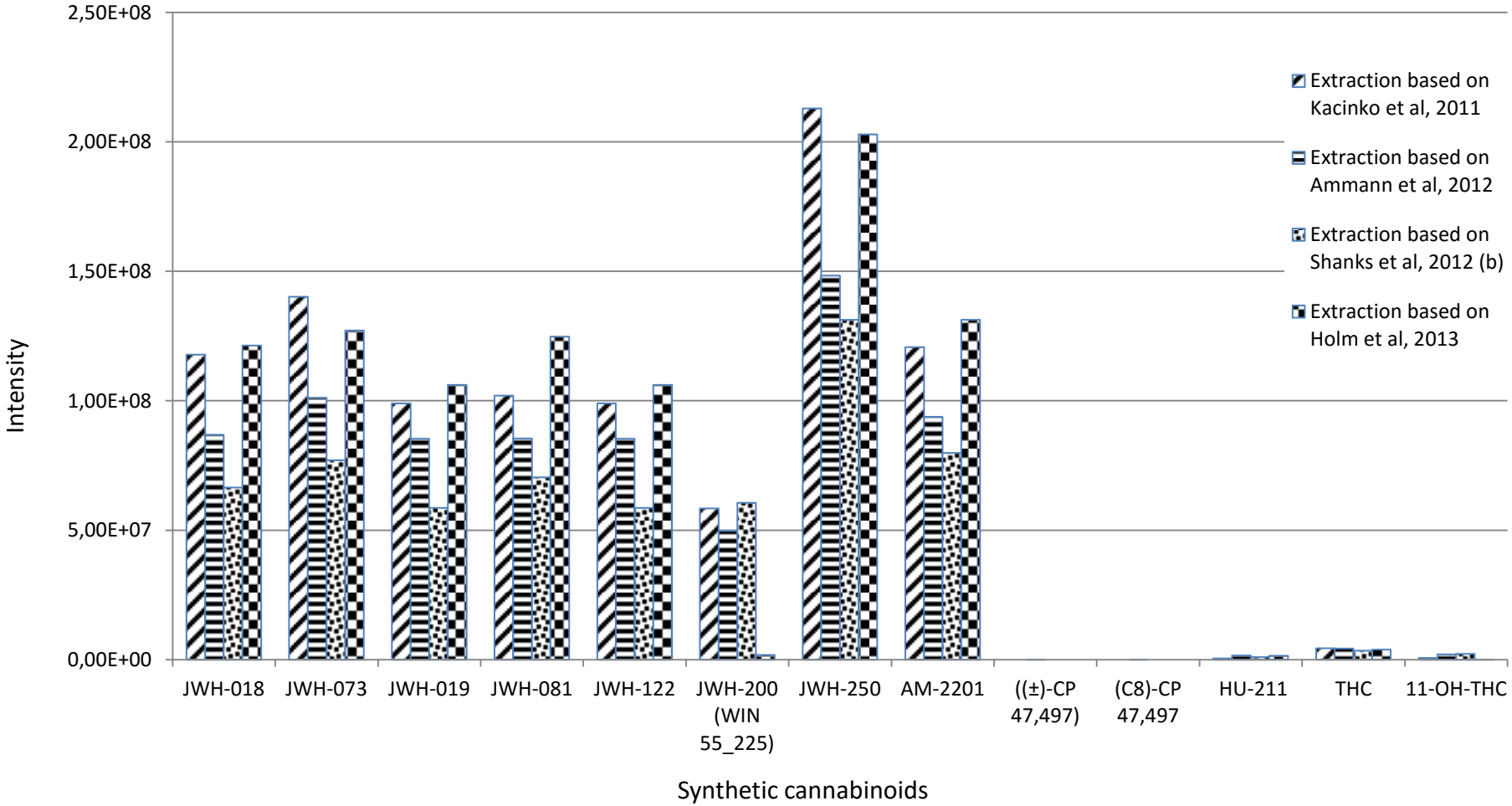


Figure 3.1. Graph showing the intensities of selected synthetic cannabinoids for the 4 extraction methods for whole blood investigated

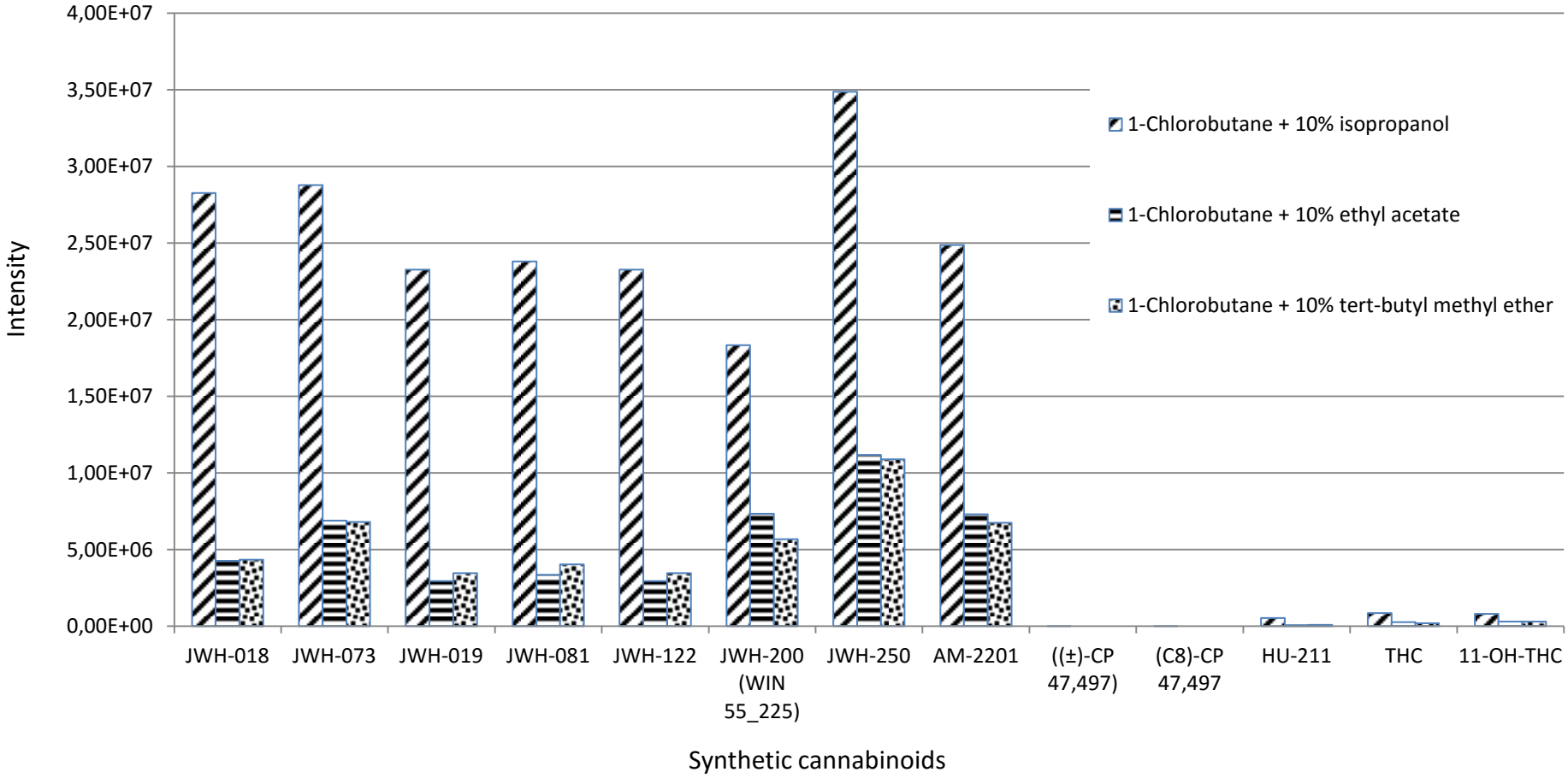


Figure 3.2. Graph showing the intensities of selected synthetic cannabinoids for the 3 extraction methods for whole blood based on Ammann et al. (2012)

3.3. Whole Blood Method Validation

The method by Ammann et al. (2012) produced acceptable results for synthetic cannabinoids in whole blood, and due to compatibility with existing forensic methods was further validated. A table containing a summary of the validation results can be found below (Table 3.4).

Additionally, due to initial analyst errors, the calibrators and quality controls were prepared at a much lower concentration than initially intended. As such, the QC Mediums and Lows were below the validated calibration range; and thus validation calculations were performed using only the QC High data.

Table 3.4: Whole Blood Validation Results

Drugs	Ionisation Mode	Retention time (min)	Accurate mass (amu)	R^2 Value	No. of Calibrators	Concentration Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
JWH-018	Positive	19,38	342,18463	0,997	6	0,675 - 20,250	0,675	0,675
JWH-019	Positive	20,02	356,20023	0,998	6	0,675 - 20,250	0,675	0,675
JWH-073	Positive	18,61	328,16898	0,994	5	0,675 - 20,250	0,675	3,375
JWH-081	Positive	19,65	372,19507	0,995	6	0,675 - 20,250	0,675	0,675
JWH-122	Positive	20,13	356,20023	0,994	6	0,675 - 20,250	0,675	3,375
JWH-200	Positive	12,65	385,19031	0,972	6	0,675 - 20,250	0,675	3,375
JWH-250	Positive	18,37	336,19513	0,997	6	0,675 - 20,250	0,675	0,675
AM-2201	Positive	17,75	360,1752	0,989	6	0,675 - 20,250	0,675	0,675
(±)-CP 47,497	Negative	19,00	317,24854	*	*	*	*	*
(C8)-CP 47,497	Negative	19,89	331,26425	*	*	*	*	*
HU-211	Positive	20,25	387,28876	0,996	6	0,675 - 20,250	0,675	3,375
THC	Positive	20,86	315,23151	0,993	5	3,375 - 20,250	3,375	3,375
11-OH-THC	Positive	17,52	331,22623	0,995	5	3,375 - 20,250	3,375	3,375
JWH-018 N-4OH pentyl metabolite	Positive	15,36	358,17938	0,991	6	0,675 - 20,250	0,675	0,675
JWH-073 N-3OH butyl metabolite	Positive	15,17	344,16388	0,994	6	0,675 - 20,250	0,675	0,675

* indicates results that did not meet the validation criteria.

LOD = lowest calibrator which showed a S/N ratio ≥ 3.

LOQ = lowest calibrator which showed a S/N ratio ≥ 10.

3.3.1. Selectivity

No interfering compounds were found in the blank whole blood samples. All the excluded ion peaks fell within the retention time (0,3 min) and accurate mass (5 ppm) limits.

3.3.2. Linearity

All of the studied compounds except (±)-CP 47,497 and (C8)-CP 47,497, showed excellent linearity (R^2 ≥ 0,970) and met the criteria for validation as can be seen in Table 3.4.

3.3.3. Limit of Detection and Quantitation

Both the LODs and the LOQs for the analytes in whole blood ranged between 0,675 ng/mL and 3,375 ng/mL (seen in Table 3.4).

3.3.4. Accuracy and Precision

A table summarising the accuracy and precision of the High QCs of the synthetic cannabinoids in whole blood can be seen below (Table 3.5) (the complete table can be found in the Appendix, Figure A9). The accuracy of the analytes ranged from 88 - 107% in whole blood.

Table 3.5: Summary of the Accuracy and Precision of the High QCs (13,5 ng/mL) of the Synthetic Cannabinoids in Whole Blood

Drugs	No. of QCs	Mean (ng/mL)	Accuracy %	SD	% RSD	CV
JWH-018	12	13,745	102	1,014	7,485	0,075
JWH-019	11	13,846	103	1,369	10,020	0,100
JWH-073	12	13,779	102	1,045	7,726	0,077
JWH-081	11	14,467	107	1,367	9,621	0,096
JWH-122	12	13,360	99	1,521	11,389	0,114
JWH-200	11	12,733	94	1,109	8,663	0,087
JWH-250	12	12,751	94	1,256	9,971	0,100
AM-2201	11	11,865	88	1,219	9,851	0,099
(±)-CP 47,497	*	*	*	*	*	*
(C8)-CP 47,497	*	*	*	*	*	*
HU-211	9	13,834	102	2,020	14,579	0,146
THC	9	14,210	105	1,889	13,306	0,133
11-OH-THC	8	13,682	101	1,645	12,197	0,122
JWH-018 N-4OH pentyl metabolite	11	12,830	95	1,052	8,196	0,082
JWH-073 N-3OH butyl metabolite	7	12,745	94	1,473	11,577	0,116

* indicates results that did not meet the validation criteria.

3.3.5. Stability

The stability results are listed in Table 3.6.

Table 3.6: Long-term Stabilities (% Recovery) of the Synthetic Cannabinoids in Whole Blood in Glass and Plastic Containers

Drugs	Medium	Temperature		
		-20°C	4°C	Freeze-Thaw
JWH-018	Glass	34,98 ± 60,58	77,21 ± 3,62	82,69 ± 6,67
	Plastic	91,21 ± 2,29	98,97 ± 9,26	92,22 ± 9,14
JWH-019	Glass	52,70 ± 45,79	51,18 ± 44,38	58,67 ± 50,91
	Plastic	105,50 ± 6,66	*	72,26 ± 62,58
JWH-073	Glass	79,41 ± 4,51	82,86 ± 2,24	58,96 ± 51,11
	Plastic	91,72 ± 3,36	97,28 ± 3,08	90,40 ± 5,44
JWH-081	Glass	24,19 ± 41,90	79,91 ± 5,07	55,14 ± 47,76
	Plastic	97,57 ± 4,49	104,67 ± 11,53	98,78 ± 12,96
JWH-122	Glass	49,89 ± 43,47	54,80 ± 47,79	55,31 ± 48,21
	Plastic	88,22 ± 4,75	29,30 ± 50,75	99,00 ± 9,34
JWH-200	Glass	63,96 ± 55,47	95,72 ± 5,87	65,19 ± 56,47
	Plastic	115,20 ± 3,57	102,44 ± 13,28	114,81 ± 9,63
JWH-250	Glass	58,46 ± 50,82	96,34 ± 6,65	64,59 ± 56,02
	Plastic	106,40 ± 1,53	122,59 ± 9,84	108,75 ± 11,20
AM-2201	Glass	25,43 ± 44,04	74,74 ± 4,57	51,61 ± 44,75
	Plastic	51,46 ± 44,66	89,43 ± 4,07	75,85 ± 4,32
(±)-CP 47,497	Glass	*	*	*
	Plastic	*	*	*
(C8)-CP 47,497	Glass	*	*	*
	Plastic	*	*	*
HU-211	Glass	*	*	*
	Plastic	*	*	*
THC	Glass	65,11 ± 57,94	110,72 ± 7,76	82,15 ± 71,17
	Plastic	80,28 ± 69,56	85,49 ± 4,79	38,69 ± 67,02
11-OH-THC	Glass	*	*	*
	Plastic	*	*	*
JWH-018 N-4OH pentyl metabolite	Glass	66,39 ± 59,79	98,61 ± 7,44	71,54 ± 61,98
	Plastic	117,24 ± 6,12	*	37,30 ± 64,61
JWH-073 N-3OH butyl metabolite	Glass	73,03 ± 65,77	71,98 ± 62,60	80,56 ± 70,06
	Plastic	39,00 ± 67,55	95,77 ± 12,28	40,44 ± 70,04

* indicates results that did not meet the validation criteria.

n = 3.

3.4. Application to Postmortem Forensic Cases

The postmortem forensic samples included 126 whole blood and 85 urine samples which were obtained over a 5 month period, from July 2014 to December 2014.

Shown below (Table 3.7) is a summary of the demographic information of the sample population.

Table 3.7: Postmortem Cases Summary – Demographic Information

Category	Sub-category	Urine % Distribution (n = 85)	Blood % Distribution (n = 126)
Age Group (years)	15 - 24	15	15
	25 - 34	39	42
	35 - 44	32	28
	45 - 54	4	6
	55+	4	3
	Unknown	7	6
Racial affinity	Black	85	83
	White	11	11
	Asian	1	2
	Coloured	4	4
Sex	Male	92	86
	Female	8	14
Mode of Death	Assault	2	2
	Fall from Height	8	6
	GSW	28	25
	Hanging	7	13
	MVA/MBA/Train	12	9
	Overdose/Poisoning	2	2
	Other	4	6
	PVA	11	10
	Stabbed	16	17
	Strangulation/Suffocation	2	2
	Unknown	7	10

GSW = Gunshot wound; MBA = Motorbike accident; MVA = Motorist vehicle accident; PVA = Pedestrian vehicle accident; others include burns electrocution, and gassing.

None of the studied synthetic cannabinoids were detected in the studied population.

3.5. Application to Real Samples from Australia

These samples (both urine and powder) were analysed using the method described in this study and processed with the ToxID database provided by Paul Zahra of Racing Analytical

Services Limited, which contained additional synthetic cannabinoids to the list validated in this study.

Using the processing method provided, the method was able to detect the newer synthetic cannabinoids PB-22 and XLR-11 (neither were subjects of this study) in the powders (both the hydrolysed and diluted samples). Several other metabolites and traces of other synthetic cannabinoids were also found indicating that the purity of the powders is questionable. These results corresponded with the qualitative findings of Racing Analytical Services Limited, Victoria in Australia.

In the urine samples several synthetic cannabinoids (JWH-018, UR-144, and XLR-11) and their urinary metabolites that had previously been confirmed were detected using the method developed. Once again several other synthetic cannabinoids and metabolites were also found in trace amounts, which were presumably impurities present in the substance consumed.

The data for the analyses of the positive controls are not shown due to confidentiality reasons.

Chapter 4: Discussion

This study aimed to assess how easily the newer synthetic cannabinoids could be integrated into existing screening methods. The method for urine specimens currently used by the Laboratory of the National Horseracing Authority of Southern Africa, and a whole blood method found in literature were found to be suitable for majority of the analytes. Both these methods are examples of commonly used methods with common solvents and techniques, allowing for their potentially easy implementation into local forensic laboratories.

This method was validated for JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-250, AM-2201, HU-211, THC, 11-OH-THC, JWH-018 N-4OH pentyl metabolite, and JWH-073 N-3OH butyl metabolite in urine and whole blood; based on selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, matrix effects, and stability.

The methods were not successfully validated for (±)-CP 47,497 and (C8)-CP 47,497 as several issues were encountered (such as ionisation efficiency and stability) that could be assessed in future work. Furthermore, future work on urine samples could include more metabolites, since this study focused mainly on the parent components and only two metabolites were analysed.

4.1. Urine Method Validation

4.1.1. Linearity

Of the several studies undertaken on synthetic cannabinoids it appears only Scheidweiler and Huestis (2014) investigated CP 47,497-type compounds, and reported good linearity. In the current study, good recoveries were initially observed for (±)-CP 47,497 and (C8)-CP

47,497 in the first batch (102 and 94 %, respectively), but the concentration declined rapidly in subsequent batches to below detection limits. This indicates that these compounds might not be stable long term or that the storage conditions were not optimal. It is possible that this analytical method could be improved for compounds (±)-CP 47,497 and (C8)-CP 47,497, by preparing fresh spiking solution with each batch, as well as including an additional internal standard. This could have implications for forensics application as it may require a specific targeted method.

4.1.2. Limit of Detection and Quantitation

Both the LODs and the LOQs for the analytes in urine ranged from 0,225 ng/mL to 3,375 ng/mL. Several of the compounds have a LOD which is the same as its LOQ. This is due to the lower calibrator not fulfilling the criteria of S/N ratio ≥ 3, while the calibrator above that had an S/N ratio ≥ 10 fulfilling both the LOD and LOQ criteria. In reality, the LOD lies somewhere between the lower and higher calibrator, however no additional calibrators were prepared in that range and the exact LOD is not quantifiable from this study.

Scheidweiler and Huestis (2014) validated a similar LC-MS method for 20 synthetic cannabinoids and 21 metabolites in urine (10 of which were in common with this project) from 0,1 µg/mL to 100 µg/mL (100 ng/mL to 100000 ng/mL), which is significantly higher than the concentration range in this study. Wohlfarth et al. (2013) validated a method for 9 synthetic cannabinoids and 20 metabolites (8 of which were in common with this project) in urine and determined their LODs in the range of 0,5 ng/mL to 10 ng/ mL, which is comparable to the LODs determined in this project (0,225 ng/mL – 3,375 ng/mL).

The highest calibrator for the urine validation was 6,750 ng/mL, which means that any values above this would fall outside the calibration range of this study. Thus a much larger error would be associated with them making the method semi-quantitative.

4.1.3. Accuracy and Precision

Due to the low concentration levels of the Low and Medium QCs (falling mostly outside the calibration range), the High QC (4,5 ng/mL) values were used for all accuracy and precision calculations. The accuracy of the analytes ranged from 95 - 109% in urine, with RSD values of between 4,9 and 11,9 % which are well within the acceptance criteria.

De Jager et al. (2012) reported accuracy for their studied synthetic cannabinoid metabolites of 73,5 – 117,5 %, while Jang et al. (2013) reported 92 – 108% accuracy. Scheidweiler and Huestis (2014) achieved an accuracy of 86,5 – 118,3 % while another study in Portugal (Simões et al., 2014) reported accuracy of 90 - 115 %. This shows that the accuracy achieved in this study is of a comparable standard to international research, while having the advantage of screening for a large number of different compounds.

4.1.4. Stability

Storage of urine specimens in glass at -20°C is best for most of the compounds as they had higher recovery and lower standard deviations. For majority of the analytes storage in plastic resulted in lower recoveries and exceptionally high standard deviations. The very high recoveries (i.e. > 100) in several of the samples could be due to factors such as matrix enhancement, but more research would be needed. Another possibility is that some of the compounds were not stable in the spiking solution. Initially, the stability samples were

prepared at the same time as the spiking solution. The spiking solution was stored frozen but was defrosted with each batch to prepare calibrators and QC samples. Thus, the stability samples might have had a higher concentration of drug than the QCs and calibrators. This might suggest a need to prepare a fresh spiking solution so as to eliminate stability issues.

Caution should be exercised as a small sample size (only three replicates) was used and thus further research would need to be performed with more replicates to corroborate these results.

Of the several studies undertaken on urine, very few have studied stability. Three groups didn't study stability at all (de Jager et al., 2012; Grigoryev et al., 2011; Simões et al., 2014). Two groups (Scheidweiler and Huestis, 2014; Wohlfarth et al., 2013) only investigated stability over 3 days; while only one (Jang et al., 2013) investigated stability over 30 days (and reported good stabilities, 96 – 106 %, for JWH-018 and JWH-073 metabolites).

4.2. Blood Extraction Method

At first glance Figure 3.1 shows clearly that the method utilised by Holm et al. (2013) produced the highest intensities for many of the compounds, followed closely by that by Kacinko et al. (2011). When assessed closer however, the method employed by Holm et al. makes use of a polymer-based, strong cation mixed mode extraction column which is specific towards basic compounds (Phenomenex Inc., 2016). This is unsuitable for a general screening method which aims to test for a large number of compounds with varying polarities. The method by Ammann et al. (2012) was ultimately chosen as it yielded good intensities and requires solvents that are routinely used for forensic applications, thus making it easier to integrate with existing screening methods. In South Africa where many

facilities lack the proper equipment, a simple sample preparation method (such as this LLE method), is ideal.

During optimisation of the Ammann et al. (2012) method it was observed that isopropanol resulted in the highest intensities. This significant difference can be attributed to the greater polarity as well as eluant strength of isopropanol (Reichardt, 2003).

4.3. Whole Blood Method Validation

4.3.1. Linearity

The results suggest that the storage conditions of the spiking solution was not ideal for compounds (±)-CP 47,497 and (C8)-CP 47,497, as all the other compounds showed good linearity, but the compounds (±)-CP 47,497 and (C8)-CP 47,497 resulted in good linearity only on the first day and very poor linearity on subsequent days.

Ammann et al. (2012) also reported several issues with (±)-CP 47,497 and (C8)-CP 47,497 as well citing possible matrix effects. These compounds require negative ionisation mode to be detected and showed a lower response possibly indicating a lower ionisation efficiency of the ESI source (Ammann et al., 2012). This implies that detecting these compounds is more challenging and may require a targeted method which isn't ideal for forensic applications.

4.3.2. Limit of Detection and Quantitation

Both the LODs and the LOQs for the analytes in whole blood ranged from 0,675 ng/mL to 3,375 ng/mL. Several of the compounds have a LOD which is the same as its LOQ. This is due to the lower calibrator not fulfilling the criteria of S/N ratio ≥ 3, while the calibrator above that had an S/N ratio ≥ 10 fulfilling both the LOD and LOQ criteria. In reality, the LOD lies

somewhere between the lower and higher calibrator however no additional calibrators were prepared in that range so the exact LOD is not quantifiable from this study.

Kacinko et al. (2011) determined the LODs for JWH-018, JWH-019, JWH-073, and JWH-250 to be between 0,005 ng/mL and 0,020 ng/mL and the LOQ to be 0,1 ng /mL, while Holm et al. (2013) determined the LOD for 13 synthetic cannabinoids (4 of which were in common with this project) to be in the 0,1 ng/mL to 0,25 ng/mL range in whole blood.

Also in whole blood, Shanks et al. (2012a) found the LOD for their studied synthetic cannabinoids (JWH-018 and JWH-073) to be 0,01 ng/mL with a linear range of 0,05 ng/mL to 50 ng/mL.

This study's higher LODs and LOQs could be attributed to the method used that had already been optimised and validated for various other drugs of abuse. The intention of the method was not to optimise for the lowest LOD, but rather to be able to add compounds to an existing screening method. Furthermore, if one was only searching for synthetic cannabinoids the method and its analytical testing parameters could be optimised to achieve lower LODs and LOQs. In the forensic setting the LODs and LOQs from this study are sufficient as most decedents associated with substances of abuse have levels of the substance that is far greater than even the LOQs.

The higher LODs and LOQs would be significant when attempting to detect trace amounts. A study conducted on postmortem cases to investigate synthetic cannabinoid use as a cause or contributory cause of death (Labay et al., 2016) included several cases where the levels of several of the synthetic cannabinoids were below the LOD of this study and would thus not be detected, which would be a significant limitation. Labay et al. (2016) studied 25 cases, 8

of which had a concentration of various synthetic cannabinoids below the LODs of this study, although 4 of them also contained additional synthetic cannabinoids which would have been detected. Synthetic cannabinoids were not reported as the cause of death in any of the case studies. They concluded that the role of synthetic cannabinoids in the cause and manner of death is still undefined as many of the cases involved alcohol as well as other drugs.

The highest calibrator for the blood validation was 20,250 ng/mL, which means that any values above this would fall outside the calibration range of this study. Thus a much larger error would be associated with them making the method semi-quantitative.

4.3.3. Accuracy and Precision

Due to the low concentration levels of the Low and Medium QCs, the High QC (13,5 ng/mL) values were used for all accuracy and precision calculations. The accuracy of the analytes ranged from 88 - 107% in whole blood, with RSD values of between 7,5 and 15,0 % which are well within the acceptance criteria. This compares well with Kacinko et al. (2011) who reported accuracy (which translates to 91,8 to 108,2 %) for all their analytes except JWH-019; Ammann et al. (2012) who reported that all their analytes fell within an acceptance interval of ± 15 % (i.e. 85 – 115 %); and Shanks et al. (2012b) who reported accuracy of 99,1 - 107,0 % for JWH-018 and 97,7 – 102,0 % for JWH-073.

4.3.4. Stability

From Table 3.6 below it appears that not many of the analytes were stable over the three months in glass. A few of them, namely JWH-018, JWH-073, JWH-081, JWH-200, and JWH-

250, appeared to be stable in plastic at all the temperatures as well as being freeze-thaw stable.

Caution should be exercised when interpreting the data in Table 3.6 as a small sample size (only three replicates) was used and thus further research would need to be performed with more replicates to corroborate these results. Analytes for which no stability data was available at all include CP 47,497, (C8)-CP 47,497, HU-211, and 11-OH-THC. The very high recoveries (i.e. > 100) in several of the samples could be due to factors such as matrix enhancement, but more research would be needed.

The stability of 11-OH-THC is not relevant as it is a metabolite of THC and will not ordinarily be found in blood. A previous stability study on THC (Christophersen, 1986) found that THC stored in plastic (polystyrene) containers suffered a significant loss (between 60 and 100%) after 4 weeks, while the amount of THC in glass containers remained unchanged. They theorised that it could be due to unknown compounds which diffuse from the plastic into the blood and form compounds which aren't detected.

Kacinko et al. (2011) analysed stability in plastic containers with several common preservatives and concluded that the collection container did not affect stability at room temperature, refrigerated (approximately 3° C) and frozen (approximately -10° C) up to 30 days, however the stability of the compounds longer than that was not investigated.

Amman et al. (2012) investigated long term stability, but only up to 42 days. They found all their studied compounds to be stable up to that point.

4.4. Application to Postmortem Forensic Cases

From Table 3.7 it can be seen that the 25 to 34 year and 35 to 44 year age groups are the most prevalent in this sample, comprising 71% of the urine cases (n = 60) and 70% of the whole blood cases (n = 88), respectively. This is very similar to the National Injury Mortality Surveillance System (NIMSS) (NIMSS was a project started in 1999 to monitor and provide information about deaths due to external causes) 2009 report which listed the 20 to 29 year age group as having the highest number of mortality cases (Medical Research Council, 2009).

Approximately 80% of victims were Black individuals and 11% were White individuals, which correlates directly with the findings of the 2011 Census for the Gauteng province (Statistics South Africa, 2012).

The sex distribution of the study sample of males to females was at a ratio of 92:8 (n = 78 and 7 respectively) for urine, and 86:14 (n = 108 and 18 respectively) for whole blood, which is a closer reflection of the NIMSS report (approximately 80:20) than of the Census results (approximately 50:50).

None of the blood or urine samples tested positive for any of the studied synthetic cannabinoids; however several samples did test positive for common drugs of abuse included in the original screening method such as THC, benzodiazepines, methamphetamines, and opioids (data not shown).

From an extensive literature search only one study has been conducted internationally on postmortem cases, and a literature search found no studies involving synthetic cannabinoids in South Africa. Shanks et al. (2012b) analysed 45 postmortem blood cases and found either

JWH-018, JWH-073, or both in 18 of the cases. Ammann et al. (2012) validated their method in both antemortem and postmortem blood samples but did not apply their method to actual samples.

Analysing postmortem specimens provides numerous challenges since there are many factors that can influence the concentrations of substances of abuse in a decedent. Among these are postmortem interval (i.e. the time that has elapsed since a person has died), the condition of the body, as well as the little knowledge of many drugs' *in vivo* stability and postmortem redistribution. In South Africa, bodies can be stored for several days in suboptimal storage conditions, where these factors play an even larger role.

From the results obtained it is clear that the scope of research should be widened to include groups such as drug rehabilitation centres and seized drugs to obtain a more accurate representation of whether these drugs are being used in South Africa. If further research amongst these groups were to yield negative results then there is a good point to be made about these synthetic cannabinoids not being used in South Africa. If, however, positive results were found, the South African Police Services Forensics Science Laboratories would need to consider adding a screening test like the one presented in this study to their testing protocols.

The use of LLE extraction is ideal for the South African environment as it is easy to perform, large numbers can be processed simultaneously, and it requires minimal equipment and apparatus (basic glassware is sufficient).

As seen from several news articles (Serrao, 2014a, 2014b) the South African forensics labs are in an exceptionally bad state and thus there might even be cases with synthetic

cannabinoids, but by the time a postmortem investigation is conducted, a sample submitted, and then analysed, any trace amounts might have degraded already.

4.5. Application to Real Samples from Australia

The use of the method on real samples obtained from Australia serves to illustrate the versatility of using LC-HRAM-MS (liquid chromatography- high resolution accurate mass-mass spectrometry) for the detection of synthetic cannabinoids. In a field where the structures of drugs are constantly changing it is difficult to obtain and expensive to manufacture certified reference materials to keep up. LC-MS with accurate mass detection allows for the screening of forensic samples provided that the molecular formula of the compound is known or can be predicted. Only once these compounds are detected with a screening method will it be necessary to obtain the relevant reference materials for confirmation and quantitation. In this case, although a different LC-MS system was used, it was still possible to detect the compounds identified previously in the Australian laboratory by merely extracting the accurate mass of the compounds.

4.6. Conclusion

Overall majority of the studied cannabinoids exhibited excellent linearity ($R^2 \geq 0{,}990$), and good LODs and LOQs, 0,225 ng/mL and 3,375 ng/mL for urine, and 0,675 ng/mL and 3,375 ng/mL for whole blood. Both THC and 11-OH-THC had higher LODs and LOQs in both matrices as their structures are significantly different from the synthetic cannabinoids and no internal standard was used for them.

The method could not be validated for two of the target compounds, (±)-CP 47,497 and (C8)-CP 47,497. This is possibly due to stability of the compounds in the spiking solution used. These compounds ionise in negative mode in LC-MS, and due to limited resources an appropriate internal standard could not be acquired. Further optimisation of negative mode parameters may also improve the signal intensity and provide better results.

The validation data indicated that the adaptation of existing routine screening methods to include several synthetic cannabinoids was mostly successful. This method provides a sensitive as well as selective way of screening for JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-250, AM-2201, HU-211, THC, 11-OH-THC, JWH-018 N-4OH pentyl metabolite, and JWH-073 N-3OH butyl metabolite in urine and whole blood.

Due to technical issues with the instrumentation some data were not obtained on Day 3 of the Whole Blood validation. The following compounds were affected: HU-211, THC, 11-OH-THC, and JWH-018 N-4OH pentyl metabolite.

Chapter 5: Conclusion

One of the main aims of this project was to adapt an existing laboratory method to include synthetic cannabinoids in the list of targeted analytes.

Although adapting existing routine methods is quite straightforward one does not always obtain the sensitivity that can be achieved with specifically developed methods. This is because the objective of a forensic investigative analytical screen test is to be able to identify a wide variety of substances, which is not possible if you tweak a method for a narrow group of compounds.

In the South African setting decedents are not routinely screened for drugs of abuse, only in cases with a history of abuse or when the pathologist suspects the involvement of drugs, will a specimen be collected for further testing. A further issue is the lack of information supplied with a decedent.

This study has shown that it is possible to add any number of compounds to existing screening methods in a forensic environment and it is a viable strategy to test and screen for newer drugs of abuse such as synthetic cannabinoids (particularly the first generation variants studied). Adapting methods which are traditionally applied to antemortem cases were adapted and applied to postmortem samples with success. The only notable exceptions were the synthetic cannabinoids (±)-CP 47,497 and (C8)-CP 47,497 for which these methods were found to be unsuitable. For the other synthetic cannabinoids the method was successfully validated, for urine the LODs and LOQS ranged from 0,225 to 3,375 ng/mL; and for whole blood the LODs ranged from 0,675 to 3,375 ng/mL.

Whole blood is easier to obtain and almost always guaranteed in postmortem cases, whereas urine is a more difficult sample to obtain as victims may either empty their bladders prior to their death or the bladder could have been ruptured. Urine has the benefit of lower LODs and LOQs (since it's a cleaner and simpler matrix), but there is the risk that many of the parent compounds of the synthetic cannabinoids might already have been metabolised. Internationally, the trend seems to be towards whole blood instead of urine, presumably for the aforementioned reasons.

The data indicates that for urine samples should be stored in glass containers, while for blood, plastic containers with preservative were more suitable for long term storage. Furthermore, the lack of convincing stability data for many of the analytes suggests that any analysis of samples suspected to contain these compounds should be undertaken as soon as possible after sampling.

One of the chief limitations of this research was that it only included postmortem cases, and was in a relatively concentrated, metropolitan area.

Future prospects include widening the scope of research to include antemortem driving under the influence cases, rehabilitation centres, as well as drugs seized by the police. Furthermore, with greater resources the research could be extended to cover the whole of South Africa.

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Appendix

Table A1: Comparison of Extraction Methods in Literature for the Analysis of Synthetic Cannabinoids in Urine

Authors	Synthetic Cannabinoids Analysed	Sample Volume (uL)	Type of Extraction	Solvent	Buffer	Notes	Linear Range (ng/mL)
(Rigdon et al., n.d.)	JWH-018, JWH-073 Metabolites: JWH-018, JWH-073		SPE	Preconditioning: ACN followed by Ammonium acetate in 0,1% acetic acid (pH 4,2). Washing: Ammonium acetate in 0,1% acetic acid. Elution: ACN followed by butyl chloride.	Ammonium acetate, pH 5,0	Hydrolysis with beta-glucuronidase (ammonium acetate, pH 5,0) performed.	1 - 500
(Grigoryev et al., 2011)	JWH-018, JWH-073 Metabolites: JWH-018, JWH-073	LLE: 2500 SPE: 3000	LLE + SPE	LLE: HCl, followed by chloroform. SPE: Preconditioning: H_2O + ACN. Washing: 10 + 40% ACN in H_2O. Elution: ACN followed by butyl chloride.			N/A
(de Jager et al., 2012)	Metabolites: JWH-018, JWH-019, JWH-073, JWH-122, JWH-200, JWH-250, JWH-398, RCS-4	500	LLE	Diethyl ether.	Ammonium acetate, pH 5.5	Hydrolysis with beta-glucuronidase (aryl sulfatase pH 5,5) performed.	0,1 - 10
(Hutter et al., 2012)	Metabolites: JWH-018, JWH-073, JWH-081, JWH-122,	1000	LLE	t-butyl methyl ether	Phosphate, pH 6; Borate, pH 9	Hydrolysis with beta-glucuronidase performed.	N/A

	JWH-210, JWH-250, RCS-4						
(Wohlfarth et al., 2013)	JWH-018, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, RCS-4, AM-2201, MAM-2201 Metabolites: JWH-018, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, JWH-250, RCS-4, AM-2201	100	Precipitation		Ammonium acetate, pH 4,0	Hydrolysis with beta-glucuronidase performed. Precipitation with ACN.	0,5 - 10

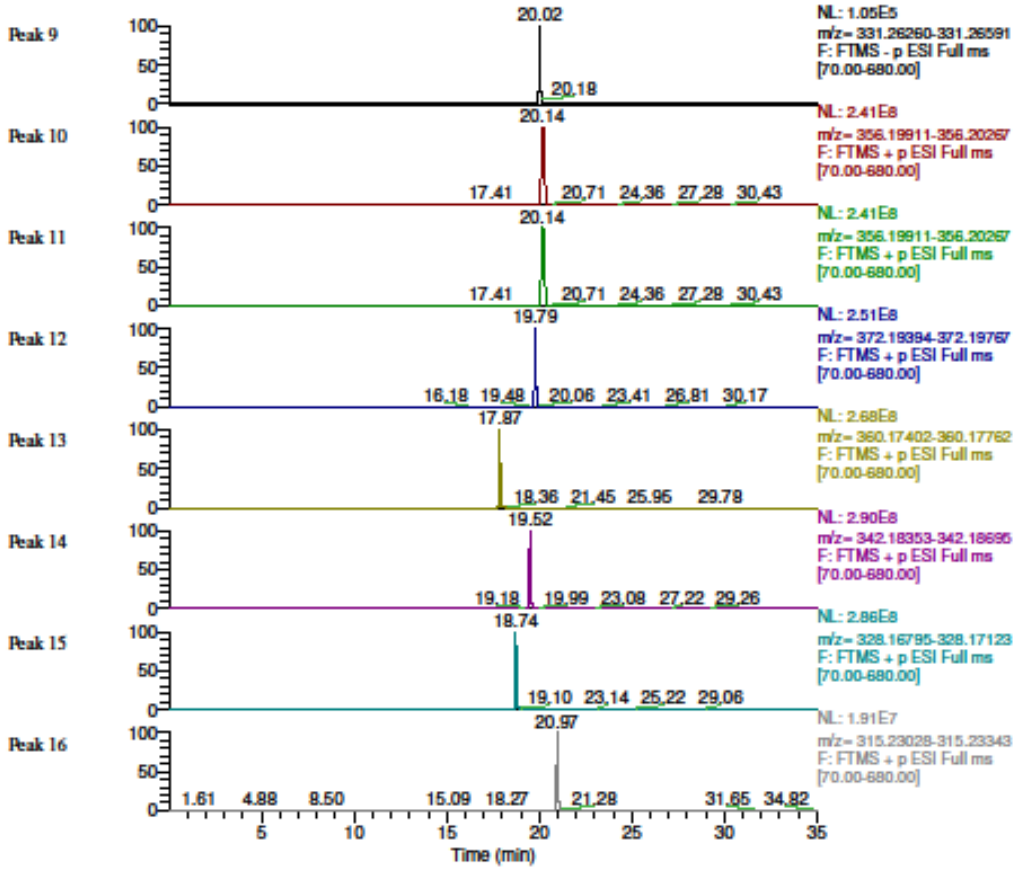
(Scheidweiler and Huestis, 2014)	JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-203, JWH-210, JWH-250, JWH-398, CP 47,497-C7, CP 47,497-C8, HU-210, RCS-4, RCS-8, AM-694, AM-2201, MAM-2201, UR-144, XLR-11 Metabolites: JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-210, JWH-250, JWH-398, RCS-4, AM-2201, MAM-2201, UR-144, CP 47,497-C7, CP 47,497-C8	SPE: 500 SLE: 200	SPE + SLE	Preconditioning: ACN followed by Ammonium acetate (pH 4,0). Washing: H_2O + ammonium acetate: ACN (80:20). Elution: 2% HAc (acetic acid) in ACN followed by hexane: ethyl acetate (90:10).	Ammonium acetate, pH 4,0	C8 and C18 SPE performed. Hydrolysis with beta-glucuronidase performed	0,05 - 50

Table A2: Comparison of Extraction Methods in Literature for the Analysis of Synthetic Cannabinoids in Whole Blood

Authors	Synthetic Cannabinoids Analysed	Sample Volume (uL)	Type of Extraction	Solvent	Buffer	Notes	Linear Range (ng/mL)
(Kacinko et al., 2011)	JWH-018, JWH-019, JWH-073, JWH-250	200	LLE	Hexane: Ethyl acetate (99:1)	Na_2CO_3, NaCl		0,1 - 20
(Ammann et al., 2012)	AM-694, AM-1241, HU-210, WIN 48,498, WIN 55,212-2 (mesylate), RCS-4, RCS-4-C-4 homolog, RCS-4 3-me homolog, RCS-4 2-me homolog, RCS-8, CP 47,497, CP 47,497-C8 homolog, JWH-007, JWH-015, JWH-018, JWH-019, JWH-030, JWH-073, JWH-081, JWH-203, JWH-210, JWH-250,	100	LLE	1-Chlorobutane + 10% isopropanol	Trizma, pH 9,2		0,5 - 100

	JWH-251, JWH-302, JWH-398						
(Shanks et al., 2012b)	JWH-018, JWH-073	500	LLE	Ethyl ether	Na_2CO_3, pH 10,2		0,05 - 50
(Holm et al., 2013)	AKB-48, AM-2201, HU-210, MAM-2201, UR-144, JWH-015, JWH-018, JWH-073, JWH-122, JWH-182, JWH-203, JWH-210, JWH-370	200	SPE	Preconditioning: 15% MeOH in ACN, followed by HAc. Washing: HAc, followed by HAc in ACN (70:30). Elution: 2% HAc in ACN.		Initial protein precipitation step (15% MeOH in ACN used).	0,25 - 10

Q Exactive Summary Report



#	Comp. Index	Compound Name	Formula	Detected m/z	Delta (ppm)	Expected RT	Actual RT	Intensity	Adducts			Fragments		
									H	2	3	1	2	3
9	9	Cannabicyclohexanol ((C8)-CP 47_497)	C22H36O2	331.26404	-0.7	20.03	20.02	104855	Y*	-	-	-	-	-
10	10	JWH-019	C25H25NO	356.20038	-1.4	20.14	20.14	241294189	Y*	-	-	-	-	-
11	11	JWH-122	C25H25NO	356.20038	-1.4	20.14	20.14	241294189	Y*	-	-	-	-	-
12	12	JWH-081	C25H25NO2	372.19528	-1.4	19.78	19.79	251165700	Y*	-	-	-	-	-
13	13	AM-2201	C24H22FNO	360.17514	-1.9	17.87	17.87	267717680	Y*	-	-	-	-	-
14	14	JWH-018	C24H23NO	342.18466	-1.7	19.51	19.52	289821150	Y*	-	-	-	-	-
15	15	JWH-073	C23H21NO	328.16898	-1.9	18.73	18.74	285640544	Y*	-	-	-	-	-
16	16	Tetrahydrocannabinol (THC)	C21H30O2	315.23138	-1.5	21.00	20.97	19142436	Y*	-	-	-	-	-

Figure A3. Example of a ToxID Report

R14/49 Mr Dale Pon

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130428

NAME: (Principal Investigator)	Mr Dale Pon
DEPARTMENT:	Division of Forensic Medicine and Pathology Medical School
PROJECT TITLE:	Synthetic Cannabinoids in Johannesburg, South Africa: A Study into their Prevalence in Post-Mortem Cases
DATE CONSIDERED:	26/04/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Guinevere Gordon
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 26/04/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Figure A4. HREC Clearance Certificate

Table A5: Table of Statistical Equations

Term	Equation
Correlation Coefficient (R)	$\frac{\sum(X_i - X_{avg})^* (Y_i - XY_{avg})}{\{\sum(X_i - X_{avg})^2 \sum(Y_i - Yavg)^2\}^{\frac{1}{2}}}$
Regression Line	$y = mx + c$
Slope (m)	$\frac{\sum(X_i - X_{avg})^* (Y_i - XY_{avg})}{\sum(X_i - X_{avg})^2}$
Intercept (b)	$Y_{avg} - bX_{avg}$
Residual Standard deviation (Standard error)	$S_{y/x} = \{\sum \frac{(Y_i - \hat{y})^2}{n-2}\}^{1/2}$
Relative Standard deviation (RSD)	$\frac{s}{\bar{x}}(100)$
Standard Deviation (s)	$\sqrt{\frac{\sum_{i=l}^{n}(X_i - \overline{x})^2}{n-1}}$
Mean	$\frac{\sum X_i}{n}$

Table A6: Linearity of the Analytes in Urine

Drugs		Retention time(min)	Slope (m)	y-Intercept	R^2 Value	No. of Calibrators
JWH-018	Day 1	19,50	0,3079	-0,006	0,999	7
	Day 2	19,45	0,04095	0,029	0,980	5
	Day 3	19,45	0,04925	-0,005	0,996	7
	Day 4	19,48	0,04648	-0,001	0,998	7
	Average	19,47	0,1112	0,004	0,993	6,5
JWH-019	Day 1	20,15	0,2552	-0,025	0,996	7
	Day 2	20,11	0,03463	0,009	0,990	5
	Day 3	20,10	0,03868	-0,007	0,995	7
	Day 4	20,12	0,03901	-0,010	0,991	5
	Average	20,12	0,09188	-0,008	0,993	6
JWH-073	Day 1	18,72	0,2249	0,006	0,999	7
	Day 2	18,67	0,03865	0,002	0,996	7
	Day 3	18,67	0,03844	-0,001	0,998	7
	Day 4	18,70	0,04040	0,000	0,991	7
	Average	18,69	0,0856	0,002	0,996	7
JWH-081	Day 1	19,78	0,2467	-0,011	0,999	7
	Day 2	19,72	*	*	*	*
	Day 3	19,73	0,03659	-0,003	0,998	7
	Day 4	19,75	0,03466	-0,006	0,995	5
	Average	19,75	0,1060	-0,007	0,997	6,33
JWH-122	Day 1	20,26	0,2525	-0,017	0,999	7
	Day 2	20,21	0,0352	0,008	0,991	7
	Day 3	20,22	0,03696	-0,007	0,994	7
	Day 4	20,24	0,03410	0,001	0,991	5
	Average	20,23	0,08969	-0,004	0,994	6,5
JWH-200	Day 1	12,59	0,1137	0,006	0,998	7
	Day 2	12,58	0,01324	0,004	0,990	6
	Day 3	12,61	0,01637	-0,003	0,996	6
	Day 4	12,65	0,02958	0,006	0,990	5
	Average	12,61	0,04322	0,003	0,994	6
JWH-250	Day 1	18,45	0,5154	0,000	0,999	7
	Day 2	18,44	0,07372	0,010	0,997	7
	Day 3	18,44	0,07727	-0,004	0,998	6
	Day 4	18,46	0,08696	0,018	0,989	5
	Average	18,45	0,1883	0,006	0,996	6,25
AM-2201	Day 1	17,86	0,2703	-0,008	0,999	7
	Day 2	17,81	0,04129	0,013	0,991	6
	Day 3	17,81	0,04845	-0,009	0,990	6
	Day 4	17,84	0,04722	0,011	0,988	5
	Average	17,83	0,10182	0,002	0,992	6

(±)-CP 47,497	Day 1	19,13	0,000113	0,000	0,992	6
	Day 2	19,08	7,88E-06	0,000	0,973	4
	Day 3	19,08	*	*	*	*
	Day 4	19,11	*	*	*	*
	Average	19,10	6,044E-05	0,000	0,983	5
(C8)-CP 47,497	Day 1	20,03	0,0001243	0,000	0,994	6
	Day 2	19,95	*	*	*	*
	Day 3	19,98	*	*	*	*
	Day 4	20,00	*	*	*	*
	Average	19,99	0,0001243	0,000	0,994	5
HU-211	Day 1	20,37	0,005749	0,000	0,992	7
	Day 2	20,29	0,0005564	0,000	0,992	7
	Day 3	20,32	0,0007053	0,000	0,990	7
	Day 4	20,33	0,0006792	0,000	0,993	7
	Average	20,33	0,001923	0,000	0,992	7
THC	Day 1	20,99	0,01757	0,000	0,996	7
	Day 2	20,96	0,001515	0,001	0,999	7
	Day 3	20,96	0,001912	-0,001	0,992	5
	Day 4	20,98	0,001707	0,000	0,984	5
	Average	20,97	0,005676	0,000	0,993	6
11-OH-THC	Day 1	17,61	0,009588	0,000	0,997	7
	Day 2	17,58	0,001009	0,000	0,996	5
	Day 3	17,55	0,001073	0,000	0,980	5
	Day 4	17,58	*	*	*	*
	Average	17,58	0,003890	0,000	0,991	5,67
JWH-018 N-4OH pentyl metabolite	Day 1	15,45	0,2072	-0,001	0,999	7
	Day 2	15,38	0,02638	0,004	0,995	6
	Day 3	15,38	0,02948	-0,008	0,987	6
	Day 4	15,42	0,04321	0,012	0,991	5
	Average	15,41	0,07657	0,002	0,993	6
JWH-073 N-3OH butyl metabolite	Day 1	15,28	0,1582	0,007	0,999	7
	Day 2	15,21	0,02446	0,000	0,999	6
	Day 3	15,21	0,02563	-0,005	0,994	6
	Day 4	15,25	*	*	*	*
	Average	15,24	0,06943	0,001	0,997	6,33

* indicates results that did not meet the validation criteria.

Table A7: Linearity of the Analytes in Whole Blood

Drugs		Retention time(min)	Slope (m)	y-Intercept	R^2 Value	No. of Calibrators
JWH-018	Day 1	19,38	0,05167	-0,023	0,998	6
	Day 2	19,37	0,04667	-0,007	0,999	6
	Day 3	19,37	0,04475	-0,019	0,992	7
	Day 4	19,38	0,05127	-0,025	0,999	6
	Average	19,38	0,04859	-0,019	0,997	6,25
JWH-019	Day 1	20,02	0,03684	-0,019	0,998	6
	Day 2	20,00	0,03521	-0,008	0,999	6
	Day 3	20,02	*	*	*	*
	Day 4	20,02	0,03685	-0,020	0,998	6
	Average	20,02	0,0363	-0,016	0,998	6
JWH-073	Day 1	18,61	0,08901	-0,029	1,000	6
	Day 2	18,59	0,03598	0,038	0,983	6
	Day 3	18,61	0,001907	-0,002	0,994	5
	Day 4	18,61	0,04364	-0,015	0,999	6
	Average	18,61	0,04263	-0,002	0,994	5,75
JWH-081	Day 1	19,66	0,04886	-0,016	0,998	6
	Day 2	19,64	*	*	*	*
	Day 3	19,64	0,0005064	0,000	0,990	6
	Day 4	19,66	0,04752	-0,014	0,998	6
	Average	19,65	0,0323	-0,010	0,995	6
JWH-122	Day 1	20,14	0,0345	-0,021	0,998	6
	Day 2	20,13	0,03147	-0,004	0,998	6
	Day 3	20,11	0,0002501	0,000	0,982	5
	Day 4	20,14	0,03448	-0,022	0,998	6
	Average	20,13	0,02518	-0,012	0,994	5,75
JWH-200	Day 1	12,49	0,06159	-0,068	0,973	6
	Day 2	12,76	*	*	*	*
	Day 3	12,86	*	*	*	*
	Day 4	12,49	0,05864	-0,063	0,971	6
	Average	12,65	0,06012	-0,066	0,972	6
JWH-250	Day 1	18,37	0,09275	-0,033	0,997	6
	Day 2	18,35	0,09478	-0,045	0,998	5
	Day 3	18,37	0,000637	-0,001	0,994	6
	Day 4	18,37	0,09111	-0,032	0,997	6
	Average	18,37	0,06982	-0,028	0,997	5,75
AM-2201	Day 1	17,75	0,06537	-0,031	0,995	6
	Day 2	17,74	0,05619	0,010	0,986	6
	Day 3	17,74	0,001718	-0,005	0,982	5
	Day 4	17,75	0,06366	-0,028	0,994	6
	Average	17,75	0,04674	-0,014	0,989	6

(±)-CP 47,497	Day 1	19,01	6,945E-05	0,000	0,988	6
	Day 2	18,99	0,0001203	0,000	0,997	5
	Day 3	*	*	*	*	*
	Day 4	19,01	6,545E-05	0,000	0,989	6
	Average	19,00	8,507E-05	0,000	0,991	5,67
(C8)-CP 47,497	Day 1	19,90	*	*	*	*
	Day 2	19,88	*	*	*	*
	Day 3	*	*	*	*	*
	Day 4	19,90	*	*	*	*
	Average	19,89	*	*	*	*
HU-211	Day 1	20,25	0,0005673	0,000	0,995	6
	Day 2	20,24	0,0006534	-0,001	0,997	5
	Day 3	*	*	*	*	*
	Day 4	20,25	0,0005635	0,000	0,997	6
	Average	20,25	0,0005947	0,000	0,996	5,67
THC	Day 1	20,87	0,001917	-0,001	0,999	6
	Day 2	20,84	0,001672	-0,002	0,989	5
	Day 3	*	*	*	*	*
	Day 4	20,87	0,001931	-0,001	0,999	6
	Average	20,86	0,00184	-0,001	0,996	5,67
11-OH-THC	Day 1	17,53	0,001846	-0,001	0,991	6
	Day 2	17,50	0,001196	0,000	0,997	6
	Day 3	*	*	*	*	*
	Day 4	17,53	0,001679	0,000	0,996	6
	Average	17,52	0,001574	0,000	0,995	6
JWH-018 N-4OH pentyl metabolite	Day 1	15,36	0,06125	-0,028	0,991	6
	Day 2	15,34	0,04054	0,014	0,992	5
	Day 3	15,37	*	*	*	*
	Day 4	15,36	0,06055	-0,028	0,989	6
	Average	15,36	0,05411	-0,014	0,991	5,67
JWH-073 N-3OH butyl metabolite	Day 1	15,18	0,04218	-0,014	0,998	6
	Day 2	15,17	0,03036	0,023	0,992	6
	Day 3	15,16	0,0004981	-0,001	0,989	6
	Day 4	15,17	0,04091	-0,012	0,998	6
	Average	15,17	0,02849	-0,001	0,994	6

* indicates results that did not meet the validation criteria.

Table A8: Accuracy and Precision of the High QCs of the Analytes in Urine

Drugs		Mean (ng/mL)	Accuracy %	SD	% RSD	CV
JWH-018	Day 1	4,517	100	0,088	1,953	0,020
	Day 2	5,045	112	0,769	15,237	0,152
	Day 3	4,504	100	0,512	11,366	0,114
	Average	4,688	104	0,456	9,519	0,095
JWH-019	Day 1	4,375	97	0,137	3,120	0,031
	Day 2	4,919	109	0,659	13,398	0,134
	Day 3	4,731	105	0,531	11,226	0,112
	Average	4,675	104	0,442	9,248	0,092
JWH-073	Day 1	4,924	109	0,361	7,324	0,073
	Day 2	4,840	108	0,530	10,957	0,110
	Day 3	4,601	102	0,109	2,367	0,024
	Average	4,788	106	0,333	6,883	0,069
JWH-081	Day 1	4,377	97	0,155	3,533	0,035
	Day 2	*	*	*	*	*
	Day 3	4,816	107	0,437	9,074	0,091
	Average	4,597	102	0,296	6,304	0,063
JWH-122	Day 1	4,573	102	0,164	3,579	0,036
	Day 2	4,998	111	0,553	11,064	0,111
	Day 3	4,803	107	0,616	12,824	0,128
	Average	4,791	106	0,444	9,156	0,092
JWH-200	Day 1	4,434	99	0,218	4,909	0,049
	Day 2	*	*	*	*	*
	Day 3	*	*	*	*	*
	Average	4,434	99	0,218	4,909	0,049
JWH-250	Day 1	4,380	97	0,318	7,253	0,073
	Day 2	*	*	*	*	*
	Day 3	4,451	99	0,165	3,702	0,037
	Average	4,415	98	0,241	5,478	0,055
AM-2201	Day 1	4,537	101	0,071	1,555	0,016
	Day 2	*	*	*	*	*
	Day 3	3,996	89	0,671	16,795	0,168
	Average	4,266	95	0,371	9,175	0,092

(±)-CP 47,497	Day 1	4,577	101,711	0,563	12,311	0,123
	Day 2	*	*	*	*	*
	Day 3	*	*	*	*	*
	Average	*	*	*	*	*
(C8)-CP 47,497	Day 1	4,227	93,941	0.065	1,526	0,015
	Day 2	*	*	*	*	*
	Day 3	*	*	*	*	*
	Average	*	*	*	*	*
HU-211	Day 1	4,663	104	0,434	9,318	0,093
	Day 2	5,405	120	0,129	2,379	0,024
	Day 3	4,587	102	0,161	3,500	0,035
	Average	4,885	109	0,241	5,066	0,051
THC	Day 1	4,347	97	0,229	5,272	0,053
	Day 2	4,527	101	0,348	7,679	0,077
	Day 3	4,845	108	0,517	10,670	0,107
	Average	4,573	102	0,365	7,873	0,079
11-OH-THC	Day 1	4,582	102	0,673	14,698	0,147
	Day 2	*	*	*	*	*
	Day 3	4,279	95	0,195	4,561	0,046
	Average	4,430	98	0,434	9,629	0,096
JWH-018 N-4OH pentyl metabolite	Day 1	4,714	105	0,241	5,109	0,051
	Day 2	*	*	*	*	*
	Day 3	4,507	100	0,575	12,757	0,128
	Average	4,610	102	0,408	8,933	0,089
JWH-073 N-3OH butyl metabolite	Day 1	4,783	106	0,302	6,305	0,063
	Day 2	*	*	*	*	*
	Day 3	3,871	86	0,674	17,410	0,174
	Average	4,327	96	0,488	11,858	0,119

* indicates results that did not meet the validation criteria.

Table A9: Accuracy and Precision of the High QCs of the Analytes in Whole Blood

Drugs		Mean (ng/mL)	Accuracy %	SD	% RSD	CV
JWH-018	Day 1	14,045	104	1,570	11,180	0,112
	Day 2	14,386	107	0,654	4,549	0,045
	Day 3	14,236	105	0,598	4,204	0,042
	Day 4	12,315	91	1,232	10,007	0,100
	Average	13,745	102	1,014	7,485	0,075
JWH-019	Day 1	13,369	99	1,889	14,132	0,141
	Day 2	14,083	104	0,650	4,613	0,046
	Day 3	14,561	108	1,010	6,940	0,069
	Day 4	13,374	99	1,925	14,394	0,144
	Average	13,846	103	1,369	10,020	0,100
JWH-073	Day 1	13,547	100	1,586	11,707	0,117
	Day 2	14,772	109	0,530	3,588	0,036
	Day 3	13,876	103	0,667	4,810	0,048
	Day 4	12,922	96	1,395	10,798	0,108
	Average	13,779	102	1,045	7,726	0,077
JWH-081	Day 1	13,889	103	1,064	7,661	0,077
	Day 2	13,916	103	2,691	19,339	0,193
	Day 3	16,096	119	0,812	5,042	0,050
	Day 4	13,967	103	0,899	6,440	0,064
	Average	14,467	107	1,367	9,621	0,096
JWH-122	Day 1	13,397	99	1,363	10,175	0,102
	Day 2	13,263	98	1,487	11,214	0,112
	Day 3	13,293	98	1,888	14,201	0,142
	Day 4	13,487	100	1,344	9,966	0,100
	Average	13,360	99	1,521	11,389	0,114
JWH-200	Day 1	12,273	91	1,388	11,306	0,113
	Day 2	12,287	91	0,317	2,584	0,026
	Day 3	13,709	102	1,335	9,737	0,097
	Day 4	12,664	94	1,396	11,025	0,110
	Average	12,733	94	1,109	8,663	0,087
JWH-250	Day 1	12,549	93	1,727	13,759	0,138
	Day 2	12,053	89	0,913	7,577	0,076
	Day 3	13,719	102	0,425	3,099	0,031
	Day 4	12,684	94	1,960	15,450	0,154
	Average	12,751	94	1,256	9,971	0,100
AM-2201	Day 1	11,861	88	1,511	12,742	0,127
	Day 2	13,283	98	1,738	13,081	0,131
	Day 3	10,151	75	0,129	1,268	0,013
	Day 4	12,163	90	1,498	12,314	0,123
	Average	11,865	88	1,219	9,851	0,099
(±)-CP 47,497	Day 1	*	*	*	*	*
	Day 2	*	*	*	*	*
	Day 3	21,898	162	3,104	14,174	0,142
	Day 4	14,807	110	1,526	10,306	0,103
	Average	18,353	136	2,315	12,240	0,123

(C8)-CP 47,497	Day 1	12,983	96	0,720	5,543	0,055
	Day 2	20,138	149	2,612	12,971	0,130
	Day 3	*	*	*	*	*
	Day 4	12,644	94	0,536	4,238	0,042
	Average	16,561	123	1,666	9,257	0,093
HU-211	Day 1	13,963	103	2,205	15,789	0,158
	Day 2	13,491	100	1,719	12,741	0,127
	Day 3	*	*	*	*	*
	Day 4	14,047	104	2,136	15,207	0,152
	Average	13,834	102	2,020	14,579	0,146
THC	Day 1	14,158	105	2,314	16,346	0,163
	Day 2	14,266	106	1,072	7,516	0,075
	Day 3	*	*	*	*	*
	Day 4	14,207	105	2,281	16,055	0,161
	Average	14,210	105	1,889	13,306	0,133
11-OH-THC	Day 1	13,184	98	1,931	14,647	0,146
	Day 2	14,512	107	0,918	6,325	0,063
	Day 3	*	*	*	*	*
	Day 4	13,349	99	2,085	15,618	0,156
	Average	13,682	101	1,645	12,197	0,122
JWH-018 N-4OH pentyl metabolite	Day 1	12,759	95	1,590	12,463	0,125
	Day 2	13,470	100	0,624	4,630	0,046
	Day 3	12,224	91	0,471	3,851	0,039
	Day 4	12,866	95	1,523	11,840	0,118
	Average	12,830	95	1,052	8,196	0,082
JWH-073 N-3OH butyl metabolite	Day 1	12,550	93	1,667	13,281	0,133
	Day 2	*	*	*	*	*
	Day 3	12,885	95	0,877	6,804	0,068
	Day 4	12,802	95	1,875	14,648	0,146
	Average	12,745	94	1,473	11,577	0,116

* indicates results that did not meet the validation criteria.

Figure A10. Turnitin Originality Report

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