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Thesis

DETECTION AND QUANTITATION OF 17 SYNTHETIC CANNABINOIDS IN HUMAN WHOLE BLOOD USING LC-MS/MS FOLLOWING SUPPORTED LIQUID EXTRACTION

by

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B.S., California State University, Los Angeles, 2014

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Master of Science

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DETECTION AND QUANTITATION OF 17 SYNTHETIC CANNABINOIDS IN HUMAN WHOLE-BLOOD USING LC-ESI+/MS/MS FOLLOWING SUPPORTED LIQUID EXTRACTION

DANIEL LEE

ABSTRACT

Synthetic cannabinoids have become a growing concern in society. The extensive list of synthetic cannabinoids and the abuse rate has drawn the attention by government agencies throughout the world. These synthetic cannabinoids can adopt a number of different structures, while still acting on endogenous cannabinoid (CB1 and CB2) receptors. In addition, due to structural modifications of these synthetic cannabinoids, many of these compounds can bind to CB1 and CB2 receptors with greater affinity causing severe adverse and life-threatening effects. Because of their structural dissimilarity to the phytocannabinoid Δ^{9-}THC, combating the rapid growth and emergence of synthetic cannabinoids with conventional THC-based methods has become an ongoing struggle.

The purpose of this research was to develop and validate a robust and reliable method to accurately identify and quantify 17 synthetic cannabinoids in human whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method was validated in accordance to SWGTOX guidelines for quantitative analysis using the following analytes: 4-cyano-CUMYL-BUTINACA, 5F-3,5-ABPFUPPYCA, 5F-ADB-PINACA, 5F- PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUNICACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, and XLR11.

With this developed method, total analysis time was 8 minutes with samples eluting from 3.8 to 5.8 minutes. Calibration curves for each analyte had acceptable R^2 values > 0.98 using a weighting factor of 1/x. A linear dynamic range of 0.5 – 25 ng/mL was used for all analytes, except for APP-PICA, XLR11 and NM2201 which were quantifiable at 0.1 ng/mL and PB-22 which used a quadratic model. Extraction of analytes using supported liquid extraction (SLE) cartridge improved sample-prep time by more than half, compared to traditional solid phase extraction (SPE) methods. Percent recovery of analytes using SLE was determined to be from 54.92 to 83.36%. Bias and Precision was assessed at 1, 3, 7, and 20 ng/mL for all analytes. All samples had acceptable calculated percent bias and percent coefficient of variation (%CV) within ±20%. No carryover was observed with this method. Matrix effect, using 10 different sources, did not have any interfering effects on detection and quantification of analytes. Ionization suppression and enhancement was observed at various levels, from -4.47 to 76.67%, but had little effect on other validation parameters. Analysis of other commonly encountered drugs (clonazepam, diazepam, (+) methadone, morphine, fentanyl, cocaine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 25I-NBOMe, and phencyclidine (PCP)) does not show any source of interference.

The overall development and validation of this method demonstrates a sensitive and reliable way to positively identify 17 different synthetic cannabinoids in human whole blood in rapid time.

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LIST OF ABBREVIATIONS

Δ^9-THC	Tetrahydrocannabinol
AAPCC	American Association of Poison Control Centers
ACN	Acetonitrile
AIDS	Acquired Immune Deficiency Syndrome
API	Atmospheric Pressure Ionization
cAMP	Cyclic Adenosine Monophosphate
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CNS	Central Nervous System
CSA	Controlled Substance Act
CV	Coefficient of Variation
CYP450	Cytochrome P450
Da	Dalton
DC	Direct Current
DEA	Drug Enforcement Administration
ELISA	Enzyme-linked Immunosorbent Assay
ESI	Electrospray Ionization
ESI+	Electrospray Ionization in Positive Mode
FA	Formic Acid
FDA	Food & Drug Administration
H_2O	Water

HEIA	Homogenous Enzyme Immunoassay
LC	Liquid Chromatography
LC-HRMS	Liquid Chromatography – High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography – Tandem Mass Spectrometry
LC-TOF/MS	Liquid Chromatography – Time of Flight Mass Spectrometry
LLE	Liquid-liquid Extraction
µg	Microgram
MDMA	3,4-Methylenedioxymethamphetamine
MeOH	Methanol
mg	Milligram
Min	Minutes
mL	Milliliter
Msec	Millisecond
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
M/Z	Mass-to-charge Ratio
NaOH	Sodium Hydroxide
NFLIS	National Forensic Laboratory Information System
ng	Nanogram
NIDA	National Institute of Drug Abuse
NPS	Novel Psychoactive Substances
PNS	Peripheral Nervous System

QC	Quality Control
RF	Radio Frequency
SALLE	Salting-out Liquid-liquid Extraction
t_R	Retention Time
TMS	Trimethylsilyl
TOF	Time of Flight
UGT’s	UDP-glucuronosyltransferase
U.S.	United States
U.K.	United Kingdom
USA	United States of America
USD	United States Dollar
V	Volts

1. Introduction

1.1 Background and Chemistry

Cannabinoids have been one of the oldest and longest abused substances in our society. Prior to the Controlled Substances Act of 1970, cannabis has been used for thousands of years to treat medical ailments.[1] There are currently 29 states that have adopted its legal medical applications.[2] Today, there are numerous applications for medical marijuana. Medical marijuana is currently used to help nausea and vomiting in chemotherapy patients, increase appetite or used for analgesic effects for acquired immune deficiency syndrome (AIDS) patients and patients with multiple sclerosis experiencing mild to moderate pain.[3,4] With the increasing demand for medical applications of cannabis and to better understand endogenous cannabinoid receptors came the emergence of synthetic cannabinoids.[5,6,7]

There are other potential research areas for medical marijuana including bronchodilation from asthma, reduction of intraocular pressure in glaucoma patients, or potentially used as anticonvulsants, anxiolytic, analgesic, or anti-inflammatory agents.[4] Synthetic cannabinoids are a class of synthetic compounds known to produce marijuana-like effects by acting on endogenous cannabinoid CB1 and CB2 receptors, hence are known to have cannabimimetic properties.[8] Unlike most other novel psychoactive substances (NPS) that are analogs or isomeric forms of their natural-producing counterpart, synthetic cannabinoids may take on a completely different structure compared to the phytocannabinoid molecule, tetrahydrocannabinol (Δ^9-THC).[9,10] It is important to note that synthetic cannabinoids can act on receptors producing cannabinoid effects (full agonist)

but there is a class of synthetic cannabinoids that can also act as antagonists; blocking receptor-activity without any cannabimimetic effects.[11] For the purposes of this transcript, we will focus primarily on synthetic cannabinoids known to have agonistic properties.

1.2 Recreational Use

1.2.1 Synthetic Cannabinoids as Designer Drugs

Designer drugs, also known as NPS, have been synthesized to provide a desired physiological and psychological effects, often similar to that of a federally controlled substance.[12,13] NPS compounds are sought out from users as an alternative method for recreational use without the fear of legal repercussions. Like some NPS, newly emerging synthetic cannabinoids are not currently scheduled under the Controlled Substances Act (CSA); therefore they are marketed as a 'legal highs.'[12] There are also many classes of synthetic cannabinoids that are structurally dissimilar to the phytocannabinoid Δ^{9}-THC which is how these NPS are able to circumvent current drug tests.[9] In addition, due to their full agonistic effects, synthetic cannabinoids have greater potency compared to Δ^{9}-THC, which poses another issue of low doses and concentrations in the body, making toxicological analysis difficult.

1.2.2 History and Scheduling

Δ^{9}-THC, the major psychoactive ingredient, was discovered and isolated in the plant species *Cannabis sativa* in 1960's.[12,14] In 1970, cannabis was placed on the CSA as a Schedule I drug, restricting its recreational and legal applications.[15] Although cannabis

was federally scheduled, many states approved its legal applications of medical marijuana. In the Late 1970's approximately 24 states approved legislation (The Controlled Substances Therapeutic Research Act) to circumvent the federal regulations set forth by the Controlled Substances Act of 1970.[16] In the 1980s, a synthetic cannabinoid (dronabinol) was approved by the Food & Drug Administration (FDA) to treat nausea and vomiting for chemotherapy patients.[4] A Belgian company, Solvay Pharmaceuticals began distributing and marketing the synthetic cannabinoid dronabinol under the trademark, Marinol.[4]

Soon after the emergence of dronabinol, researchers began synthesizing cannabinoids to better understand endogenous cannabinoid receptors.[5,6] One of the earliest synthesized cannabinoids was HU-210, which was synthesized in 1988 by Raphael Mechoulam from Hebrew University (HU-compounds).[12,17] John W. Huffman, a professor from Clemson University is also prized with synthesizing hundreds of synthetic cannabinoids commonly known as JWH-compounds.[6] Others include Pfizer who synthesized the cyclohexylphenol series (CP-compounds) and Alexandros Markriyannis who synthesized the AM-compounds.[18,19]

In the early 2000's, synthetic cannabinoids began being sold and distributed online in European countries, marketed under the name 'Spice' (also known as K2) in specialized smoke-shops (headshops) and online markets.[20] Prior to government scrutiny, these Spice-products were readily available online, being sold without any age restrictions, and sold as 'legal highs' or 'herbal highs'.[12,21] Of the earliest known emergence of Spice (K2) was sold in 2004, predominantly in European countries. By 2008, synthetic cannabinoids gained

enormous popularity in many European countries (Austria, Germany, France, Poland and Sweden).[12] In that same year, two major compounds synthetic cannabinoids (CP-47,497 and JWH-018) were identified as the psychoactive ingredient in Spice (K2) products.[22,23]

In early 2009, high abuse rate of Spice (K2) products gained the attention of the European Monitoring Centre for Drugs and Drug Addiction's (EMCDDA) early warning system on newly emerging drugs.[24] Shortly after the early detection of synthetic cannabinoids, growing concerns from the Advisory Council on the Misuse of Drugs (ACMD)[25] forced the European government to make amendments to the Misuse of Drugs Act 1971 where they classified synthetic cannabinoids as a controlled substance in the United Kingdom (U.K.).[26] These compounds or any Spice-related products were restricted by the European government and no longer accessible through headshops and online-markets.[12] However, the recent amendments did not deter clandestine laboratories from manufacturing and distributing; instead, various other synthetic cannabinoids not yet classified have been substituted in Spice products. In 2009 the synthetic cannabinoid HU-210 was identified.[27] In 2010, JWH-015 was identified in a spice-related product called 'Topaz' along with other newly emerged derivative compounds, JWH-122, which was a methyl-derivative of JWH-018.[28]

As synthetic cannabinoids grew in popularity, spice-related products eventually made its way into the United States (U.S.) in 2008; advertised online as 'K2' and other various names (e.g. Spice, Spice Gold, Spice Diamond, Dream, Silver, and Genie).[11,29] The first known appearance of synthetic cannabinoids in the U.S. was in Dayton, Ohio.[30] in late 2008, U.S. Customs and Border Protection noticed large shipments of Spice (K2) products

being shipped into the USA.[29] The increasing popularity of synthetic cannabinoids in the U.S. raised concerns in various states including Alabama, Arkansas, Georgia, Kansas, Kentucky, and Missouri. In November 2010, the U.S. Drug Enforcement Administration (DEA) temporarily placed five synthetic cannabinoids (JWH-018, JWH-073, JWH-200, CP-47,497 and CP-47,497 C8 homologues) as Schedule I substances; and permanently scheduled in 2011.[31] By 2011, Spice (K2)-related products have been reported all around the world; predominantly in the UK, USA, Germany, Japan, Austria, Ireland and Latvia).[32]

Due to a plethora of different synthetic cannabinoids, once a cannabinoid compound is scheduled, a different synthetic cannabinoid can easily replace the recently scheduled compound. This endless cycle of newly emerging synthetic cannabinoids is a growing concern for the community and local law enforcements.

1.2.3 Marketing, Use and Distribution

Synthetic cannabinoids are produced in laboratories and are dissolved in a solvent, which can then be sprayed onto non-psychoactive plant-matter, dried and packaged for distribution.[12,33] Most typical plant materials used in with synthetic cannabinoids are Damiana (*Turnera diffusa*), Marshmallow (*Althaea officinalis*), Mugwort (*Artemisia vulgaris*) or Mullein (*Verbascum thapsus*).[34] Spice (K2) has also been sold in powder or liquid forms.[35] In both Europe and U.S., a typical 3-gram dried plant material typically costs €20–€30 Euros, and $30–$40 U.S. Dollars (USD), respectively.[36] The dried leaf products sprayed with synthetic cannabinoids can eventually be smoked using a pipe or rolled with cigarette paper.[37,36] In a worldwide survey conducted by Vandrey *et al.*

including 13 countries and 42 U.S. States, majority of self-administered individuals favored the smoking routes of administration (via pipe, bong and cigarette paper) but also included oral, rectal, and vaporized via e-cigarette routes of administration.[38]

What first started out as 'Legal Highs,' easily accessible without any age restrictions with specific labels 'K2' or 'Spice' soon turned into something much more dangerous.[12,21] Due to government scrutiny and scheduling of the first five synthetic cannabinoids (JWH-073, JWH-200, CP-47,497 and CP-47,497-C8 homologue),[39] many headshops and online-markets began advertising and packaging spice-related products under different names such as 'Insense,' 'fragrance' and 'potpourri' with additional warning labels, 'not for human consumption' in order to circumvent legal regulations and detection.[4,36] These Spice (K2) products with mis-labelled packaging, without any indication of type active-ingredients,[36] cause users to speculate the actual concentration of Spice (K2) products; often taking more than a person should consume. In 2011, the National Institute on Drug Abuse (NIDA) reports indicated that synthetic cannabinoids were second highest abused substance, just after cannabis.[40]

The growing use of Spice (K2) increased at an alarming rate. According to the National Poison Data System from the American Association of Poison Control Centers' (AAPCC), in 2009 there were 112 calls of exposure to Spice (K2). The number of callers increased to 2,915 in 2010,[41] 11,561 calls in 2012,[42] 32,653 in 2013, 33,653 in 2014, and 29,588 in 2015.[43]

The other concern is also the target consumers. Due to its easy accessibility, Spice (K2) products are used amongst the youth in the U.S.[36] A survey conducted in 2011 from

hundreds of high schools nationwide showed approximately 11.4% of 12th grade seniors, 8.8% of 10th graders, and 4.4% of 8th graders admitted to using synthetic cannabinoids.[44] Although the recreational use of synthetic cannabinoids has subsided in recent years, the National Forensic Laboratory Information System (NFLIS) annual reports indicate that two synthetic cannabinoid (XLR11 and AB-CHMINACA) are among the top 25 substances that are still currently being abused throughout the U.S.[45]

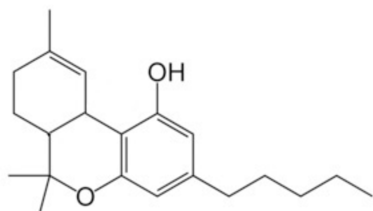
The most concerning aspect of recreational use of Spice (K2) products are the potential active ingredients. It has been reported that Spice-products may not only contain synthetic cannabinoids as its primary active ingredient, but may also contain other NPSs (e.g. synthetic opioids).[46] Other concerns of Spice products include hypnotic-inducing ingredients, or even a combination of numerous synthetic cannabinoids present in a single Spice product.[47,48] The active ingredients found in Spice products can vary greatly depending on the country of origin.[12] The ambiguity of listed active ingredients or lack-there-of in Spice (K2) products is another concern for the general public.

1.3 Chemical structure

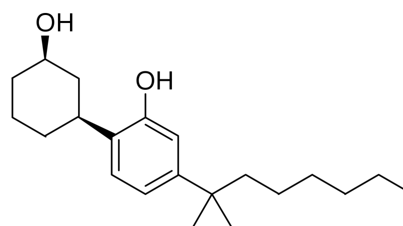
1.3.1 Structure of Synthetic Cannabinoids

There are seven major structural groups that synthetic cannabinoids can be classified under. Napthoylindoles (e.g. JWH-018), Napthylmethylindoles, Naphthopyrroles, Naphthylmethylindenes, Phenylacetylindoles (e.g. JWH-250), Cyclohexylphenols (e.g. CP47,497), and Classical cannabinoids (e.g. HU-210).[49] The commonly abused synthetic cannabinoids, found in Spice (K2)-products can be classified into four major categories

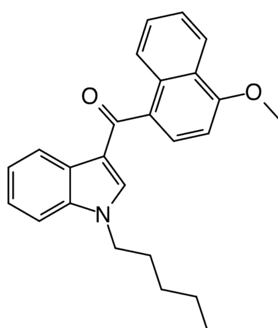
known as Classical cannabinoids (e.g. HU-210), cyclohexylphenols (e.g. CP-47,497), phenylacetylindoles (e.g. JWH-250) , and naphthoylindoles (e.g. JWH-018).[9,10] The structures of these four major categories are shown in Figure 1.



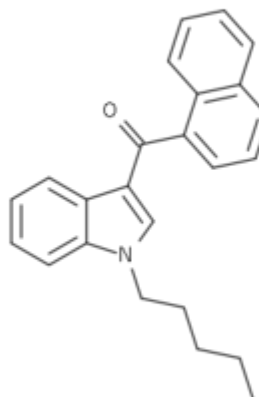
1) Classical Cannabinoid (HU-210)



2) Cyclohexylphenol (CP-47,497)



3) Phenylacetylindoles (JWH-250)



4) Napthoylindoles (JWH-018)

<u>Figure 1</u>. Major Structures. Four commonly abused synthetic cannabinoid core structures are 1) classical cannabinoids, 2) cyclohexylphenols, 3) phenylacetylindoles, and 4) naphthoylindoles.

1.4 Cannabinoid Receptors (CB1 and CB2)

1.4.1 Properties of CB1 and CB2 Receptors

The earliest known discovery of cannabinoid (CB1) receptors was in 1984 by Howlett *et al*. demonstrating that cannabinoid activity decreased cyclic adenosine monophosphate (cAMP) concentrations in the brain, primarily in the neuroblastoma cells.[50] CB1 receptors have been determined to be a part of the rhodopsin-family of G-protein-coupled receptors (GPCR).[51] GPCR's, in general, are characterized by a transmembrane-unit with alpha helices coupled with several G-proteins (Gα, Gβ and Gγ), which produces a cascade effects upon activation by a protein ligand.[52] The initial discovery of CB receptors were later confirmed in rat brain in 1988 and successfully cloned from rat tissues in 1990.[53] The discovery of CB1 receptors allowed researchers to understand the pharmacological effects of cannabinoid agonists; the CB1 activity in the basal ganglia and cerebellum have been proven to be associated with the person's gait, and activity in the cerebral cortex and hippocampus have been proven to be associated with effects on cognition and memory.[54]

Upon studying the CB1 receptors further, came the discovery of CB2 receptors. In 1993, studying the immunomodulatory effects of cannabis enabled researchers to identify and isolate a second class of cannabinoid receptors, the CB2 receptor.[55] Although, CB1 and CB2 receptors share nearly half of its protein sequence, CB1 receptors have been isolated predominantly in the central nervous system (CNS) with minor peripheral nervous system (PNS)-activity.[56,57] For many year, CB2 receptors have been known as PNS cannabinoid receptors due to its high spleen and immune cell activity and absence of

expression in the brain.[58] It was later determined in 2005, that both CB1 and CB2 receptors are localized in both the CNS and PNS.[59] At this point, there is now convincing evidence of two types of cannabinoid receptors, CB1 and CB2 receptors.

1.5 **Pharmacology of Synthetic Cannabinoids**

Although metabolic studies involving synthetic cannabinoids began in the early 2000's, it was not studied in depth until 2010, when an alarming rate of exposures were reported by the AAPCC.[60] However, due to the recent emergence, the diverse chemical compounds, and the rapid introduction of differing synthetic cannabinoids, pharmacological studies are limited to only a handful number of synthetic compounds. Of the several hundreds of different synthetic cannabinoids available, recent pharmacological studies include AB-001, AM694, AM2201, CP55,940, JWH-015, JWH-018, JWH-019, JWH-073, JWH-210, PB-22, RCS-8, UR-144, and XLR-11.[61] Because there is currently no FDA approved studies, many pharmacology studies have been observed through animal studies, self-experiments,[61] clinical reports, and toxicological reports.

1.5.1 Pharmacodynamics

Pharmacodynamics is the study of a drugs particular mechanism of action and how it affects the body.[62] As mentioned previously, synthetic cannabinoids have been known to have cannabimimetic effects by acting on the CB1 and CB2 receptors. Surprisingly, synthetic cannabinoids have been reported to act on CB1 and CB2 receptors as full agonists with greater affinity compared to the phytocannabinoid Δ^9-THC.[5] Many synthetic

cannabinoids have been synthesized over the years to fully understand cannabinoid receptors and to improve its medicinal applications.

In 2016, Bow and Rimoldi helped shed some light on the structural relationship between synthetic cannabinoids and receptor activity. Although primarily focusing on the classical cannabinoid structure, Bow and Rimoldi demonstrate the affinity and selectivity on CB1 and CB2 receptors is directly correlated to the functional-group substitutions; for example, increasing C3 alkyl side-chains, substitution of C1 phenol groups, and modifications on C9/C11 groups can all impact the selectivity on cannabinoid receptors, sometimes having an upwards of 300-times selectivity; causing greater adverse effects.[51] To fully understand the structure-affinity of cannabinoids and receptor activity, there needs to be a larger focus which include other classes of synthetic cannabinoids (e.g. cyclohexylphenols, phenylacetylindoles, and naphthoylindoles).

Many users seek recreational use of synthetic cannabinoids for its cannabis-like effects, which may include elevated mood and sense of wellbeing.[12] Alarmingly, synthetic cannabinoids are known to be more potent than cannabis due to their full agonist properties.[6] However, another reason may be due to the lack of cannabidiol (CBD) in synthetic cannabinoids. CBD is naturally produced in cannabis species, and may act as an antagonist of CB1 and CB2 receptor agonists.[63] Unfortunately, there is not sufficient data in human trials and very few in animals to fully grasp the effects of synthetic cannabinoids. Many known side-effects are primarily known through case reports, clinical reports, or self-administered reports through online forums.[12] Although synthetic cannabinoids are known to have cannabimimetic effects (e.g. euphoria and elevated mood), there are many

common adverse effects, which may be directly related to synthetic cannabinoid ingestion. The types of side-effects associated with synthetic cannabinoids can vary depending on the user or the type of Spice (K2) products used; nevertheless the list of potential effects is alarmingly extensive.[12]

For acute exposures, synthetic cannabinoids can cause euphoric effects, feeling of well-being and relaxation with mild memory impairments. However, acute exposures can still have adverse effects, which may include neurological effects (i.e. dizziness, ataxia and nystamus), cardiovascular issues (e.g. chest pain, hypertension, palpitations) and gastrointestinal issues (e.g. nausea, vomiting).[64] Acute users are also prone to psychotic reactions, although has not been consistently reported for every case.[65] Chronic users are more likely to experience any number of these effects with greater risks. Of the most common symptoms observed from recreational users include nausea, anxiety, agitation, panic attacks, tachycardia, paranoia, and hallucinations.[64] In addition, recent studies have demonstrated tolerance, withdrawal, and dependencies involved in chronic users.[66,67] Chronic users are also prone to lethal effects which may include seizures, coma, and suicidal thoughts.[64,68]

1.5.2 Pharmacokinetics

Pharmacokinetics is the study of a drug's movement, interaction, and time-course within the body including its bioavailability, absorption, distribution, metabolism, and elimination.[62] Due to lack of available resources and the large array of synthetic compounds, pharmacokinetic information is limited. However, general conclusions can be

drawn from synthetic cannabinoid metabolism. For example, synthetic cannabinoids have been seen to undergo extensive oxidation and conjugation metabolism.[69] The sequence of forming metabolites for the process of elimination in the human body begins via oxidation by a superfamily of enzymes called cytochrome P450 (CYP450) which are typically associated with liver metabolism, which is then conjugated with glucuronic acid via UDP-glucuronosyltransferases (UGTs).[69]

With phytocannabinoids for example, metabolism of Δ^9-THC is oxidized by cytochrome P-subtypes (e.g. CYP2C9 and CYP3A4), which forms the major psychoactive metabolite, 11-hydroxy- Δ^9-THC and later excreted via conjugation.[70] Similarly, synthetic cannabinoids metabolism also involves various CYP-subtypes. CYP2C9 and CYP1A2 have been isolated as the primary oxidative enzyme in the metabolism of JWH-018 and AM2201.[71] Other CYP-subtypes have also been identified in a tissue specific manner. CYP2C9 is found high expressed in the intestine (for oral ingestion ROA) and CYP1A2 is found in the lungs (for smoking ROA). Although the liver is the primary role in drug and xenobiotic metabolism, it has been reported that the liver plays a minimal role in oxidative metabolism of synthetic cannabinoids.[69] Although the pharmacology of many synthetic cannabinoid compounds are unanswered, a handful of known metabolic bio-transformations include hydroxylation, carboxylation, dehydrogenation, N-dealkylation, dihydrodiol formation, ketone formation, O-demethylation, oxidative defluorination, and ring opening in phase I metabolism, and glucuronic acid conjugation in phase II metabolism.[61]

One of the major issues surrounding synthetic cannabinoids include low dosage and concentrations in the body. This is due to having a greater potency, which requires less amount of substance to achieve a "high". In addition to the low dosage, the rapid metabolism of synthetic cannabinoids poses issues in toxicological analysis. Unlike Δ^9-THC, where parent compounds can be detected in plasma for up to 7 days and metabolites in urine for up to 12 days or longer in chronic users,[72] synthetic cannabinoids have a shorter window of detection. Synthetic cannabinoids have been reported to decrease in concentration of parent compounds drastically in human serum within 21 hrs and approximately 3 days for metabolites.[61] Because of this, detectability of synthetic cannabinoids is dependent on the amount of time that has elapsed since ingestion and time of bodily fluid collection.

2. Current Detection Methods

Due to structural dissimilarities of synthetic cannabinoids, conventional THC screening and confirmatory analysis are ineffective. Duquenois color test, a standard screening technique used to determine the presence of Δ^9-THC has been practiced for many years.[73] However, due to dissimilar functional groups of Δ^9-THC and other synthetic cannabinoids, Duquenois color test cannot presumptively identify presence of these NPS compounds. Another screening technique was the presence of botanical features found on the plant *Cannabis sativa L.;* for example the presence of cystolith hairs, resin glands, glandular hairs and trichomes.[4] GC-MS and LC-MS, the gold standards in toxicological analysis, may not always identify these synthetic compounds. Because of the structural

dissimilarities, without certified reference standards, positively identifying or reporting synthetic cannabinoids can be near impossible.

In the past several years, certified reference standard suppliers have been manufacturing and supplying certified reference materials enabling laboratories to develop and validate analytical methods to positively identify the presence of synthetic cannabinoids in human bodily fluids. To-date, there are various analytical methods that have been developed: enzyme-linked immunosorbent assay (ELISA), gas chromatography–mass spectrometry (GC-MS), liquid chromatography–tandem mass spectrometry (LC-MS/MS), positive and negative ESI ionization LC-MS/MS, liquid chromatography–time-of-flight mass spectrometry (LC-TOF/MS), liquid chromatography–high resolution mass spectrometry (LC-HRMS), and homogenous EMIT-type assay (HEIA).[61] In addition to various analytical and immunoassay techniques, various sample preparation techniques have been developed for the analysis of synthetic cannabinoids: hydrolysis, liquid-liquid extraction (LLE), solid-phase extraction (SPE), dilution, TMS-derivatization and –acylation, NaOH digestion, base hydrolysis, protein precipitation, salting-out liquid-liquid extraction and supported-liquid extraction (SLE).[61]

3. Research Objective

Recreational use of synthetic cannabinoids has become a global issue since 2008.[11,60] Although the Drug Enforcement Administration has had a positive impact on identifying illicit activities, clandestine laboratories are continuously modifying the structure of synthetic cannabinoids to circumvent legal regulations.[45] The alarming rate at

which these synthetic cannabinoids are reaching the market has become a never-ending battle for forensic laboratories. Therefore, the goal of this research project is to provide the forensic community with a newly developed method for accurately and reliably identifying and quantifying commonly encountered synthetic cannabinoid compounds.

Utilizing the sensitivity and resolving power of the liquid chromatography– tandem mass spectrometry, this method positively identified and quantified the presence of 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-ABPFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUBINACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, and XLR11. Figure 2 shows the structures the synthetic cannabinoids and Figure 3 shows the internal standards. Validation of this method was performed in accordance to the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines, which includes determining the limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision, carryover, dilution integrity, matrix interference, internal standard interference, and ionization suppression and enhancement.[74]

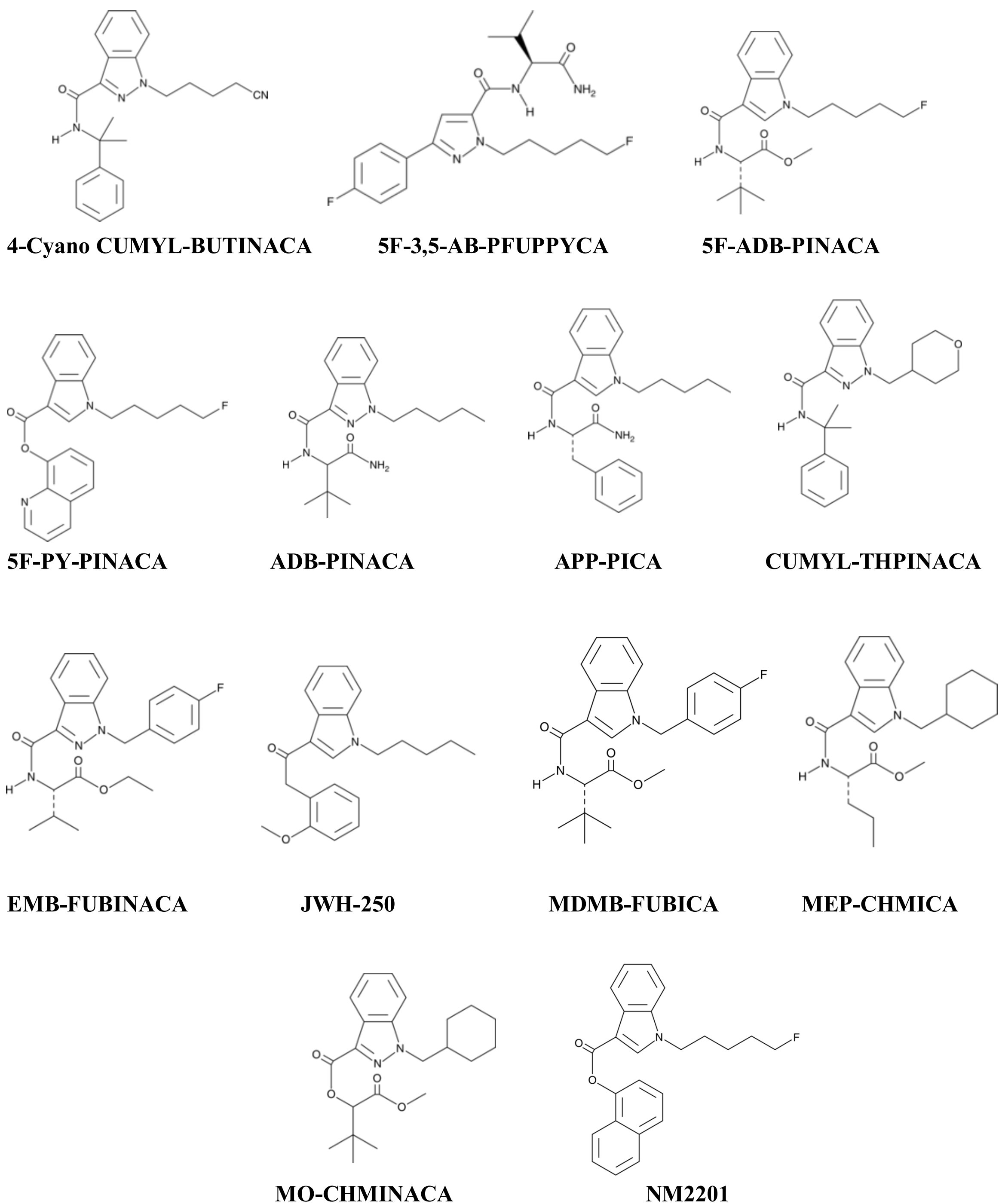


Figure 2. Chemical Structure of All Reference Standards. Chemical structure of 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-AB-PFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUBINACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, and XLR11.

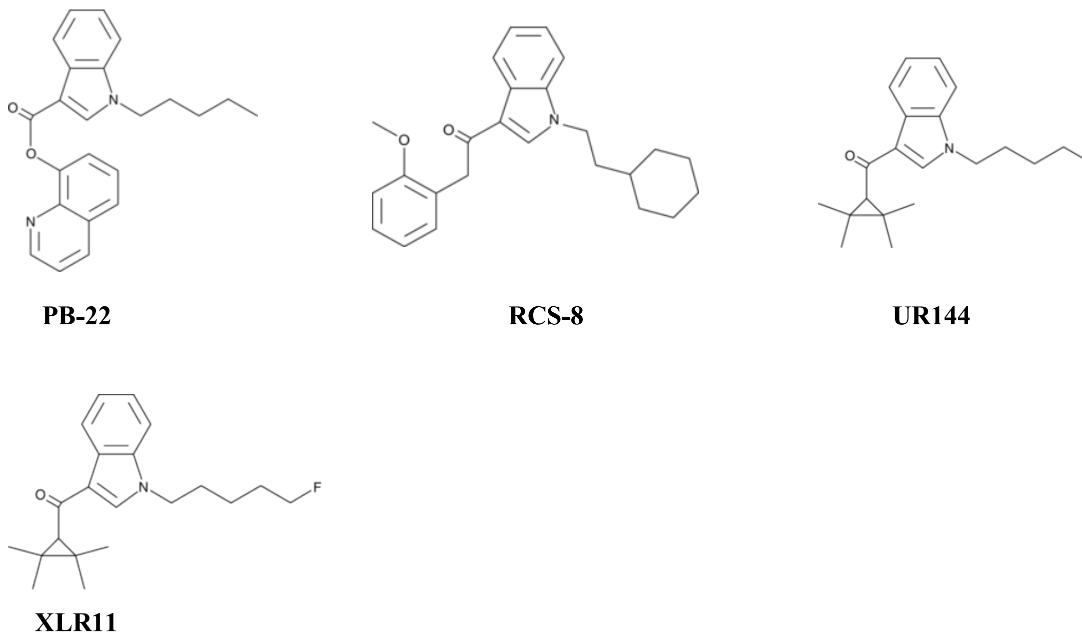


Figure 2. Chemical Structure. (cont.) Chemical structure of 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-AB-PFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUBINACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, and XLR11.

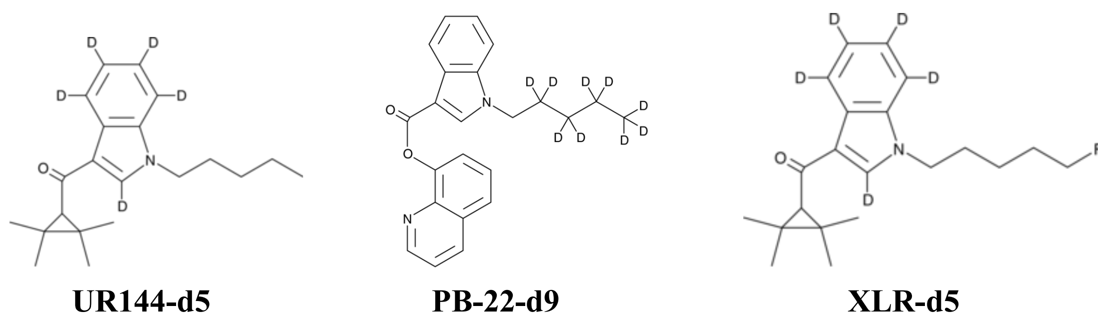


Figure 3. Chemical Structure for All Internal Standards. Chemical structures of all deuterated internal standards UR144-d5, PB-22-d9, and XLR11-d5.

4. Materials and Methods

4.1 Theory of Instrumentation

4.1.1 Liquid Chromatography

Liquid chromatography (LC) is a technique commonly used to separate components in a mixture by exploiting their physical and chemical affinities for a solid stationary phase and a liquid mobile phase.[75] The solid stationary phase, commonly referred to as the "LC column," is a cylindrical tube where the separation of components occurs. The liquid mobile phase is typically an organic solvent, which is used to help carry the components through the LC column.[75] Once a mixture of components is introduced into the column, the various components will separate as they travel through the column due to their intermolecular interactions (van der Waal forces, hydrogen bonding, or dipole-dipole interactions) associated between the LC column and the mobile phase. Analyte components that have a higher affinity (greater attraction) to the stationary phase travel through the column longer than analytes with little affinity and vice versa for analytes with lower affinity to the LC column. These individual analytes are then measured by their retention time (t_R), which is the amount of time it takes for each analyte to exit or eluted out from the column.[62]

Liquid chromatography systems are comprised of various components: solvent reservoir to house the mobile phase(s), a degassing unit to remove any air bubbles or dissolved gasses from the mobile phase line, an auto-sampler for autonomous injections, an oven that houses the solid-phase column, and a detector used to measure the t_R of eluting analytes.[62] The varying components of the system include the mobile phase(s) and the LC

column. Depending on the type of chemistry and the desired separation, different mobile phases are used (e.g. methanol, acetonitrile, water, and other buffers).[75] In addition, a complementary LC column is used to achieve the desired separation of analytes. These columns can come in various forms (e.g. column dimensions, pore size, and particle shape and size).[75] The detector located at the end of the LC column measures the t_R in the form of a chromatograph; a plot of time versus intensity. With these underlying theories, the LC system is an effective analytical instrument that uses high pressure to carry the mobile phase through the solid stationary phase, resulting in a more efficient separation.[62]

4.1.2 Mass Spectrometry

Mass Spectrometry (MS) is an analytical technique used to elucidate the chemical structure of compounds by analyzing their mass to charge (m/z) ratio. The MS system consists of an ionization source used to fragment and charge ions, mass-analyzer used to separate fragmented ions, and a detector to measure the m/z ratio of the separated ions.[76] Of the many ionization sources currently available, the most conventional is the electron impact ionization source. This specific source produces a stream of high-energy directly on analytes as they enter the source through a high-pressure vacuum.[76] The stream of high-energy electrons causes the bonds to break in the molecule resulting in fragmentation of charged ions. These charged fragments enter the mass-analyzer and separated. As these separated charged ions exit the mass-analyzer, the detector analyzes them and the data is generated in the form of a mass spectrum (e.g. m/z vs. intensity).

A tandem mass spectrometer (MS/MS), similarly to the MS, consists of an

ionization source, mass analyzer, and a detector, all of which is housed under high-pressure vacuum. Compared to an MS system, the difference lies in the mass analyzer for the MS/MS system. Of the many types of mass analyzers available, the most conventional is the quadrupole, which consists of three components: Q1 mass analyzer, Q2 collision cell, and Q3 a second mass analyzer.[76] The MS/MS system offers a higher degree of discriminating power by producing two types of charged ions: precursor (parent) ions and product ions, unlike the MS system, which only produces only single ions. This is a result of fragmentation occurring twice in the MS/MS system.

The first fragmentation of analytes occur at the source, typically in an electrospray ionization (ESI) source which produces precursor (parent) ions.[76] These precursor ions enter the mass analyzer under a high-pressure vacuum. The mass analyzer consists of four separate rods or quadrupoles; two of which have applied direct current (DC) and the other two with applied radio frequency (RF). These rods alternate in DC and RF, which allows ions of specific m/z to separate. As these precursor ions exit the Q1, it undergoes second fragmentation in Q2, which produces product ions. These products are then funneled into the Q3 mass analyzer where a second series of separation occurs and measured by a detector in the form of a mass spectra.[76]

4.1.3 Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS)

Mass spectrometers are typically coupled with chromatography instruments, which act as a detector for eluting compounds. In this case, the coupled instrument is commonly referred to as liquid chromatography – tandem mass spectrometry (LC-MS/MS).[62] Here, a mixture of different analytes is injected into the LC system, in which a mobile phase is used to carry the analytes through the LC column. Each individual analyte is then separated based on their physical and chemical affinity to the LC column. A piece of peek tubing connects the end of the LC column to the ionization source of the MS system, allowing eluting analytes to enter directly into the MS source.

Electrospray ionization (ESI) is a soft ionization technique typically used in tandem mass spectrometry to produce multiple charged fragmented ions.[62] Because the eluting analytes exiting the LC column is still in solution form, a high voltage probe is used to evaporate the solution into a fine aerosol. This charged aerosol then enters a low pressure, high temperature vacuum chamber coupled with pure nitrogen gas to vaporize the eluent of any remaining mobile phase, leaving only the gas-phase ions to enter the mass spectrometer.[62] The gas phase ions enter the MS system and are accelerated via a series of rods with alternating direct current and radio frequency which allow these ions to travel through the first mass analyzer, undergo fragmentation in the collision cell, and filter through the second mass analyzer to reach the detector.[62]

4.2 Materials

4.2.1 Standards/Reagents

Milli-Q water from an Ultrapure (type 1) water system from Millipore Sigma (Burlington, MA, USA) was used throughout this study. Optima grade methanol (MeOH), optima grade isopropanol, optima grade formic acid (FA), and optima grade acetonitrile (ACN), ACS certified hexane, optima grade ethyl acetate, and ammonium formate were purchased from Thermo Fisher Scientific (NY, USA). 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-ABPFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro MDMB-PICA, 5-fluoro PB-22, 5-fluoro PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUNICACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, XLR11, UR144-d5, PB-22-d9, and XLR11-d5 were all purchased from Cayman Chemical Company (Ann Arbor, MI, USA) as standards of 1 mg neat solids (prepared in 1 mL of methanol) or 1 mg/mL and 100 µg/mL DEA exempt preparations in either MeOH or ACN. Table 1 list the Cayman Chemical certified materials (CRM) and lot numbers. Certified drug-free human whole blood was purchased from UTAK Laboratories, Inc. (Valencia, CA) and Equitech-Bio Inc. (Kerrville, TX). The Oakton 700 pH meter used throughout this project was purchased from Thermo Fisher Scientific (NY, USA).

Table 1. Lot numbers of CRM). All CRM were stored at -20°C.

Product	Company	Lot Numbers
4-cyano CUMYL-BUTINACA	Cayman Chemical	0490444-17
5F-3,5-AB-PFUPPYCA	Cayman Chemical	0472185-17
5F-ADB-PINACA	Cayman Chemical	0485273-10
5F-PY-PINACA	Cayman Chemical	048712
ADB-PINACA	Cayman Chemical	0468161
APP-PICA	Cayman Chemical	0470126-13
CUMYL-THPINACA	Cayman Chemical	0467623-26
EMB-FUBINACA	Cayman Chemical	0473498-25
JWH-250	Cayman Chemical	0509389-01
MDMB-FUBICA	Cayman Chemical	0488504-12
MEP-CHMICA	Cayman Chemical	0506714-4
MO-CHMINACA	Cayman Chemical	0465397-25
NM2201	Cayman Chemical	0464900-37
PB-22	Cayman Chemical	0504390
RCS-8	Cayman Chemical	0491176-1
UR144	Cayman Chemical	0499668
XLR11	Cayman Chemical	0498175-1
UR144-d5	Cayman Chemical	0516864-1
PB-22-d9	Cayman Chemical	0516372-1
XLR11-d5	Cayman Chemical	0513591-1

4.2.2 UFLC-ESI-MS/MS Instrument Hardware and Software

All analyses were performed on a Shimadzu UFLC Chromatography System with LC-20AD model pumps and SIL-20AC model auto-sampler (Kyoto, Japan). The LC column was a XBridge™ C18 3.5uM, 2.1x50mm column (Waters, Milford, MA, U.S.A.). Analytes were detected on a SCIEX 4000 QTRAP tandem mass spectrometer with a Turbo V™ ESI (Framingham, MA, U.S.A.). Data were collected using Analyst® (version 1.6.2) software and quantitation was performed with MultiQuant™ 3.0 (version 3.0.5373.0) software (SCIEX).

4.3 Methods

4.3.1 Compound/ Source Optimization

Manual optimization was performed by directly infusing each analyte into the mass spectrometer and data was reviewed in real-time using Analyst® software. Each CRM including all internal standards were serially diluted to a final concentration of 100 ng/mL and 10 ng/mL in a mixture 50% LC-grade methanol and 50% millipore water with 0.1% formic acid. Starting with 10 ng/mL, each analyte was loaded in a clean syringe and all air bubbles were removed. The syringe was placed onto a syringe pump and connected directly to the MS/MS source via peek tubing. The infusion was set at a flow rate of 10 uL/min with only the mass spectrometer hardware profile activated.

In Analyst® software, under "Manual Tuning," the "Q1 MS" scan type was selected under positive ionization mode to determine approximate ranges of expected molecular weight; a range of ±50 Da of the expected molecular weight for each analyte. Dwell time

was set to 1 second and the "MCA" box was checked. The scan was acquired for 10 minutes. Intensities of each analyte were monitored with an expected intensity of $1^{e5} – 1^{e7}$. If the intensity of any analyte fell below 1^{e5}, infusion was repeated with 100 ng/mL. Once an appropriate intensity was stable in the total ion chromatograph, the m/z value (in Da) of the precursor (Q1) mass was reported.

Keeping the same analyte and infusion rate, the scan type was changed to "Product Ion MS2." The "MCA" box remained checked, and the previously obtained Q1 mass was entered in the column "product of." The Q3 mass scan ranges were set at 50 Da (start) to approximately 20 Da above the obtained Q1 mass (stop). The Dwell time was kept at 1 second, and collision energy (CE) ramp was applied to help induce fragmentation of Q3 product ions. The m/z values of the two most intense product ions in the extracted ion chromatograph, were recorded as Q3 (1) and Q3 (2).

Declustering potential (DP) was optimized next. Changing scan type to "MRM," masses of Q1 and Q3 were entered in the MRM table. "Declustering Potential" was selected and scans were set at 150 msec dwell time. The voltages of the greatest intensity were observed for both product ions and recorded. The CE and collision cell exit potential (CXP) for each product ion Q3 (1) and (2) were evaluated next. Keeping the MRM scan type, only one Q1/ Q3 ions were evaluated separately. The Q1/Q3 values and DP voltage were entered in the MRM table, dwell time remained at 150 msec. Entrance potential (EP) was set at 10, and CE ramp was applied. The average maximum voltage of three scans was recorded as the CE. This CE value was added into the information window and CXP) ramp was applied. The scan was acquired three times, and the average apex of the greatest

voltage was recorded as the CXP. The CE and CXP were determined for each Q3 (1) and (2) product ions. This compound optimization procedure for determining the Q1, Q3, DP, CE, and CXP was repeated for all analytes and internal standards.

Source optimization was performed on all analytes to optimize the curtain gas, collision gas, ion spray voltage, temperature and ion sources 1 and 2. Each analyte was diluted to a final concentration of 100 ng/mL in optima-grade methanol and loaded into a 1 mL syringe. The syringe was fastened onto a syringe pump at a pump flow rate of 10 uL/min. The infusion of the syringe was connected to the MS ion source and attached to the output of the LC column via a T-tube adaptor peek tubing. The hardware profile in Analyst® software "LC/MS" was activated. In manual tune mode, the autosampler was shut off, pumping mode set to binary flow, the LC flow rate was set to 0.4 mL/min, the oven temperature set to 40 °C, and the mobile phase B concentration was set to 50%. In the MS method, "MRM" scan type was selected and all analyte ions and their respective DP, CE, and CXP values were entered. The source and gas parameters were set to the starting conditions: ion spray voltage set at 2500 V, temperature set at 350 °C, gas 1 and 2 set at 30 °C, and curtain gas set at 20 °C. The duration of the scan was set to 20 minutes. After each scan, each of the parameters were optimized individually until the value that produced the best signal in the total ion chromatogram was obtained.

4.3.2 LC-MS/MS instrument Parameters

Both compound and source optimized parameters obtained from the previously described method were used to build the LC-MS/MS acquisition method. The parameters that were established from the compound optimization and source optimization above were established and shown in Tables below; illustrating the final parent (Q1) masses, two product (Q2) masses, DP, CE, and CXP for each product ions. Table 2 shows the final MRM table used for all analytes, which were conducted in positive ionization mode. The MRM scan duration was set to 8 minutes. Table 3 shows optimized source and gas parameters.

Table 2. Compound Optimization MRM Table.

Analyte	Retention Time	Q1 Mass (Da)	Q3 (1) Mass (Da)	Q3 (2) Mass (Da)	DP (V)	Q3 (1) CE (V)	Q3 (1) CXP (V)	Q3 (2) CE (V)	Q3 (2) CXP (V)
4-cyano CUMYL-BUTINACA	4.40	361.2	226.2	119.2	62	29.71	39.59	33.31	19.95
5F-3,5-AB-PFUPPYCA	4.10	393	189.2	134.2	100	52.63	32.75	89.11	22.8
5F-ADB-PINACA	3.98	363.3	346.3	233.2	56	14.14	9.91	35.06	38.99
5F-PY-PINACA	4.10	304.3	233.3	145.1	78	27.50	39.7	50.80	24.16
ADB-PINACA	4.39	345.2	328.3	215.3	52	13.33	19.05	35.06	36.79
APP-PICA	4.25	378.2	361.3	214.3	56	14.12	10.83	26.66	36.84
CUMYL-THPINACA	4.47	378.2	260.2	243.3	65	16.02	14.52	28.77	43.91
EMB-FUBINACA	4.88	398.2	324.3	109.2	72	21.90	18.20	57.14	18.16
JWH-250	5.08	336.3	121.2	91.2	87	28.83	20.52	65.18	15.21
MDMB-FUBICA	4.66	397.2	252.3	109.2	61	21.03	43.99	52.42	18.53
MEP-CHMICA	4.93	371.3	240.4	55.3	60	20.8	39.96	82.38	7.68
MO-CHMINACA	5.55	387.3	241.3	145.2	70	22.71	41.62	49.53	24.59
NM2201	5.24	376.2	232.3	144.3	55	15.34	41.02	52.16	23.53
PB-22	5.03	359.2	214.3	144.2	53	18.043	36.93	50.99	24.63
RCS-8	5.54	376.2	121.3	91.2	104	32.85	20.22	74.63	15.05
UR144	5.62	312.3	125.1	55.3	97	31.85	21.96	60.216	8.41
XLR11	5.18	330.3	232.3	125.3	95	33.95	39.08	33.03	22.65

Table 3. Optimized Ion Source/ Gas Parameters.

Curtain Gas (^oC)	Collision Gas (^oC)	Ion Spray Voltage (V)	Temperature (^oC)	Ion Source Gas 1 (^oC)	Ion Source Gas 2 (^oC)
25	High	2500	550	45	45

This LC method used 0.1% formic acid in Millipore water (mobile phase A) and 0.1% formic acid in LC grade acetonitrile (mobile phase B). In the LC parameter setting, max pressure was set to 5,000 psi, flow rate was set to 0.6 mL/min, and the starting percentage was set at 5% mobile phase B. Autosampler parameters are shown in Table 4.

Table 4. Auto Sampler Parameters

Rinsing Vol.	Needle stroke	Rinsing Speed	Sampling Speed	Purge Time	Rinse Dip Time	Cooler Temp.
1000 uL	52 mm	35 uL/sec	3 uL/sec	25 min	5 sec	15 ^oC

The method begins with a 10 min pre-equilibration to ensure the instrument and column conditions are met with each run. After a 10 uL sample injection, the %B concentration begins at 5% and ramps up to 95% B over time of 5.5 minutes as shown in Table 5. After 8 minutes, the %B concentration returns down to 5% starting condition and is held there for re-equilibration for approximately 1.0 min.

Table 5. LC Time Program

Time (min)	Module	Event	Parameter (%)
0.01	Pump B Conc.	Pump B Conc.	5
0.50	Pump B Conc.	Pump B Conc.	5
5.50	Pump B Conc.	Pump B Conc.	95
7.50	Pump B Conc.	Pump B Conc.	95
8.00	Pumps	Pump B Conc.	5
9.00	Controller	Stop	

4.3.3 Sample Preparation

All CRM standards and internal standards were purchased from Cayman Chemical Company in either as neat solids (1 mg) or received in solution form (in either methanol or acetonitrile) reference standards in either 1mg/mL or 100 µg/mL. All neat samples were prepared with 1 mL of methanol to give a final concentration of 1 mg/mL. Stock solutions were prepared for each reference standard at a final concentration of 10,000 ng/mL and serially diluted to 1,000 ng/mL and 100 ng/mL. These stock solutions were used to prepared working solutions for calibration standards (0.1, 0.5, 1, 2, 5, 10, 15, and 25 ng/mL) and quality control (QC) standards (1, 3, 7, and 20 ng/mL) for the validation studies and shown in Table 6. The internal standards concentration was 7.5 ng/mL. .

Table 6. Preparation of Calibration Curve and QC Samples. The table shows the final concentration of the calibrators and QC's prepared prior to SLE sample preparation. All calibrator and QC samples contained each analyte. The internal standard solutions had a final concentration of 7.5 ng/mL containing three deuterated internal standards.

Calibrators/ QC's name	Concentration (ng/mL)	Internal Standard (ng/mL)
Cal 1	0.1	7.5
Cal 2	0.5	7.5
Cal 3	1	7.5
Cal 4	2	7.5
Cal 5	5	7.5
Cal 6	10	7.5
Cal 7	15	7.5
Cal 8	25	7.5
Lower Limit QC	1	7.5
Low QC	3	7.5
Medium QC	7	7.5
High QC	20	7.5

4.3.3.1 Sample Preparation – Supported Liquid Extraction (SLE)

ISOLUTE SLE (1 mL capacity cartridges) from Biotage (Charlotte, NC)was performed on all samples for the removal of unwanted matrix components from human whole blood and to concentrate analytes of interest. Prior to SLE, each sample (300 uL) was pretreated with 300 uL of 50 mM ammonium acetate (pH 4) and was vortexed for approximately 30 seconds. The entire pre-treated sample (total vol. less than 1mL) was added to the SLE cartridge. Slight pressure was applied to each sample-containing SLE cartridge to help initiate absorption onto the sorbent bed. Samples were allowed to sit for 5 minutes on the cartridge. Afterwards, each sample was eluted with 2.5 mL of ethyl acetate, allowed to sit for 5 minutes, and repeated elution with an additional 2.5 mL of ethyl acetate. The entire eluted sample was dried under compressed nitrogen at 40°C and reconstituted in 100 uL of 50:50 mobile phase. These samples were transferred into autosampler vials and placed in the autosample chamber (15°C).

4.3.4 Method Validation

All validation parameters were performed in similar to SWGTOX guidelines to determine the limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision, carryover, dilution integrity, matrix interference, internal standard interference, and ionization suppression and ionization enhancement.[74] Quantitative data analysis was performed using MultiQuant™ software (ver. 3.0.5373.0). UR144-d5, PB-22-d9, and XLR11-d5 deuterated internal standards were used to quantify 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-ABPFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro PY-

PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUNICACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, NM2201, PB-22, RCS-8, and UR144.

4.3.4.1 Calibration Curve, LLOQ, LOD, Dilution Integrity and Carryover

The calibration model was developed using a working range of concentrations for the calibration curve (8 non-zero calibrators) and evaluating the statistical parameters so that concentrations of unknown synthetic cannabinoids in human whole blood could be accurately and reproducibly calculated. Once the calibration model was developed, it was confirmed by performing five additional separate runs using this same calibration range.

Once the calibration model was set, the lower limit of quantitation (LLOQ) was determined for each analyte in blood samples. The lowest non-zero calibrator for each analyte (n=9) was used to determine the LLOQ over nine separate linearity runs. Using the developed calibration model, the LLOQ concentrations for each of the samples were examined. Following SWGTOX recommendations,[74] all quantitative values were defined within 20% accuracy in all three samples.

LOD was determined for each analyte in three different blood lots. Four concentrations were used to determine the LOD (0.5, 0.1, 0.05, 0.025 ng/mL). These samples were prepared in duplicates and ran three times (n=24). The average (X_{blank}) and standard deviation (S_{blank}) of the signal from the blank matrix samples were used to determine the LOD. The calculations used to determine the LOD are given below.

$$LOD = X_{blank} + 3.3(S_{blank})$$

Dilution integrity of analytes was assessed for accuracy and reproducibility. Blood samples were spiked at 100 ng/mL and performed 1:50 and 1:10 for a final concentration of 2 ng/mL and 4 ng/mL, respectively. The dilutions were extracted in triplicate over five separate runs (n=15 at each dilution).

Analyte carryover was assessed by analyzing blank matrix samples in triplicate after the highest calibrator (n=6). The highest calibrator was 25 ng/mL for all except MO-CHMINACA at 15 ng/mL. All samples underwent SLE, reconstituted and run on the LC-MS/MS method with subsequent blank samples in between each high concentration sample. The highest concentration that did not produce carryover in the blank samples were further confirmed by running the same sample three times with subsequent blank samples in between the high concentration sample.

4.3.4.2 Bias and Precision

Bias and precision studies were conducted to determine the accuracy and reproducibility of the method when calculating the concentrations of synthetic cannabinoids in human whole blood. Here we evaluated bias by preparing samples with concentrations of 1, 3, 7, and 20 ng/mL. Each sample was run five separate times. With the concentrations obtained, the grand mean and grand standard deviation in each run was calculated. The formula used to calculate bias is shown below:

$$Bias\ (\%) = \left[\frac{Grand\ mean\ of\ calculated\ conc. - Nominal\ conc.}{Nominal\ concentration}\right] x\ 100$$

The precision was determined in this method by assessing within-run precision and between-run precision by calculating the mean and the standard deviation of each concentration separately for each of the five runs. The calculations were used for each analyte present in the blood sample. Calculations for within-run and between-run precision is shown below:

$$Within\ run\ \%CV = \frac{\sqrt{MSwg}}{Grand\ mean\ for\ each\ conc.}\ x\ 100$$

$$Between\text{-}run\ \%CV = \frac{\sqrt{MSbg+(n+1)*MSwg}}{Grand\ mean\ for\ each\ conc.}\ x\ 100$$

Where MS_{wg} *is mean square within groups,* MS_{bg} *is the mean square between groups, and n is the number of observations in each group.*

4.3.4.3 Matrix Effects – Ionization Suppression/Enhancement & Recovery

The effects of matrix on ionization suppression or enhancement of analyte signal intensity was investigated at low (0.5 ng/mL) and high concentrations (15 ng/mL). This experiment involves observing signal intensity in three different sets of samples: Neat samples, pre-extraction samples, and post-extraction addition of analytes in human whole blood. Neat samples were prepared at low and high concentrations, with internal standard, in mobile phase and injecting 6 times onto the LC-MS/MS.

A pre-extraction samples was performed using pooled blood samples. The pre-extracted samples involved spiking human blood at low and high concentrations, in triplicates, with 10 uL of internal standard, following normal SLE procedure, reconstituting in 50:50 (mobile phase A: mobile phase B), and injecting into the LC-MS/MS.

A post-extraction addition of analytes was also performed with 10 individual lots of whole blood samples. This approach can yield quantitative estimations of ionization suppression or enhancement that may potentially occur in a new quantitative method by comparing neat standards (Area set 1) with post-extracted samples (Area set 2). Here, each drug-free blood lot underwent normal SLE procedures, reconstituted in neat low (0.5 ng/mL) and neat high (15 ng/mL), with internal standard and injected into the LC-MS/MS. Ion suppression or enhancement was observed if $\geq \pm 25\%$.

$$\textit{Ionization Suppression or Enhancement (\%)} = \left(\frac{Area\ of\ set\ 2}{Area\ of\ set\ 1} - 1\right) x\ 100$$

Matrix recovery was evaluated using the same pre-extraction and post-extraction addition samples. By comparing pre-extraction addition samples to post-extraction addition samples, the percent recovery of each analyte can be assessed with our current method. The calculations used to determine percent recovery is shown below:

$$\textit{Percent Recovery (\%)} = \frac{Area\ pre\text{-}extraction\ addition}{Area\ post\text{-}extraction\ addition}\ x\ 100$$

4.3.4.4 Interference – Matrix Effects/ Internal standard/ Other Analytes

Interference studies were performed to assess the effects of blood matrices, internal standards, and other commonly encountered analytes. To determine potential matrix interferences, analyte-free blood samples (without internal standards) was prepared using 10 different blood lots. This was performed by aliquoting 300 uL of drug-free blood and performing supported-liquid extraction, eluting, evaporating, reconstitution in 100uL in 50:50 (mobile phase A: mobile phase B) and injecting 10uL into the LC-MS/MS. The signal of analytes was monitored to observe the presence or absence of any false-positive identification, with an acceptable criterion below the LOQ.

To assess the effects of internal standard, two sets of blood were prepared: one blood matrix analyte-free with internal standard, and one blood matrix with analytes at high calibrator concentration (15 ng/mL) without internal standard. Each sample was prepared via supported-liquid extraction, reconstituted in 50:50 (mobile phase A: mobile phase B) and injected 10uL into the LC-MS/MS. The signal of analytes was observed in both sets to see if any stable isotopes posed as interferences in the LC-MS/MS method, with an acceptable criterion below the LOD.

Other commonly encountered drugs were assessed to determine if they interfered with the LC-MS/MS method by causing false positives or suppressing the signal of expected analytes. The analytes involved in this study include: clonazepam, diazepam, (+)methadone, morphine, fentanyl, cocaine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 25I-NBOMe, and PCP. 25I-NBOMe was purchased from Lipomed. All other standards were purchased from Cerilliant. All standards had a stock concentration of 1 mg/mL and serially diluted to a working stock concentration of 30,000 ng/mL. 20uL of this working stock was added to drug-free human blood (at a final concentration of 2,000 ng/mL), with 10uL of internal standard and proceeded with supported-liquid extraction, reconstituted in 50:50 (mobile phase A: mobile phase B) and injected 10uL into the LC-MS/MS. The signal of analytes was observed to see if any analytes posed as a false positive interference in the LC-MS/MS method, with an acceptable criterion below the LOD.

Table 7. List of Interference Analytes. 10 drug compounds commonly encountered in forensic case work were chosen for the interference study.

Interference Compounds	Company	Lot Number	Stock Concentration (ng/mL)
Clonazepam	Cerilliant	FE07131603	1mg/mL
Diazepam	Cerilliant	FE05201602	1mg/mL
(+) Methadone	Cerilliant	FE06221502	1mg/mL
Morphine	Cerilliant	FE03191402	1mg/mL
Fentanyl	Cerilliant	FE022508-01	1mg/mL
Cocaine	Cerilliant	FE051012-01	1mg/mL
Amphetamine	Cerilliant	FE07011403	1mg/mL
MDMA	Cerilliant	FE043013-02	1mg/mL
25I -NBOMe	Lipomed	1557.1B1.1L1	1mg/mL
PCP	Cerilliant	FE05291401	1mg/mL

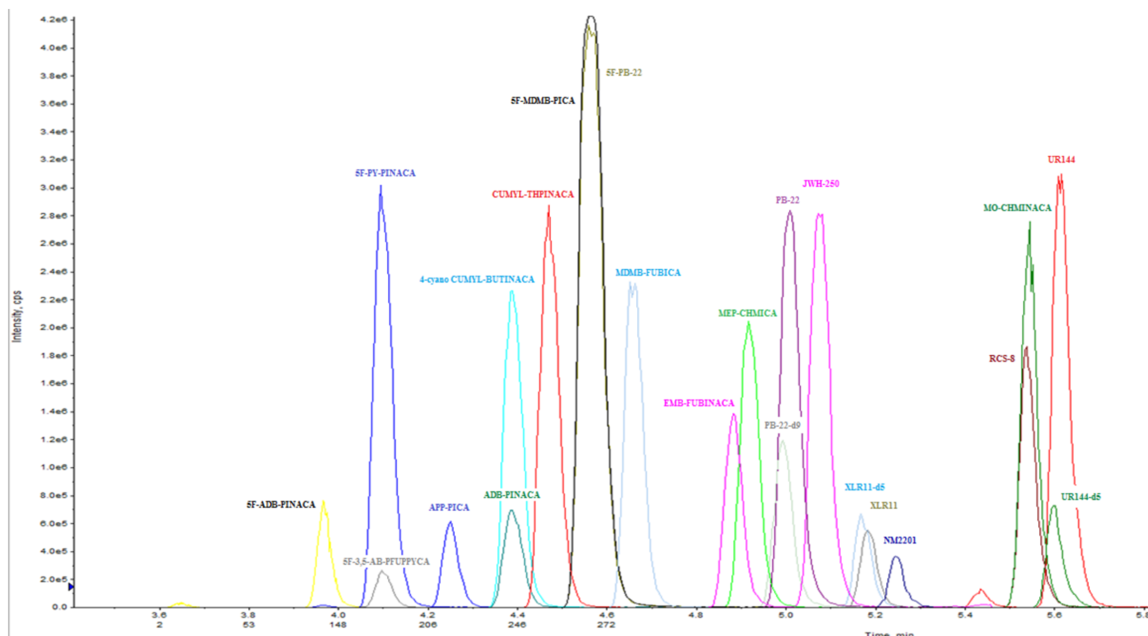
5. Results

5.1 Detection of Analytes

The total method length is 8 minutes and all analytes eluted between 3.8 and 5.8 minutes. Overlap of chromatographic peaks was observed for similarly structured analytes (e.g. 5F-3,5-AB-PFUPPYCA and 5F-PY-PINACA), however, all analytes were positively identified via mass spectrometry fragmentation of precursor and product ions. Maximum ion ratio variance did not exceed 20%.

Table 8. Retention Time for All Analytes.

Analyte	Retention time (min)
5F-ADB-PINACA	3.98
5F-3,5-AB-PFUPPYCA	4.10
5F-PY-PINACA	4.10
APP-PICA	4.25
ADB-PINACA	4.39
4-cyano CUMYL-BUTINACA	4.40
CUMYL-THPINACA	4.47
MDMB-FUBICA	4.66
EMB-FUBINACA	4.88
MEP-CHMICA	4.93
PB-22-d9	5.00
PB-22	5.03
JWH-250	5.08
XLR11-d5	5.16
XLR11	5.18
NM2201	5.24
RCS-8	5.54
MO-CHMINACA	5.55
UR144-d5	5.59
UR144	5.62



<u>Figure 4. Total Extracted Ion Chromatogram in Blood</u>. Results show the total extracted chromatogram (XIC) of all analytes including the internal standards in blood. All compounds elute between 3.8 minutes and 5.8 minutes. Analytes are eluted in the following order: 5-fluoro ADB-PINACA, 5-fluoro PY-PINACA, 5-fluoro-3,5-ABPFUPPYCA, APP-PICA, ADB-PINACA, 4-cyano CUMYL-BUTINACA, CUMYL-THPINACA, MDMB-FUBICA, EMB-FUBINACA, MEP-CHMICA, PB-22-d9, PB-22, JWH-250, XLR11-d5, XLR11, NM2201, RCS-8, MO-CHMINACA, UR144-d5, UR144.

5.2 Method Validation

5.2.1 Calibration Model

Calibration curves containing all analytes were run over 5 separate runs in spiked drug-free human whole blood. A working range of 0.1 ng/mL to 25 ng/mL for analytes was established for APP-PICA and NM2201. Other analytes had an established working range of 0.5 ng/mL to 25 ng/mL which includes: 4-cyano CUMYL-BUTINACA, 5-fluoro-3,5-ABPFUPPYCA, 5-fluoro ADB-PINACA, 5-fluoro PY-PINACA, ADB-PINACA, CUMYL-THPINACA, EMB-FUNICACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, PB-22, RCS-8, UR144, XLR11. MO-CHMINACA had an established working

range of 0.5 ng/mL to 15 ng/mL. Three internal standards used in this study include UR144-d5, XLR11-d5, PB-22-d9 using a weighting factor of 1/x. All analytes were set at linear models, except for PB-22, which were set as a quadratic model. The average R^2 values for each analyte was above of 0.998. A summary of these results is shown in Tables 9 and 10.

Table 9. Working Linear Dynamic Range and Regression Model for Each Analyte.

Analytes	Working Range (ng/mL)	Regression Model
4-cyano CUMYL-BUTINACA	0.5 - 25	Linear
5F-3,5-AB-PFUPPYCA	0.5 - 25	Linear
5F-ADB-PINACA	0.5 - 25	Linear
5F-PY-PINACA	0.5 - 25	Linear
ADB-PINACA	0.5 - 25	Linear
APP-PICA	0.1 - 25	Linear
CUMYL-THPINACA	0.5 - 25	Linear
EMB-FUBINACA	0.5 - 25	Linear
JWH-250	0.5 - 25	Linear
MDMB-FUBICA	0.5 - 25	Linear
MEP-CHMICA	0.5 - 25	Linear
MO-CHMINACA	0.5 - 15	Linear
NM2201	0.1 - 25	Linear
PB-22	0.5 - 25	Quadratic
RCS-8	0.5 - 25	Linear
UR144	0.5 - 25	Linear
XLR11	0.5 - 25	Linear

Table 10. Average R^2 values of Five Calibration Curves of all Analytes in Blood.

Analyte	Run 1	Run 2	Run 3	Run 4	Run 5	Average
4-cyano CUMYL-BUTINACA	0.99859	0.99933	0.9979	0.99971	0.99995	0.9990
5F-3,5-AB-PFUPPYCA	0.99952	0.99757	0.99929	0.99779	0.9992	0.9987
5F-ADB-PINACA	0.99952	0.99844	0.99970	0.99824	0.99832	0.9988
5F-PY-PINACA	0.99967	0.99910	0.99955	0.99967	0.99970	0.9995
ADB-PINACA	0.99859	0.99933	0.99979	0.99971	0.99995	0.9995
APP-PICA	0.99968	0.99900	0.99981	0.99920	0.99969	0.9996
CUMYL-THPINACA	0.99772	0.99964	0.99988	0.99949	0.99944	0.9992
EMB-FUBINACA	0.99763	0.99659	0.99920	0.99908	0.99815	0.9981
JWH-250	0.99942	0.99963	0.99959	0.99987	0.99959	0.9996
MDMB-FUBICA	0.99792	0.99998	0.99946	0.99910	0.99482	0.9983
MEP-CHMICA	0.99788	0.99747	0.99954	0.99952	0.99774	0.9984
MO-CHMINACA	0.99970	0.99927	0.99980	0.99995	0.99977	0.9997
NM2201	0.99969	0.99977	0.99896	0.99966	0.99895	0.9994
PB-22	0.99961	0.99983	0.99995	0.99989	0.99994	0.9998
RCS-8	0.99825	0.99931	0.99988	0.99955	0.99981	0.9994
UR144	0.99918	0.99957	0.99985	0.99930	0.99961	0.9995
XLR11	0.999859	0.99823	0.99976	0.99981	0.99925	0.9991

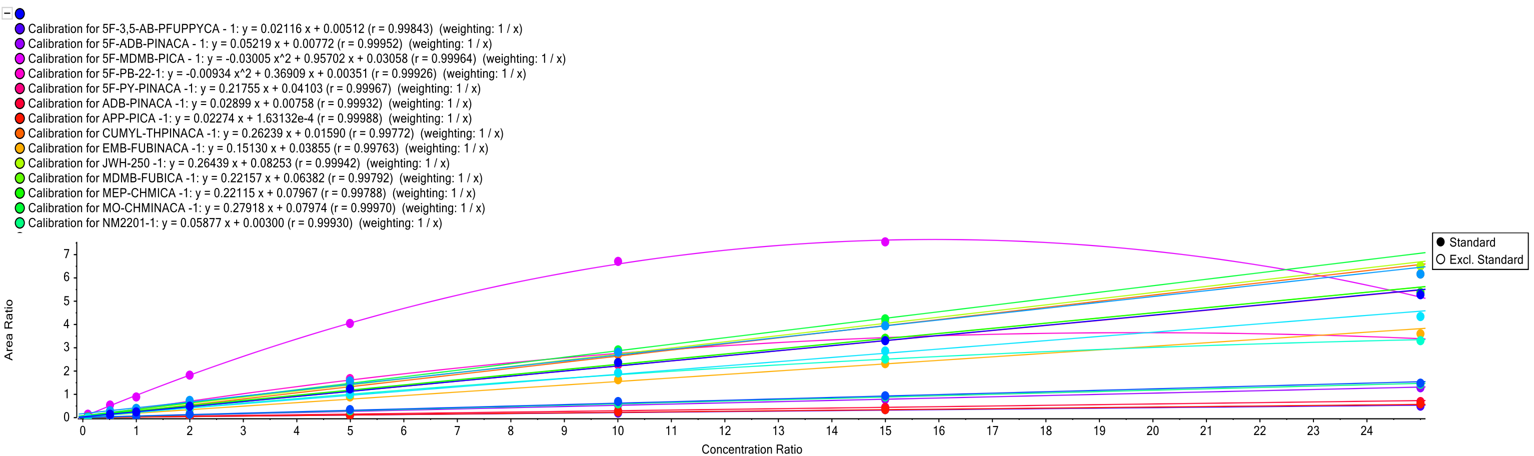


Figure 5. Calibration Curve of All analytes in Blood. The R^2 value for each analyte was above the minimum accepted values of 0.98.

5.2.2 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Using the previously established calibration model with a working range of 0.1 ng/mL to 25 ng/mL, the limit of quantitation (LOQ) was determined for each analyte. The LOQ was determined as the lowest concentration that could be quantified within a 20% accuracy in spiked drug-free human whole blood. A LOQ of 0.1 ng/mL was determined for analytes APP-PICA, EMB-FUBINACA, NM2201, and XLR11. A LOQ of 0.5 ng/mL was determined for analytes 4-cyano CUMYL-BUTINACA, 5F-3,5-AB-PFUPPYCA, 5F-ADB-PINACA, 5F-PY-PINACA, ADB-PINACA, CUMYL-THPINACA, JWH-250, MDMB-FUBICA, MEP-CHMICA, MO-CHMINACA, PB-22, RCS-8, and UR144.

LOD was determined for each analyte by running each analyte at decreasing concentrations: 0.5, 0.1, 0.05, 0.025 ng/mL to determine a signal greater than the average signal of the blank matrix sample plus 3.3 times the standard deviation. The LOD was determined to be 0.1 ng/mL for analyte 4-cyano CUMYL-BUTINACA, 0.05 ng/mL for analytes MDMB-FUBICA and MEPCHMICA, and 0.025 ng/mL for analytes 5F-3,5-AB-PFUPPYCA, 5F-ADB-PINACA, 5F-PY-PINACA, ADB-PINACA, APP-PICA, CUMYL-THPINACA, EMB-FUBINACA, JWH-250, MDMB-FUBICA, JWH-250, MO-CHMINACA, NM2201, PB-22, RCS-8, UR144, and XLR11. LOD and LOQ data are summarized in Table 11 below.

Table 11. Limit of Quantitation and Limit of Detection for Each Analyte.

Analytes	Limit of Quantitation (ng/mL)	Limit of Detection (ng/mL)
4-cyano CUMYL-BUTINACA	0.5	0.1
5F-3,5-AB-PFUPPYCA	0.5	0.025
5F-ADB-PINACA	0.5	0.025
5F-PY-PINACA	0.5	0.025
ADB-PINACA	0.5	0.025
APP-PICA	0.1	0.025
CUMYL-THPINACA	0.5	0.025
EMB-FUBINACA	0.5	0.025
JWH-250	0.5	0.025
MDMB-FUBICA	0.5	0.05
MEP-CHMICA	0.5	0.05
MO-CHMINACA	0.5	0.025
NM2201	0.1	0.025
PB-22	0.5	0.025
RCS-8	0.5	0.025
UR144	0.5	0.025
XLR11	0.1	0.025

5.2.3 Analyte Carryover

Determination of carryover was assessed by monitoring double blank samples (blood matrix samples that were fortified without analytes and internal standards) that were run immediately after the highest calibrator. Signal intensity of all analytes in the double blank was determined to be lower than the established limit of detection for each analyte. No carryover was observed in any of the double blank matrix samples.

5.2.4 Dilution Integrity

Dilution integrity of fortified matrix was investigated in cases where samples have exceedingly high concentrations that are beyond the linear dynamic range. In this case, fortified matrix was spiked at 100 ng/mL and diluted by 1:50 and 1:10, with final concentrations at 2 ng/mL and 10 ng/mL, respectively. Dilution samples were prepared in triplicates and ran over the course of 5 separate runs. According to SWGTOX guidelines, accuracy and %CV and bias must fall within ±20%. All analytes, with the exception of NM2201 1:50 accuracy (125.08%), met these conditions as shown in table 12.

Table 12. Dilution integrity of fortified matrix at 1:50 (2 ng/mL) and 1:10 (10 ng/mL). Dilution integrity of each analyte was investigated over a course of 5 separate runs.

	1:50 (2 ng/mL)				1:10 (10 ng/mL)			
Analyte	**Average (ng/mL)**	**STDEV (ng/mL)**	**Accuracy**	**%CV**	**Average (ng/mL)**	**STDEV (ng/mL)**	**%Accuracy**	**%CV**
4-cyano CUMYL-BUTINACA	2.03	0.17	101.33	8.46	9.60	0.83	98.83	8.68
5F-3,5-AB-PFUPPYCA	1.90	0.17	95.17	9.04	9.54	1.28	95.40	13.43
5F-ADB-PINACA	1.91	0.19	95.04	10.11	9.52	1.14	94.42	11.98
5F-PY-PINACA	1.90	0.19	94.88	10.27	9.40	0.50	94.03	5.28
ADB-PINACA	1.86	0.15	92.89	8.24	9.35	0.73	93.52	7.76
APP-PICA	1.76	0.15	88.06	8.67	9.15	0.90	91.50	9.84
CUMYL-THPINACA	1.90	0.13	95.18	6.90	9.40	0.72	93.95	7.67
EMB-FUBINACA	2.37	0.21	118.57	8.78	11.11	0.92	111.11	8.28
JWH-250	2.06	0.10	102.83	4.79	10.18	0.27	101.77	2.62
MDMB-FUBICA	2.16	0.13	108.01	6.01	11.12	0.51	111.18	4.58
MEP-CHMICA	2.25	0.15	112.50	6.58	10.83	0.64	108.33	5.93
MO-CHMINACA	2.00	0.24	99.92	12.14	9.69	0.87	96.92	8.95
NM2201	2.50	0.18	125.08	7.25	12.50	1.55	115.25	12.40
PB-22	2.20	0.10	110.00	4.60	10.70	0.41	106.95	3.84
RCS-8	2.25	0.11	112.30	4.78	10.43	0.59	104.28	5.62
UR144	2.28	0.07	113.87	3.29	10.75	0.41	107.54	3.81
XLR11	2.19	0.05	109.61	2.39	11.00	0.67	109.98	6.05

5.2.5 Bias and Precision

Concentrations at 1.0 ng/mL, 3.0 ng/mL, 7.0 ng/mL, and 20 ng/mL were evaluated for bias and precision in fortified blood matrix. Each sample concentration was prepared in triplicates and analyzed over the course of five separate runs. Bias is the measure of the closeness in value to the true expected value, and precision is the measure of reproducibility. Within-run precision is calculated for each concentration separately for each of the five runs and is the precision between the three replicates within a single run at each concentration. Between-run precision is calculated for each concentration over five runs and is the precision between all the replicates at each concentration.

Table 13. Bias and Between-Run Precision for all Analytes in Fortified Matrix Blood.

1.0 ng/mL	Average (ng/mL)	STDEV (ng/mL)	Bias (%)	CV (%)
4-cyano CUMYL-BUTINACA	1.05	0.08	4.92	7.29
5F-3,5-AB-PFUPPYCA	1.05	0.10	4.86	9.55
5F-ADB-PINACA	1.10	0.06	10.19	5.53
5F-PY-PINACA	1.06	0.10	6.14	9.07
ADB-PINACA	1.08	0.08	8.16	7.21
APP-PICA	1.07	0.08	7.04	7.14
CUMYL-THPINACA	1.04	0.08	3.72	8.03
EMB-FUBINACA	1.09	0.08	8.79	7.31
JWH-250	1.04	0.08	3.78	7.57
MDMB-FUBICA	1.07	0.08	6.72	7.87
MEP-CHMICA	1.08	0.05	7.75	4.89
MO-CHMINACA	0.98	0.10	-1.72	10.48
NM2201	1.08	0.06	7.59	5.90
PB-22	1.05	0.05	5.19	4.63
RCS-8	1.02	0.09	1.98	8.47
UR144	1.06	0.06	5.83	6.04
XLR11	1.04	0.07	4.01	6.65

Table 13. Bias and Between-Run Precision for all Analytes in Fortified Matrix Blood. (cont.)

3.0 ng/mL	Average (ng/mL)	STDEV (ng/mL)	Bias (%)	CV (%)
4-cyano CUMYL-BUTINACA	3.16	0.14	5.35	7.29
5F-3,5-AB-PFUPPYCA	3.11	0.26	3.68	8.22
5F-ADB-PINACA	3.23	0.19	7.71	5.92
5F-PY-PINACA	3.25	0.16	6.14	4.97
ADB-PINACA	3.20	0.14	6.58	4.46
APP-PICA	3.14	0.22	4.60	6.86
CUMYL-THPINACA	3.19	0.15	6.19	4.81
EMB-FUBINACA	3.35	0.20	11.52	5.89
JWH-250	3.21	0.13	7.01	4.11
MDMB-FUBICA	3.27	0.16	8.96	4.99
MEP-CHMICA	3.33	0.17	7.75	5.03
MO-CHMINACA	3.05	0.31	1.63	10.08
NM2201	3.09	0.27	2.87	8.77
PB-22	3.04	0.17	1.22	5.62
RCS-8	3.11	0.13	3.54	4.14
UR144	3.34	0.17	11.45	5.12
XLR11	3.12	0.25	3.86	7.92
7.0 ng/mL	**Average (ng/mL)**	**STDEV (ng/mL)**	**Bias (%)**	**CV (%)**
4-cyano CUMYL-BUTINACA	7.34	0.32	4.88	4.38
5F-3,5-AB-PFUPPYCA	7.29	0.47	4.12	6.39
5F-ADB-PINACA	7.55	0.65	7.92	8.56
5F-PY-PINACA	8.03	1.20	14.73	14.89
ADB-PINACA	7.42	0.31	5.95	4.13
APP-PICA	7.25	0.37	3.55	5.08
CUMYL-THPINACA	7.49	0.21	7.00	2.78
EMB-FUBINACA	7.35	0.36	8.79	4.83
JWH-250	7.47	0.33	6.65	4.47
MDMB-FUBICA	7.35	0.46	5.07	6.31
MEP-CHMICA	7.64	0.28	9.08	3.62
MO-CHMINACA	7.18	0.46	2.61	6.45
NM2201	7.24	0.37	3.42	5.13
PB-22	7.11	0.26	1.58	3.68
RCS-8	7.16	0.16	2.29	2.19
UR144	7.81	0.31	11.60	4.01
XLR11	7.25	0.48	3.63	6.67

Table 13. Bias and Between-Run Precision for all Analytes in Fortified Matrix Blood. (cont.)

20 ng/mL	Average (ng/mL)	STDEV (ng/mL)	Bias (%)	CV (%)
4-cyano CUMYL-BUTINACA	19.84	1.12	-0.81	5.62
5F-3,5-AB-PFUPPYCA	20.12	1.95	0.60	9.67
5F-ADB-PINACA	21.11	1.52	5.54	7.18
5F-PY-PINACA	21.38	1.23	6.92	5.75
ADB-PINACA	20.27	0.71	1.34	3.48
APP-PICA	20.39	0.35	1.93	1.70
CUMYL-THPINACA	20.19	0.74	0.95	3.65
EMB-FUBINACA	19.37	1.39	-3.17	7.18
JWH-250	19.97	0.94	-0.16	4.69
MDMB-FUBICA	19.52	1.52	-2.37	7.77
MEP-CHMICA	19.93	1.24	-0.36	6.24
MO-CHMINACA	N/A	N/A	N/A	N/A
NM2201	19.21	1.56	-3.93	8.09
PB-22	20.42	1.15	2.11	5.63
RCS-8	19.24	1.16	-3.80	6.05
UR144	20.17	0.37	0.85	1.81
XLR11	19.31	1.19	-3.44	6.17

Table 14. Within-Run Precision Results for All Analytes in Matrix Blood.

1.0 ng/mL	%CV Run 1	%CV Run 2	%CV Run 3	%CV Run 4	%CV Run 5
4-cyano CUMYL-BUTINACA	4.97	6.05	8.88	4.64	0.83
5F-3,5-AB-PFUPPYCA	3.52	1.64	3.36	3.85	2.50
5F-ADB-PINACA	3.52	2.46	8.11	2.17	8.42
5F-PY-PINACA	3.54	3.19	5.11	3.90	4.30
ADB-PINACA	5.84	4.69	8.03	4.72	4.43
APP-PICA	1.09	3.19	8.30	11.34	7.10
CUMYL-THPINACA	4.72	2.76	7.09	9.31	1.38
EMB-FUBINACA	0.41	5.07	5.28	0.67	3.51
JWH-250	4.33	7.55	1.54	2.81	3.31
MDMB-FUBICA	2.88	6.46	4.93	1.35	10.42
MEP-CHMICA	4.54	3.94	4.47	6.51	5.60
MO-CHMINACA	6.74	6.25	7.26	7.32	6.84
NM2201	0.98	5.29	3.67	6.32	9.30
PB-22	1.76	4.77	3.59	4.43	5.65
RCS-8	6.23	7.22	6.16	1.56	3.11
UR144	2.33	3.84	1.40	5.71	4.74
XLR11	0.59	4.91	4.80	1.05	4.02
3.0 ng/mL	**%CV Run 1**	**%CV Run 2**	**%CV Run 3**	**%CV Run 4**	**%CV Run 5**
4-cyano CUMYL-BUTINACA	4.04	0.49	5.22	1.93	2.93
5F-3,5-AB-PFUPPYCA	0.44	2.90	3.46	3.80	5.49
5F-ADB-PINACA	0.44	0.82	6.62	4.76	3.47
5F-PY-PINACA	1.15	1.39	3.86	2.38	1.67
ADB-PINACA	6.94	2.95	2.15	0.30	0.06
APP-PICA	5.99	0.54	2.54	2.07	6.97
CUMYL-THPINACA	3.98	2.42	3.68	4.96	3.01
EMB-FUBINACA	5.54	2.57	3.39	0.74	6.32

Table 14. Within-Run Precision Results for All Analytes in Matrix Blood. (cont.)

JWH-250	1.29	0.40	2.27	0.57	3.27
MDMB-FUBICA	3.24	3.68	1.60	3.42	6.07
MEP-CHMICA	3.62	4.88	4.35	2.90	3.16
MO-CHMINACA	2.38	2.59	3.94	2.16	0.43
NM2201	1.41	7.98	5.44	1.64	7.09
PB-22	3.15	1.74	2.18	4.07	3.83
RCS-8	4.79	3.95	2.47	2.77	3.11
UR144	4.00	1.92	3.29	3.30	3.39
XLR11	3.96	4.31	2.86	3.54	3.62
7.0 ng/mL	**%CV Run 1**	**%CV Run 2**	**%CV Run 3**	**%CV Run 4**	**%CV Run 5**
4-cyano CUMYL-BUTINACA	6.30	2.07	0.40	3.00	0.64
5F-3,5-AB-PFUPPYCA	6.10	2.63	2.08	4.80	1.78
5F-ADB-PINACA	1.44	3.41	6.43	0.97	5.82
5F-PY-PINACA	1.00	5.02	2.89	4.18	6.22
ADB-PINACA	2.12	4.37	3.09	1.35	4.64
APP-PICA	5.41	3.07	1.04	6.14	5.05
CUMYL-THPINACA	3.82	1.44	4.25	2.03	1.86
EMB-FUBINACA	3.55	1.37	1.81	1.47	0.72
JWH-250	1.17	4.30	3.83	1.91	3.63
MDMB-FUBICA	3.39	2.43	0.008	3.49	3.00
MEP-CHMICA	0.73	1.90	3.43	1.89	2.32
MO-CHMINACA	2.83	5.79	2.21	1.56	4.39
NM2201	1.24	2.28	7.64	1.12	8.94
PB-22	3.20	1.66	2.46	1.58	0.89
RCS-8	1.67	1.17	2.57	1.48	4.13
UR144	6.32	2.49	0.33	1.16	4.79
XLR11	5.23	3.40	1.54	1.29	4.76
20.0 ng/mL	**%CV Run 1**	**%CV Run 2**	**%CV Run 3**	**%CV Run 4**	**%CV Run 5**
4-cyano CUMYL-BUTINACA	1.46	3.28	1.99	5.73	5.75
5F-3,5-AB-PFUPPYCA	3.12	1.68	3.11	8.58	1.90
5F-ADB-PINACA	3.12	1.97	3.15	5.25	4.69
5F-PY-PINACA	0.47	3.19	1.07	3.67	1.96
ADB-PINACA	3.44	2.08	0.80	1.10	4.25
APP-PICA	2.04	0.35	2.39	2.00	1.46
CUMYL-THPINACA	2.93	4.09	0.93	4.10	5.56
EMB-FUBINACA	3.25	0.81	3.42	5.89	6.12
JWH-250	3.26	1.74	3.93	4.19	6.58
MDMB-FUBICA	1.80	3.96	1.28	9.51	4.08
MEP-CHMICA	3.10	0.96	2.92	11.05	2.78
MO-CHMINACA	N/A	N/A	N/A	N/A	N/A
NM2201	1.21	3.79	5.13	14.36	10.44
PB-22	6.08	3.78	0.20	2.24	4.21
RCS-8	2.57	2.22	1.26	0.92	5.01
UR144	0.87	1.51	0.31	0.81	0.72
XLR11	4.18	1.54	0.49	8.04	4.27

5.2.6 Matrix Effect – Ionization Suppression/Enhancement & Recovery

Matrix suppression was observed at low and high concentrations with analytes 4-cyano CUMYL-BUTINACA, 5F-PY-PINACA, CYMYL-THPINACA, NM2201, and RCS-8. Suppression of ionization was observed at high concentration for JWH-250 and MEP-CHMICA (Table 15).

Percent extraction recovery is shown in Table 16. Percent recovery ranged from 57% UR144 to 85% for ADB-PINCA at the low concentration. Percent recovery ranged from 55% EMB-FUBINACA to 83% 5F-ADB-PINACA at the high concentration.

Table 15. Matrix Effect – Ionization Suppression/ Enhancement Results.

	Low Concentration (0.5 ng/mL)	High Concentration (15 ng/mL)
Analyte	Supp/Enh. (%)	Supp/Enh. (%)
4-cyano CUMYL-BUTINACA	-46.47	-43.71
5F-3,5-AB-PFUPPYCA	2.22	-0.06
5F-ADB-PINACA	-0.63	-5.87
5F-PY-PINACA	-11.38	-28.60
ADB-PINACA	-6.20	-11.68
APP-PICA	-2.94	-15.18
CUMYL-THPINACA	-41.95	-39.71
EMB-FUBINACA	-47.39	-41.20
JWH-250	-53.47	-34.12
MDMB-FUBICA	-25.66	-12.00
MEP-CHMICA	-40.87	-23.07
MO-CHMINACA	-33.70	-4.24
NM2201	-57.78	-58.02
PB-22	-46.80	-17.32
RCS-8	-43.99	-32.52
UR144	-13.99	-10.69
XLR11	-59.03	-54.26

Table 16. Percent Extraction Recovery Results for All Analytes.

	Low Concentration (0.5 ng/mL)			High Concentration (15 ng/mL)		
Analyte	Pre-Extraction Average Area	Post-Extraction Average Area	Extraction Recovery (%)	Pre-Extraction Average Area	Post-Extraction Average Area	Extraction Recovery (%)
4-cyano CUMYL-BUTINACA	4.38E+05	6.19E+05	70.81	2.79E+06	3.75E+06	74.58
5F-3,5-AB-PFUPPYCA	3.43E+04	3.43E+04	76.83	2.33E+05	2.98E+05	78.44
5F-ADB-PINACA	1.19E+05	1.49E+05	80.20	8.51E+05	1.02E+06	83.36
5F-PY-PINACA	1.45E+06	1.87E+06	77.62	6.54E+06	9.68E+06	67.59
ADB-PINACA	5.92E+05	6.96E+05	85.09	3.27E+06	4.08E+06	80.13
APP-PICA	1.15E+05	1.44E+05	79.49	6.43E+05	9.39E+05	68.41
CUMYL-THPINACA	6.04E+05	8.26E+05	73.10	3.62E+06	3.62E+06	77.85
EMB-FUBINACA	3.03E+05	4.04E+05	75.19	1.52E+06	2.77E+06	54.92
JWH-250	7.89E+05	1.36E+06	58.01	4.05E+06	6.47E+06	62.52
MDMB-FUBICA	5.31E+05	7.75E+05	68.59	2.91E+06	4.09E+06	69.87
MEP-CHMICA	5.74E+05	8.22E+05	69.87	2.87E+06	4.25E+06	67.38
MO-CHMINACA	6.94E+05	9.51E+05	73.00	3.32E+06	5.57E+06	59.57
NM2201	1.04E+05	1.50E+05	69.00	6.38E+05	1.01E+06	63.37
PB-22	9.12E+05	1.32E+06	68.89	4.30E+06	4.30E+06	59.21
RCS-8	5.01E+05	5.01E+05	60.82	2.89E+06	4.63E+06	62.43
UR144	9.59E+05	1.67E+06	57.45	5.45E+06	7.18E+06	60.56
XLR11	1.21E+05	1.80E+05	67.08	7.09E+05	1.01E+06	70.54

5.2.7 Interference from Matrix, Internal Standard, and Other Analytes

5.2.7.1 Matrix Interference

Ten different lots of blood was used and analyzed without the presence of analytes and internal standards to determine any presence of matrix interferences that would produce a false positive identification of analytes. The acceptance criteria for matrix interference for each blood lot was determined by having signal intensity less than that of the limit of detection. Results show that each of the 10 blood lots did not produce any interference (table 17).

Table 17. Interference – Matrix Effect Results. Signal intensities of 10 blood matrices have accepted ranges below each analyte's LOD level.

Analyte	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	Lot 10
4-cyano CUMYL-BUTINACA	4.42E+03	4.60E+03	3.16E+03	4.75E+03	2.05E+03	1.42E+03	3.21E+03	3.82E+03	3.38E+03	1.04E+04
5F-3,5-AB-PFUPPYCA	6.96E+02	6.40E+02	7.89E+02	9.44E+02	1.31E+04	2.12E+03	6.59E+02	4.41E+02	1.52E+03	7.51E+02
5F-ADB-PINACA	6.39E+03	3.62E+03	3.69E+03	5.50E+03	3.80E+03	5.81E+03	1.63E+03	7.29E+03	5.30E+03	4.31E+03
5F-PY-PINACA	3.51E+02	2.63E+02	1.80E+03	4.28E+03	4.61E+03	8.69E+02	2.67E+03	6.11E+02	5.84E+03	1.51E+04
ADB-PINACA	1.03E+04	1.85E+04	8.44E+03	1.62E+04	1.22E+04	8.71E+03	7.82E+03	4.95E+03	7.26E+03	1.15E+04
APP-PICA	1.81E+03	2.09E+03	1.02E+04	8.65E+03	3.15E+03	2.31E+03	2.68E+03	1.37E+04	3.39E+03	7.93E+03
CUMYL-THPINACA	2.03E+02	2.60E+02	7.72E+02	8.29E+02	2.91E+03	7.82E+02	1.84E+03	8.72E+02	5.14E+03	1.18E+04
EMB-FUBINACA	2.66E+03	5.98E+03	5.16E+03	1.98E+03	8.34E+03	3.01E+03	2.81E+03	2.05E+03	1.90E+03	4.50E+03
JWH-250	7.77E+03	1.19E+03	3.79E+03	1.53E+04	3.14E+03	1.91E+04	2.09E+03	8.05E+03	3.64E+03	9.25E+03
MDMB-FUBICA	1.58E+02	1.51E+02	4.22E+02	5.94E+02	1.24E+03	3.15E+02	3.77E+02	1.08E+03	2.25E+03	7.66E+03
MEP-CHMICA	5.78E+02	4.40E+02	2.44E+02	4.90E+02	7.70E+02	7.00E+02	3.85E+02	6.63E+02	1.96E+03	6.58E+03
MO-CHMINACA	2.05E+03	4.89E+03	2.00E+03	1.52E+03	3.79E+03	3.19E+03	9.25E+02	6.18E+03	3.85E+03	1.20E+04
NM2201	1.77E+02	3.11E+02	3.68E+02	1.56E+02	2.56E+02	2.76E+02	1.38E+02	2.55E+02	1.75E+02	1.31E+03
PB-22	1.90E+03	1.91E+03	5.21E+02	1.18E+03	2.44E+03	3.28E+03	4.06E+02	2.26E+03	5.24E+03	9.39E+03
RCS-8	8.45E+03	8.00E+03	3.97E+03	2.13E+04	2.51E+04	8.41E+05	1.36E+03	3.71E+03	2.95E+03	8.77E+03
UR144	1.65E+03	6.13E+03	2.40E+03	4.97E+02	5.91E+03	1.44E+04	2.29E+03	9.67E+03	1.91E+04	1.19E+04
XLR11	9.61E+02	1.36E+03	1.42E+03	1.20E+03	1.30E+03	4.79E+03	1.69E+03	4.59E+03	6.47E+03	6.28E+03

5.2.7.2 Internal Standard Interference

To determine if the presence of any stable isotope would interfere with the current method, internal standards were used without the presence of any analytes. The signal intensity for each analyte was observed, with acceptable levels below the LOD. No interferences were present from analysis of deuterated internal standards.

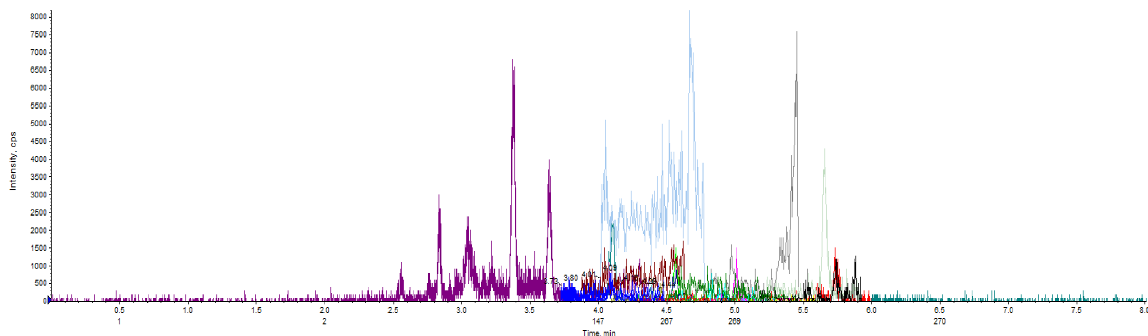


Figure 6. Total Extraction Ion Chromatograph of Blank Blood with Only Internal-standards.

5.2.7.3 Other Analyte Interference

Interferences of other commonly encountered compounds were used to assess the potential of any false positive signals. Here, 10 different drug compounds were used in this study at 2,000 ng/mL, which include: clonazepam, diazepam, (+) methadone, morphine, fentanyl, cocaine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 25I-NBOMe, and PCP. Signal intensity was observed to determine if compounds produced a signal intensity greater than the established LOD for each analyte. Chromatographic data show interference signal with ADB-PINACA.

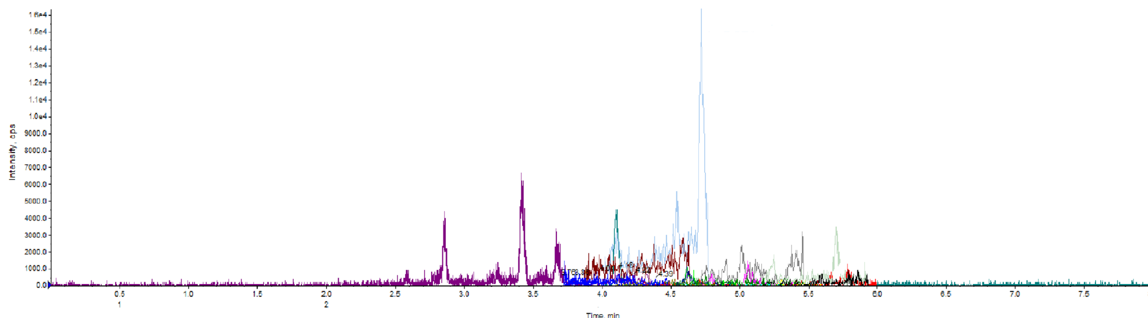


Figure 7. Interference of Other Analytes. Total Extracted Ion Chromatograph of Post-spiked blood with 10 commonly encountered drugs at high concentrations (2,000 ng/mL). Results show no indication of false identification.

6. Discussion

6.1 Detection of Analytes

Due to the large number of synthetic cannabinoids, all analytes chosen for this study were based off the popularity and reoccurrence of certain synthetic cannabinoid compounds. Unfortunately, because many compounds are relatively new, many of these compounds do not have an associated certified reference deuterated internal standard.

In this method, two fragmented transition ions were used for the detection of each analyte. Two transition ions were used in this method to determine the best signal-to-noise ratio; the first most abundant signal-to-noise ratio used for quantitative analysis and the second most abundant signal-to-noise ratio was used for qualitative analysis. With this method, all analytes eluted between 3.8 and 5.8 minutes, with a total method length of 8 minutes.

In the beginning of this study, method development was assessed looking at 19 synthetic cannabinoids. However, two compounds (5F-MDMB-PICA and 5F-PB-22)

shared similar retention time and had similar parent (Q1) and transition (Q3) ions. Because of these qualities, the two compounds could not be distinguished from one another.

6.2 Method Validation Parameters

The calibration model was assessed using a range of concentrations that produced the best linear or quadratic regression which was also accurate and reproducible. A weighting of 1/x was used with all calibration models in both linear and quadratic curves due to variation at lower concentrations. Only PB-22 produced the best calibration curve using quadratic regression models. The rest had linear regression models.

The limit of quantitation was determined as the lowest quantifiable point on the calibration model. Synthetic cannabinoids have been determined to have a very short half-life in the human body.[72] Therefore, there is a need for analytical methods to detect the presence of synthetic cannabinoids at sub-nano levels. Most analytes had a determined limit of quantitation of 0.5 ng/mL; other analytes were quantifiable at 0.1 ng/mL as shown in table 9. Most analytes had an upper-limit of 25 ng/mL and others had 15 ng/mL. According to SWGTOX guidelines, a calibration model is required to have 6 non-zero calibration points. Although some analytes failed to quantify at 0.1 ng/mL (cal 1) and others at 25 ng/mL (cal 8), dropping these calibration points did not affect the acceptance criteria.

The limit of detection was determined for each analyte by preparing pre-extraction addition samples at decreasing intervals of 0.5, 0.1, 0.05, and 0.025 ng/mL. The signal-to-noise that produce an intensity of 3.3 times the noise of the blank was determined to be the

LOD. Most analytes were determined to have an LOD of 0.025 ng/mL; very few had higher LOD's. MDMB-FUBICA and MEP-CHMICA both had a limit of detection of 0.05 ng/mL and 4-cyano CUMYL-BUTINACA had an LOD of 0.1 ng/mL as shown in table 11.

Carry-over was assessed in this method by observing blank matrix samples immediately following the highest calibration point. Carry-over was not observed in this method. However, further evaluation of analyte carry-over should be assessed at higher concentrations to determine the maximum concentration where carry-over may be an issue.

Bias and precision was assessed for all analytes. The acceptance criterion was such that the %Bias and %CV for all analyte did not exceed ±20%. As shown in table 13, the bias and precision for all analytes fell within accepted ranges. , MO-CHMINACA had an upper-limit of 15 ng/mL and therefore does not have precision and accuracy calculations at 20 ng/mL QC. .

Extraction recovery was assessed with this method to determine the efficiency of the SLE procedure. Because sample preparation techniques may exhibit some sample loss, percent recovery was assessed to determine the amount of sample recovery. This was done by evaluating all analytes at low (0.5 ng/mL) and high (15 ng/mL) concentrations for both pre-extraction- and post-extraction addition samples. Although other sample preparation techniques may offer greater sample recovery (e.g. liquid-liquid extraction), it is a time-costly method. Other conventional sample preparation methods include solid-phase extraction. However, with this developed SLE extraction method, the total sample preparation time was cut down by more than half. This was possible by eliminating the need for conditioning and washing steps in the SLE sample preparation method.

Ionization suppression and enhancement was evaluated in this study to determine any interference from matrix effects. Table 15 shows some of variation (analyte specific) in ionization suppression. Two analytes (ADB-PINACA and APP-PICA) show minor ionization enhancement; the rest of the analytes exhibited minor ionization suppression. One analyte, NM2201, had notably significant ionization suppression. However, the degree of ionization suppression and enhancement did not affect the other validation parameters.

6.2.1 Interferences

Interference studies involving matrix effect, internal standard, and other analytes were also assessed to determine the reliability of the method. Ten different sources of blood matrix were run on the method without the presence of any analytes or internal standards to assess the matrix effect. As shown in table 17, the results show no potential source of matrix interferences, with signal intensities less than the established LOD for each analyte. However, chromatography and mass spectral data show slight presence of JWH-250 or the presence of RCS-8, which were lot specific, as shown in Appendix C. However, because the signal intensities calculated fall below the expected limit of detection, we can conclude there is no matrix interferences present.

Interferences of internal standard was also assessed by running samples fortified with only internal standards without the presence of analytes to determine if analytes would be falsely identified. No interference was observed from internal standard assessment. Interference of other compounds was chosen based off drugs commonly encountered in forensic and clinical laboratories. In this case, clonazepam, diazepam, (+)methadone,

morphine, fentanyl, cocaine, amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 25I-NBOMe, and PCP were chosen for this particular study. To ensure no false positive identifications would arise, these compounds were tested at exceedingly high concentrations (2000 ng/mL). It can be concluded that there are no interferences with other commonly encountered substances.

7. Conclusion

7.1 Summary of Findings

As shown by all the results of this study, an analytical method was developed and validated for the identification and quantification of 17 synthetic cannabinoids in human whole blood.. The method was developed with a total run time of 8.013 minutes and compounds eluting within 3.8 to 5.8 minutes. The calibration models in this method exhibited an R^2 value of > 0.98 with a weighting factor of 1/x for all analytes. A linear dynamic range was established from 0.5 ng/mL to 25 ng/mL for all compounds, except for APP-PICA and NM2201, which were all quantifiable at lower levels (0.1 ng/mL). Percent recovery ranged from 54.92 to 83.36% with the developed SLE method. Bias and precision were assessed at 1, 3, 7, and 20 ng/mL for all analytes, except for MO-CHMINACA. All analytes were calculated within ±20% accuracy and ±20% CV. Carryover was not observed in this method, with the highest calibration concentration of 25 ng/mL. Ionization suppression and enhancement was observed in blood matrix from a range of -4.47 to 76.67%. Matrix effect was determined to be insignificant in this method. Stable isotopic analysis of deuterated internal standards showed no sign of interference with analytes of

interest. Lastly, one analyte, ADB-PINACA was observed from analysis of other commonly encountered drugs. Further analysis of each of the common drugs showed three drugs (MDMA, 25I-NBOMe, and PCP) to falsely identify ADB-PINACA.

7.2 Significance of Findings

Because of the rapid growth and emergence of synthetic cannabinoids, updated analytical methods for the detection of these compounds are sorely needed. The recent emergence and the lack of published materials for these compounds have also made it difficult to establish appropriate levels of identification in biological specimens. As new designer drugs continue to flood the market, more analytical methods need to be developed and updated. Therefore, it is imperative that lawmakers and scientists are constantly up-to-date with these emerging novel psychoactive substances.

This method has demonstrated the sensitivity, reliability, and robust capabilities of detecting synthetic cannabinoids in human whole blood. The results of this study and its fully validated parameters allow it to be adopted and used for identification and quantification in forensic and clinical casework.

8. Future Studies

8.1 Method Expansion

As new synthetic cannabinoids emerge, it may be necessary to reevaluate or even expand the method to include more synthetic cannabinoids. Metabolites and other biomarkers are also important to consider and incorporate in this method. In addition, this

method should be expanded to test for synthetic cannabinoids in other biological matrices (e.g., urine, saliva, plasma, etc.).

8.2 Stability Studies

Other avenues to consider with future studies include long-term stability studies. Many testing labs may store specimens for long-term storage before initial screening or even re-testing. Therefore, stability of these compounds needs to be assessed in long-term storage in various conditions such as room temperature, refrigerator (1-10°C) and freezer (-20 ^{o}C) conditions. Analytes should be prepared in the appropriate matrix and stored and tested at various time points to determine the level of degradation that these compounds can undergo. Another stability study that may need to be considered is also freeze-thaw cycles. These studies will help scientists better understand the properties and characteristics of these compounds.

APPENDIX A: LIMIT OF QUANTITATION CHROMATOGRAPHIC DATA

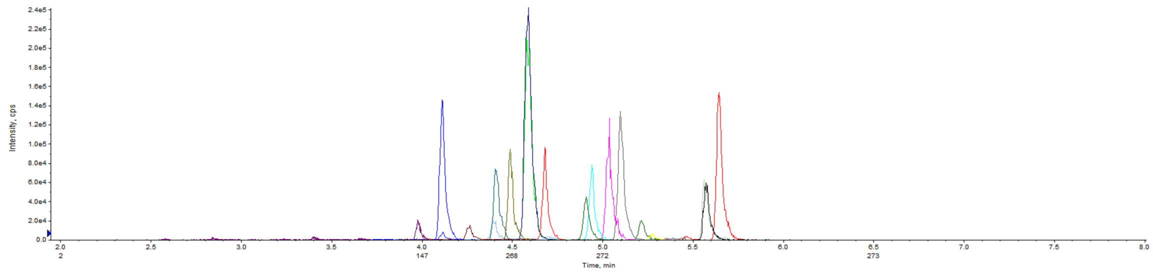


Figure A: 0.5 ng/mL Sample of All Analytes in Blood

APPENDIX B: CARRYOVER STUDY CHROMATOGRAPHIC DATA

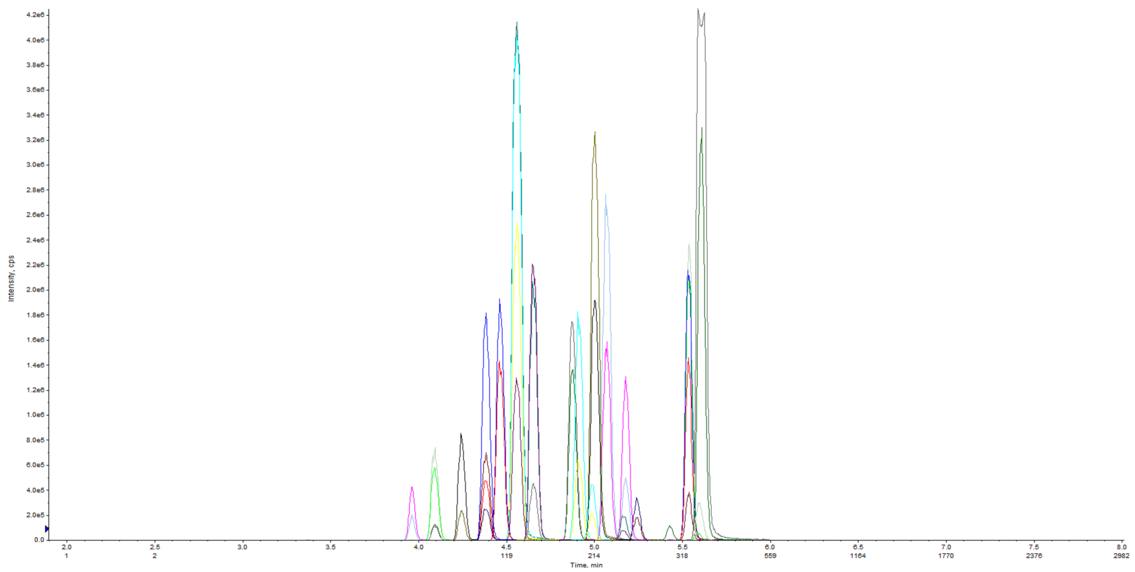


Figure B1: 25 ng/mL Sample of All Analytes in Blood. Complete chromatographic separation of all analytes.

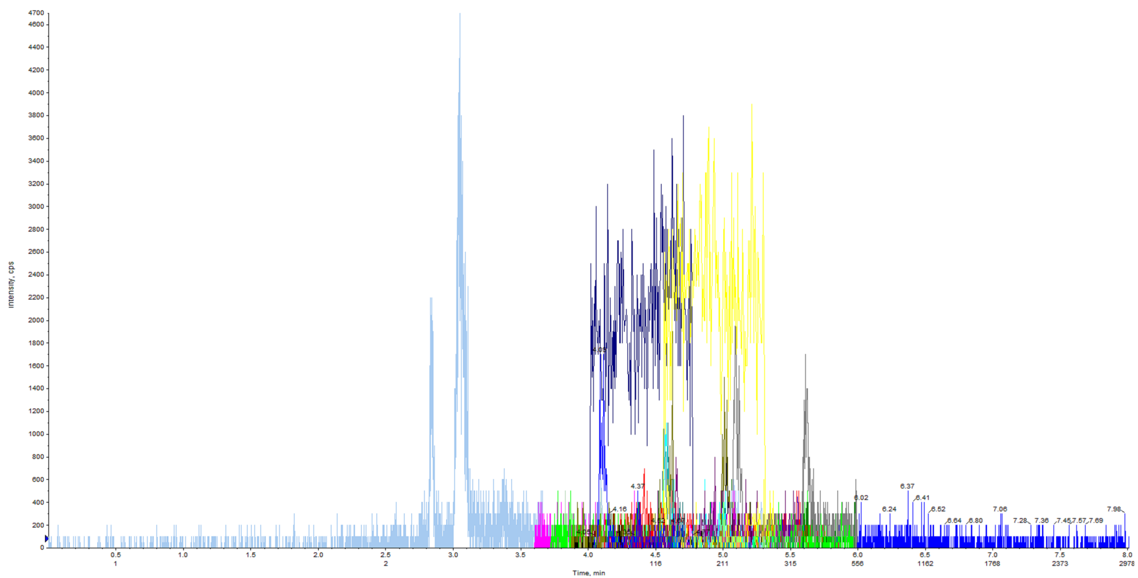


Figure B2: Double Blank (Blood Matrix) Following 25 ng/mL Sample in Blood.

APPENDIX C: MATRIX EFFECT STUDY CHROMATOGRAPHIC DATA

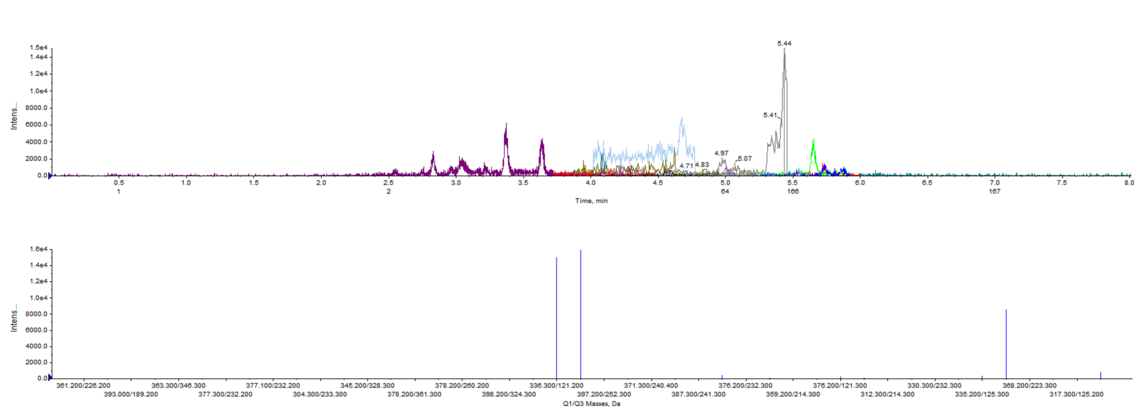


Figure C1: Double Blank from Blood Matrix Lot 1. Slight signal intensity is present for JWH-210 with associated mass ions 336.3/121.2 (Q1/Q3). However, the average signal area for JWH-210 was calculated to be less than the LOD.

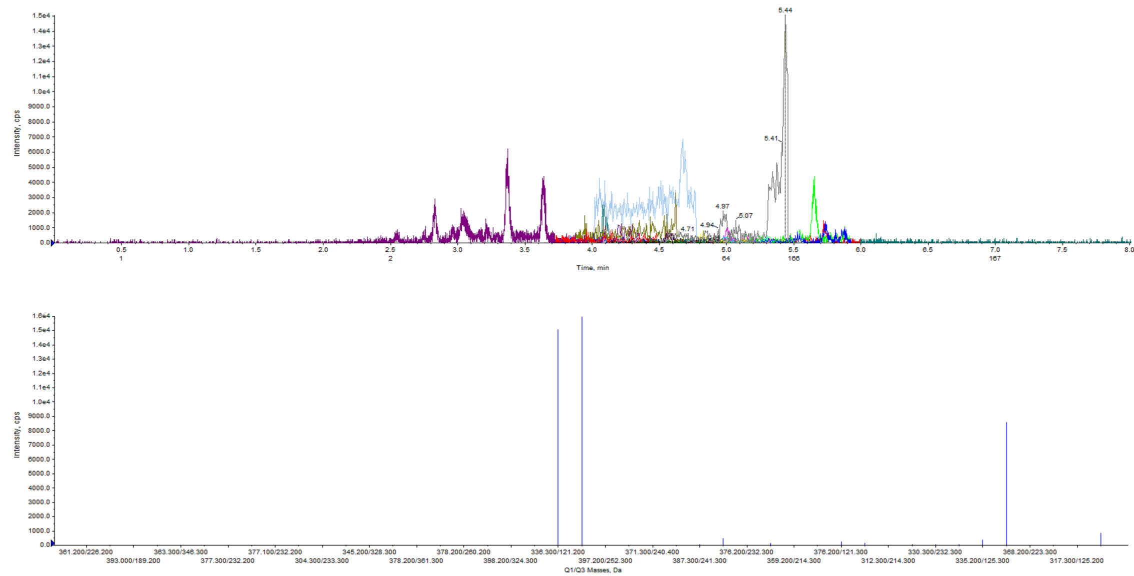


Figure C2: Double Blank from Blood Matrix Lot 2. Slight signal intensity is present for JWH-210 with associated mass ions 336.3/121.2 (Q1/Q3). However, the average signal area for JWH-210 was calculated to be less than the LOD.

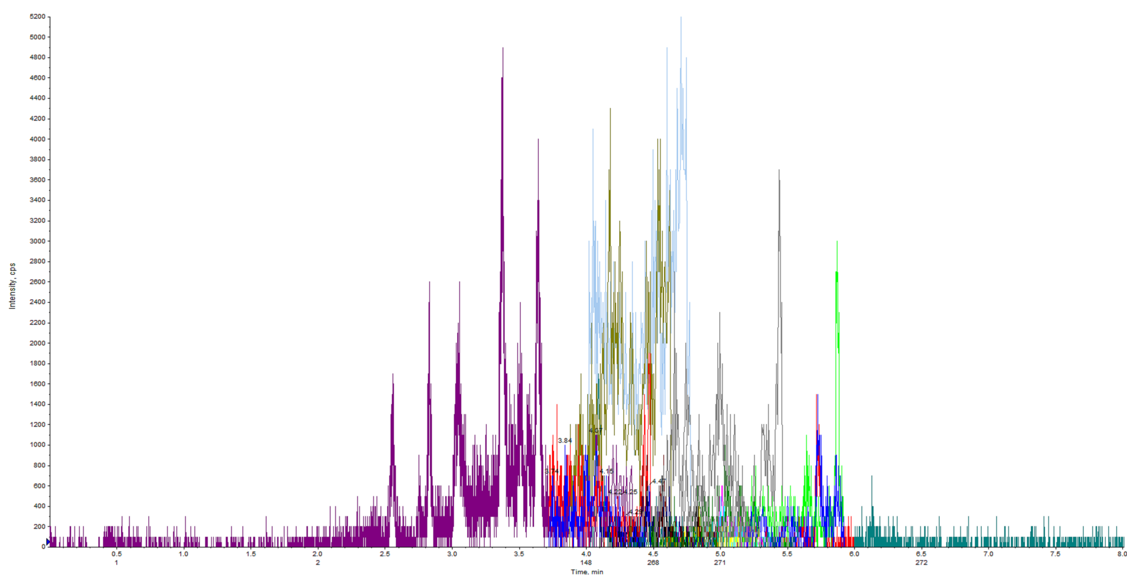


Figure C3: Double Blank from Blood Matrix Lot 3. No interference present in blank matrix.

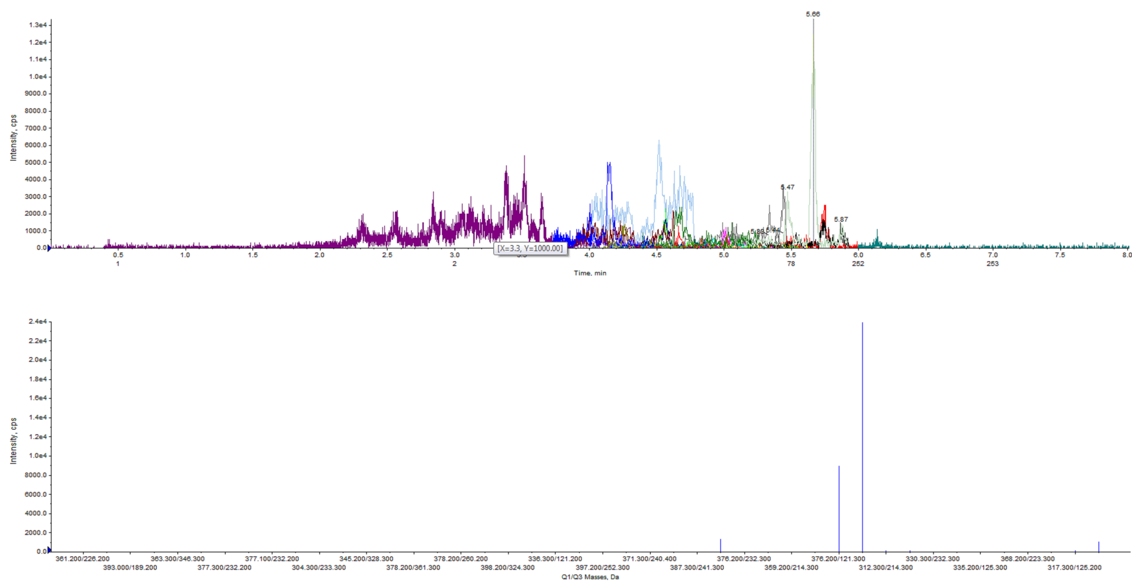


Figure C4: Double Blank from Blood Matrix Lot 4. Slight signal intensity is present for JWH-210 but the associated mass ions do not indicate confirmation. However, the average signal area for JWH-210 was calculated to be less than the LOD.

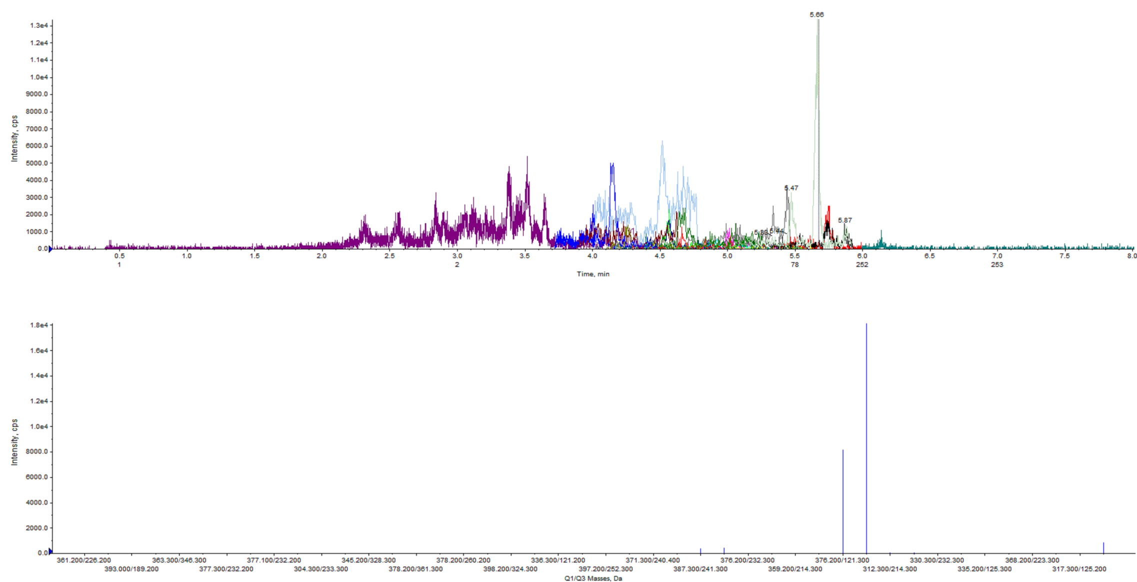


Figure C5: Double Blank from Blood Matrix Lot 5. Slight signal intensity is present for RCS-8 with associated mass ions 376.2/121.3 (Q1/Q3). However, the average signal area for RCS-8 was calculated to be less than the LOD.

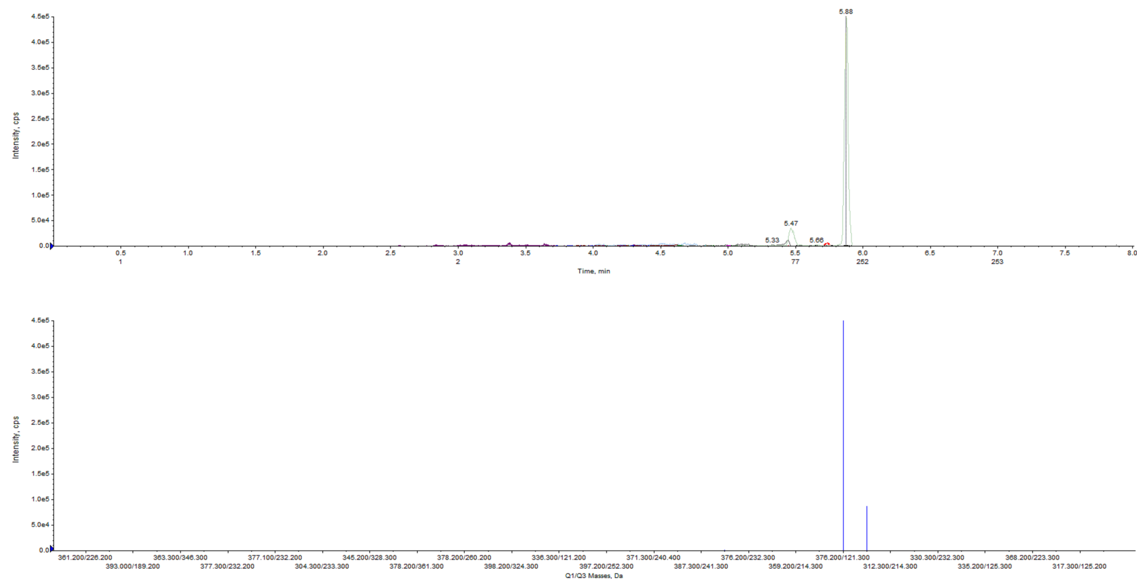


Figure C6: Double Blank from Blood Matrix Lot 6. Slight signal intensity is present for RCS-8 with associated mass ions 376.2/121.3 (Q1/Q3). However, the average signal area for RCS-8 was calculated to be less than the LOD.

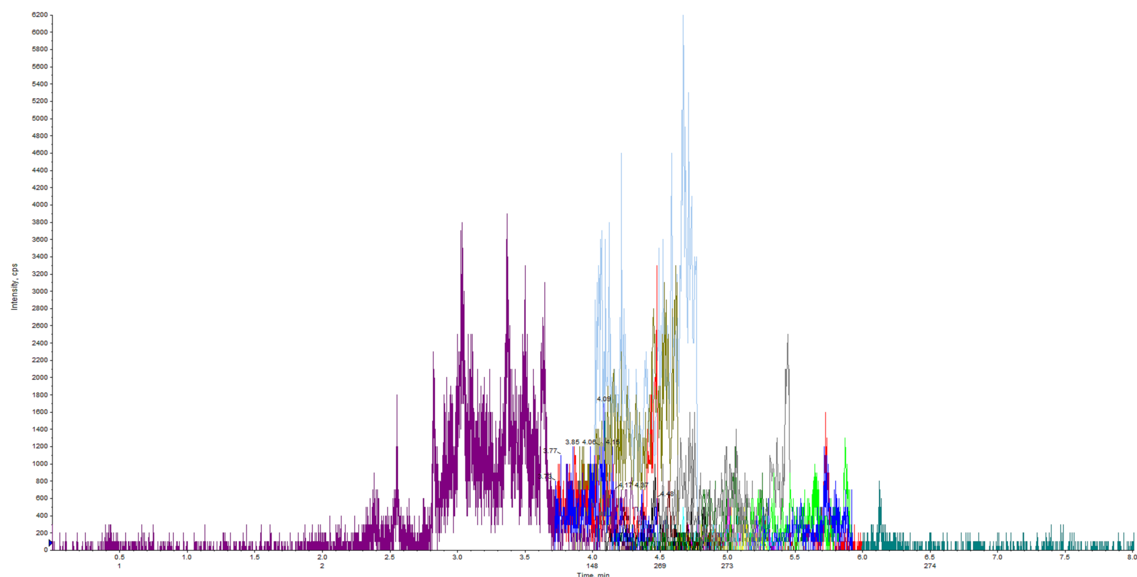


Figure C7: Double Blank from Blood Matrix Lot 7. No interferences present in blank matrix.

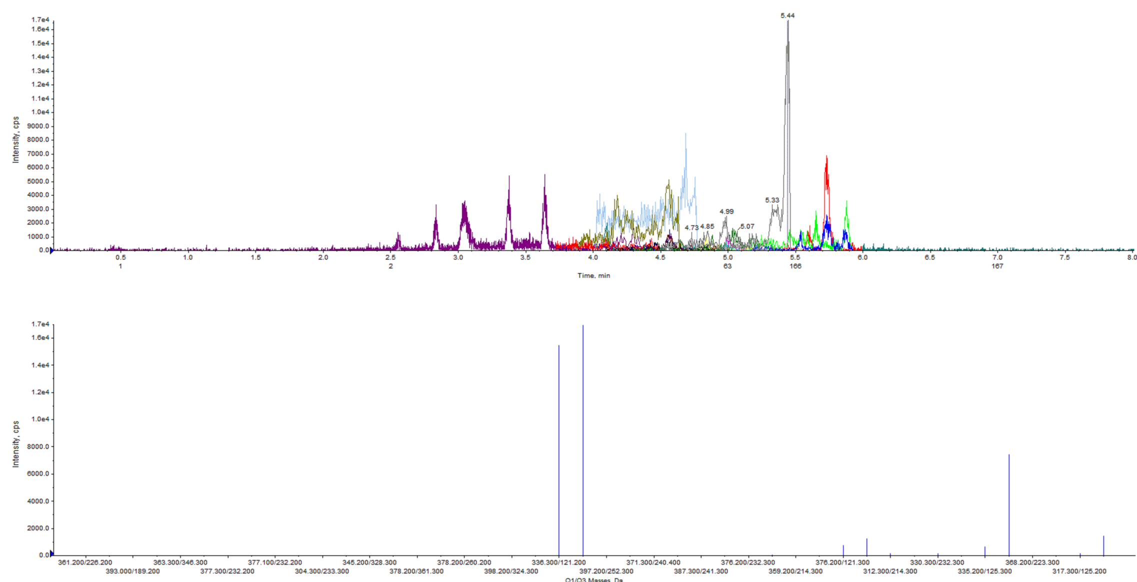


Figure C8: Double Blank from Blood Matrix Lot 8. Slight signal intensity is present for JWH-210 with associated mass ions 336.3/121.2 (Q1/Q3). However, the average signal area for JWH-210 was calculated to be less than the LOD.

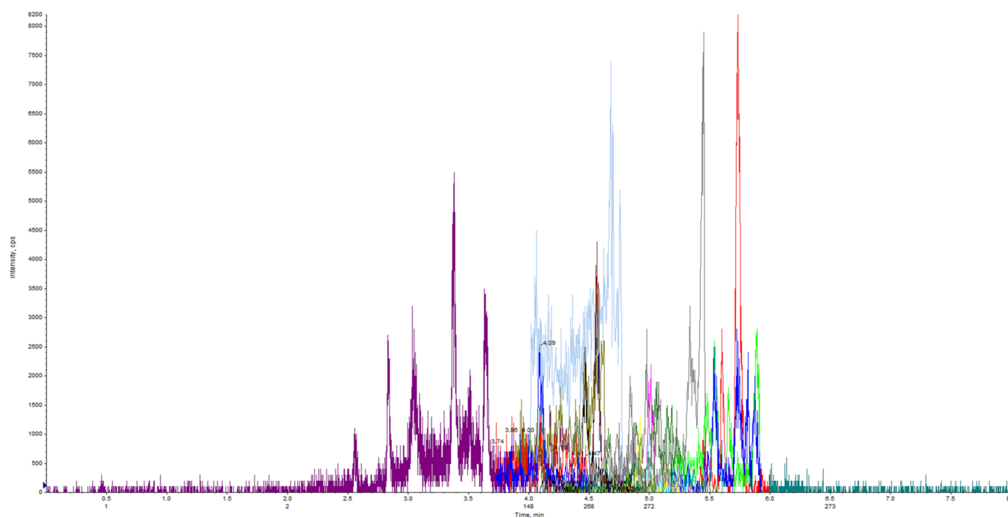


Figure C9: Double Blank from Blood Matrix Lot 9. No interferences present in blank matrix.

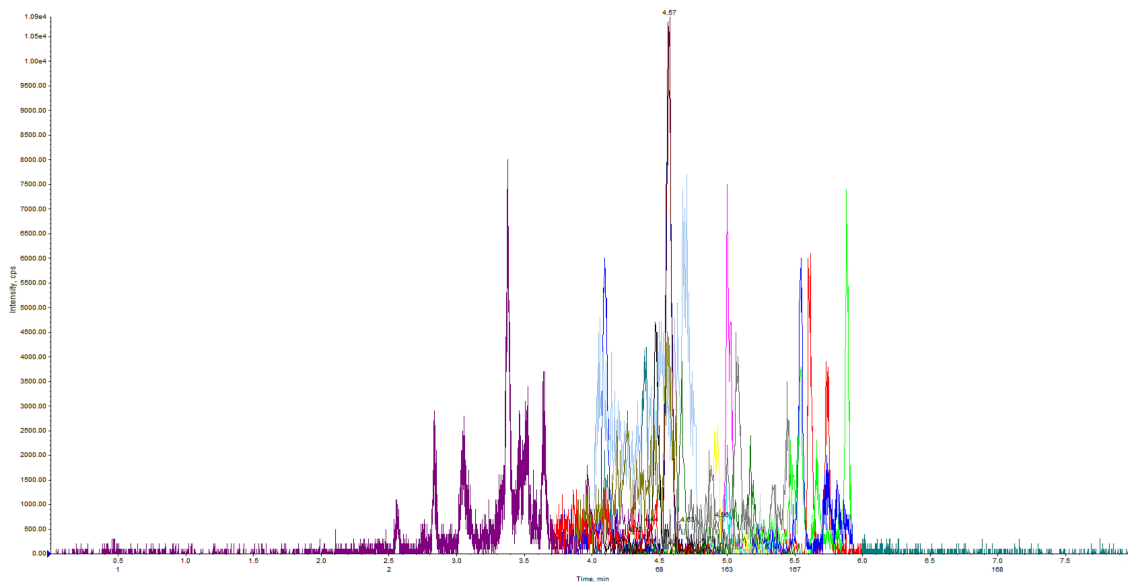


Figure C10: Double Blank from Blood Matrix Lot 10. No interferences present in blank matrix.

APPENDIX D: IONIZATION SUPPRESSION AND ENHANCEMENT & MATRIX RECOVERY CHROMATOGRAPHIC DATA

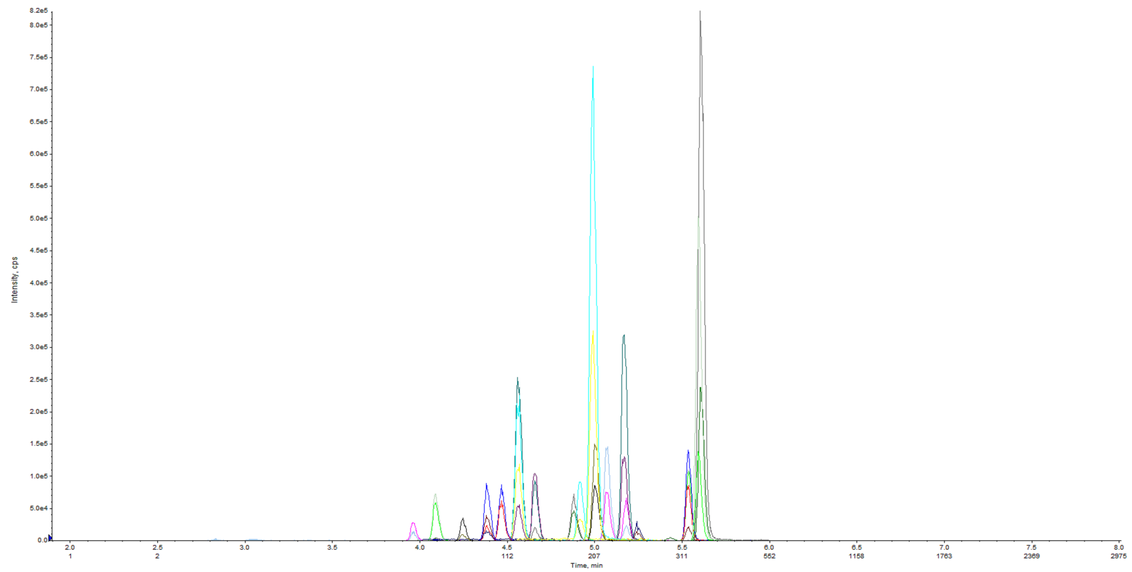


Figure D1: 0.5 ng/mL Neat Sample of All Analytes.

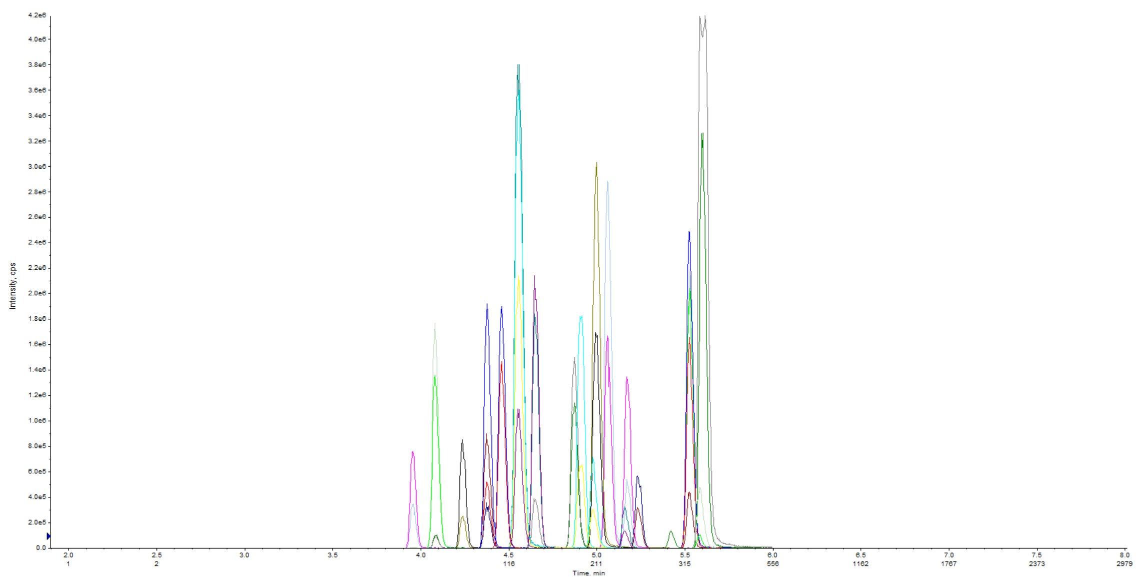


Figure D2: 15 ng/mL Neat Sample of All Analytes.

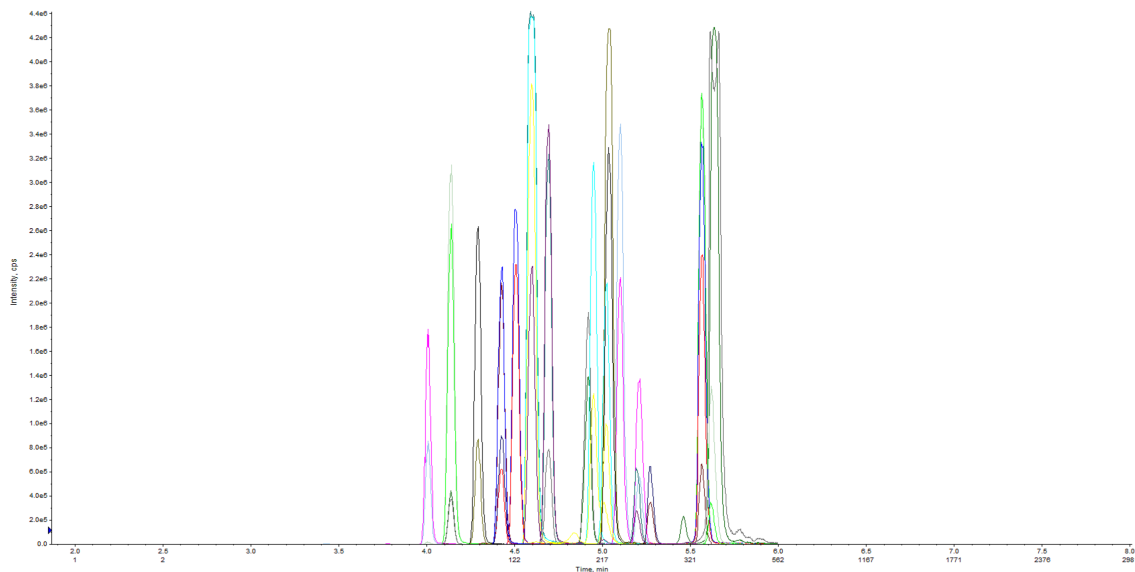


Figure D3: 0.5 ng/mL Post-Extracted Samples of All Analytes.

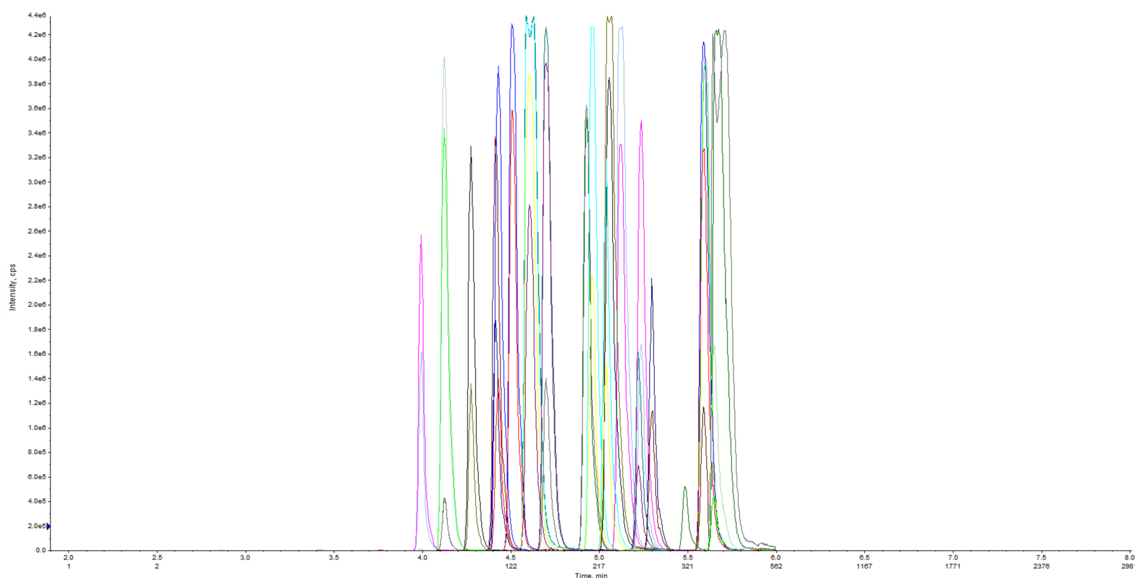


Figure D4: 15 ng/mL Post-Extracted Samples of All Analytes.

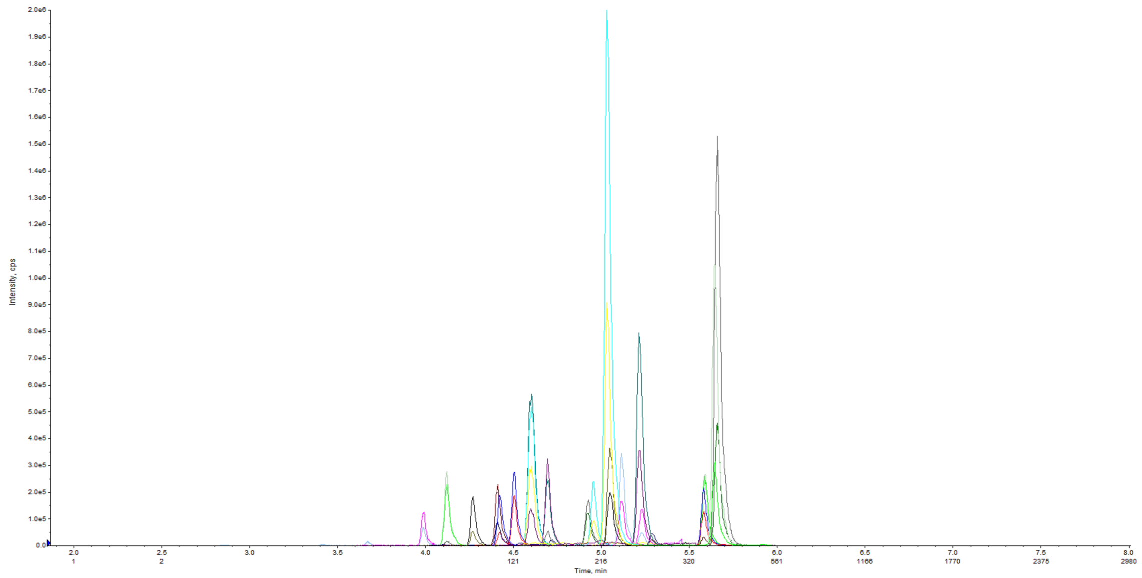


Figure D5: 0.5 ng/mL Pre-Extracted Samples of All Analytes.

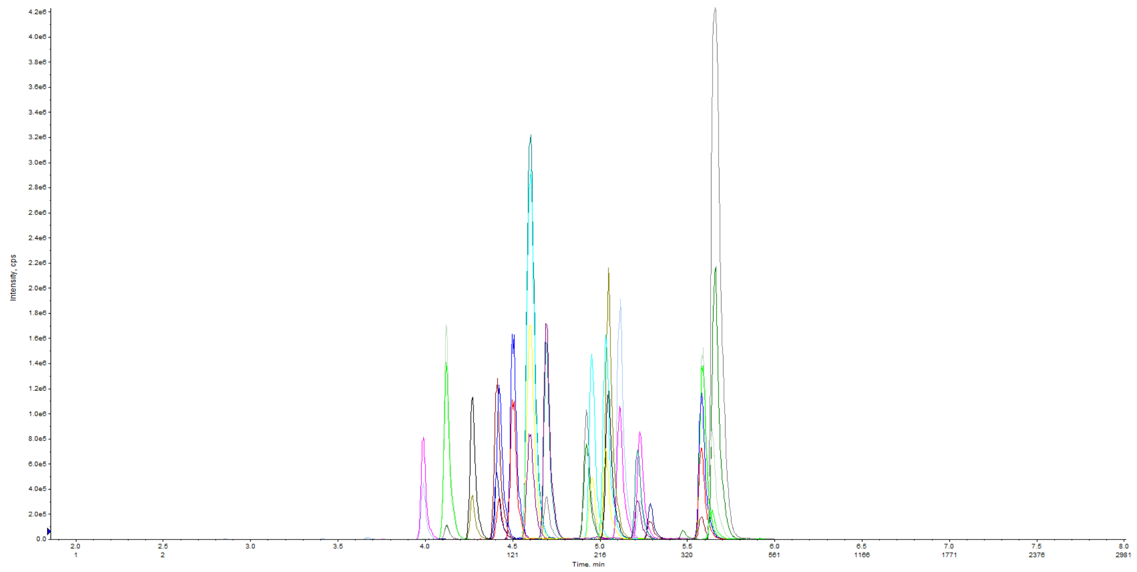


Figure D6: 15 ng/mL Pre-Extracted Samples of All Analytes.

BIBLIOGRAPHY

1. Abrams DI. Integrating cannabis into clinical cancer care. Current Oncology 2016;23(March):S8–14.

2. (NCSL) National Conference of State Legislatures. State Medical Marijuana Laws [Internet]. NCSL. 2017 [cited 2017 Jan 1];Available from: http://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx

3. Kumar RN, Chambers WA, Pertwee RG. Pharmacological actions and therapeutic uses of cannabis and cannabinoids. Anaesthesia 2001;56(11):1059–1068.

4. Barceloux D. Marijuana (Cannabis sativa L.) and Synthetic Cannabinoids. In Medical Toxicology of Drug Abuse: Synthesized Chemical Psychoactive Plants (pp. 886–931); Hoboken, NJ: John Wiley & Sons, Inc.: 2012.

5. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacology & Therapeutics 1997;74(2):129–180.

6. Huffman JW, Zengin G, Wu M-J, Lu J, Hynd G, Bushell K, et al. Structure–activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB1 and CB2 receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB2 receptor agonists. Bioorganic & Medicinal Chemistry [Internet] 2005;13(1):89–112. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0968089604007679

7. UNODC. Synthetic cannabinoids in herbal products. [Internet]. Available from: https://www.unodc.org/documents/scientific/Synthetic_Cannabinoids.pdf

8. Vardakou I, Pistos C, Spiliopoulou C. Spice drugs as a new trend: Mode of action, identification and legislation. Toxicology Letters [Internet] 2010;197(3):157–162. Available from: http://dx.doi.org/10.1016/j.toxlet.2010.06.002

9. Appendino G, Minassi A, Taglialatela-Scafati O. Recreational drug discovery: natural products as lead structures for the synthesis of smart drugs. Natural Product Reports [Internet] 2014;31(7):880. Available from: http://xlink.rsc.org/?DOI=c4np00010b

10. Shevyrin V, Melkozerov V, Endres GW, Shafran Y, Morzherin Y. On a New Cannabinoid Classification System: A Sight on the Illegal Market of Novel Psychoactive Substances. Cannabis and Cannabinoid Research [Internet] 2016;1(1):186–194. Available from: http://online.liebertpub.com/doi/10.1089/can.2016.0004

11. Rinaldi-Carmona M, Barth F, Millan J, Derocq J-M, Casellas P, Congy C, et al. SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. Journal of Pharmacology and Experimental Therapeutics 1998;284(2): 644–650.

12. Fattore L, Fratta W. Beyond THC: The New Generation of Cannabinoid Designer Drugs. Frontiers in Behavioral Neuroscience [Internet] 2011;5(September):1–12. Available from: http://journal.frontiersin.org/article/10.3389/fnbeh.2011.00060/abstract

13. Gilani F. Novel psychoactive substances: The rising wave of legal highs. British Journal of General Practice 2016; 66(642):8–9.

14. Zuardi AW, Crippa JAS, Hallak JEC, Moreira FA, Guimarães FS. Cannabidiol, a Cannabis sativa constituent, as an antipsychotic drug. Brazilian Journal of Medical and Biological Research 2006;39(4):421–429.

15. U.S. Dept. of Justice, Drug Enforcement Administration, Diversion Control Division. Title 21 United States Code (USC) Controlled Substances Act, Subchapter I – Control and Enforcement: Substances C, Reporting P, Provisions E, Date E, Provisions G. Regulatory Information Controlled Substances Act. (1). [Internet]; Available from: https://www.deadiversion.usdoj.gov/21cfr/21usc/812.htm

16. Roffman RA. The Controlled Substances Therapeutic Research Act in the State of Washington. Journal of Clinical Pharmacology 1981;21:133S–140S.

17. Martin-Calderon JL, Munoz RM, Villanua MA, Del Arco I, Moreno JL, De Fonseca FR, et al. Characterization of the acute endocrine actions of (-)-11-hydroxy-delta8-tetrahydrocannabinol-dimethylheptyl (HU-210), a potent synthetic cannabinoid in rats. European Journal of Pharmacology 1998;344(1):77–86.

18. Lan R, Lu Q, Fan P, Gatley J, Volkow ND, Fernando SR, Pertwee R, Makriyannis, A. Design and synthesis of the CB1 selective cannabinoid antagonist AM281: A potential human SPECT ligand. AAPS Journal [Internet] 1999;1(2): 39–45. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2761119/pdf/12248_2008_Article_120039.pdf

19. Buchler I, Hayes JM, Hedge GS, Hockerman LS, Jones ED, Kortum WS, Rico GJ, Tenbrink RE, Wu KK. Indazole Derivatives [Internet]. 2009; Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2009106982&recNum=140&docAn=IB2009000432&queryString=(PA/Pfizer) AND (IC/C07) &maxRec=1750

20. Bilici R. Synthetic cannabinoids. Northern Clinics of Istanbul [Internet] 2014;1(2):121–126. Available from: http://www.journalagent.com/z4/download_fulltext.asp?pdir=nci&plng=eng&un=NCI-44153

21. Kikura-Hanajiri R, Uchiyama N, Goda Y. Survey of current trends in the abuse of psychotropic substances and plants in Japan. Legal Medicine 2011;13(3):109–115.

22. Auwärter V, Dresen S, Weinmann W, Müller M, Pütz M, Ferreirós N. “Spice” and other herbal blends: harmless incense or cannabinoiddesigner drugs? Journal of Mass Spectrometry 2008;44:832–837.

23. Brents, LK and Prather PL. The K2/Spice phenomenon: Emergence, identification, legislation and metabolic characterization of synthetic cannabinoids in herbal incense products. Drug Metabolism Reviews 2014;46(1):72–85.

24. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). 2009 Annual Report: The State of the Drugs Problem in Europe [Internet]. Luxembourg: Publications Office of the European Union. 2009; Available from: http://www.emcdda.europa.eu/system/files/publications/970/EMCDDA_AR2009_EN.pdf

25. Advisory Council on the Misuse of Drugs (ACMD). Consideration of the major cannabinoid agonists [Internet]. 2008; Available from: http://www.namsdl.org/library/E2E91AB8-1372-636C-DDFC2BA64093B64C/

26. United Kingdom, House of Parliament. The Misuse of Drugs Act 1971 (Amendment) Order 2009 [Internet]. 2009; Available from: https://www.legislation.gov.uk/uksi/2009/3209/pdfs/uksi_20093209_en.pdf

27. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Understanding the “Spice” phenomenon. 2009; Available from: http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cannabinoids

28. L. Ernest, H-M. Shiebel, C. Theuring, R. Lindigkeit TB. Identification and characterization of JWH-122 used as new ingredient in “Spice-like” herbal incenses. Forensic Science International 2011;208:e31–35.

29. Mason M. Lethal Highs. Frontline: U.S. Customs and Border Protection 2013; 6(3):11–16. Available from: https://www.cbp.gov/sites/default/files/documents/Frontline_Vol6_Issue3.pdf

30. Drug Enforcement Administration (DEA). Diversion Control - Controlled Substance Schedules. 2012; https://www.deadiversion.usdoj.gov/schedules/

31. United States Dept. of Justice, Drug Enforcement Administration (DEA). Schedules of Controlled Substances: Temporary Placement of Five Synthetic Cannabinoids Into Schedule I. Federal Register 2011;76(40):11075–11078.

32. United Nations Office on Drugs and Crime (UNODC). World Drug Report 2013. 2013; Available from: https://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf

33. Dresen S, Kneisel S, Weinmann W, Zimmermann R, Auwärter V. Development and validation of a liquid chromatography–tandem mass spectrometry method for the quantitation of synthetic cannabinoids of the aminoalkylindole type and methanandamide in serum and its application to forensic samples. Journal of Mass Spectrometry 2011;46:163–171.

34. Grabenauer M, Krol WL, Wiley JL, Thomas BF. Analysis of synthetic cannabinoids using high-resolution mass spectrometry and mass defect filtering: Implications for non-targeted screening of designer drugs. Analytical Chemistry 2012;84(13):5574–5581.

35. Kikura-Hanajiri, Uchiyama N GY. Survey of current trends in the abuse of psychotropic substances and plants in Japan. Legal Medicine 2011;3:109–115.

36. Spaderna M, Addy PH, D'Souza DC. Spicing things up: Synthetic cannabinoids. Psychopharmacology 2013;228(4):525–540.

37. Musah RA, Domin MA, Walling MA SJ. Rapid identification of synthetic cannabinoids in herbal samples via direct analysis in real time mass spectrometry. Rapid Communications in Mass Spectrometry: RCM 2012;26(9):1109–1114.

38. Vandrey R, Dunn KE, Fry JA, Girling ER. A survey study to characterize use of Spice products (synthetic cannabinoids). Drug and Alcohol Dependence 2012; 120(1–3):238–241.

39. United States Dept. of Justice, Drug Enforcement Administration (DEA). Schedules of Controlled Substances: Temporary Placement of Five Synthetic Cannabinoids Into Schedule I. Federal Register 2011;76(40):11075–11078

40. NIDA (National Institute on Drug Abuse). Synthetic Cannabinoids (Nationwide Trends) [Internet]. 2015 [cited 2018 Jul 1];Available from: https://d14rmgtrwzf5a.cloudfront.net/sites/default/files/drugfacts_nationtrends_6_15.pdf

41. American Association of Poison Control Centers (AAPCC). AAPCC Issues Statement On the Synthetic Drug Abuse Prevention Act. 2012;(July). Available

from:
https://aapcc.s3.amazonaws.com/pdfs/releases/Statement_on_Signing_of_Synthetic_Drug_Act_7.2012.pdf

42. Wood KE. Exposure to Bath Salts and Synthetic Tetrahydrocannabinol from 2009 to 2012 in the United States. Journal of Pediatrics 2013;163(1):213–216.

43. U.S. Drug Enforcement Administration; Diversion Control Division. Synthetic Cannabinoids and Synthetic Cathinones Reported in NFLIS, 2013–2015. 2016;(December):2013–5. Available from: https://www.nflis.deadiversion.usdoj.gov/DesktopModules/ReportDownloads/Reports/NFLIS-SR-SynthCannabinoidCathinone.pdf

44. NIDA (National Institute on Drug Abuse). Monitoring the Future Survey: High School and Youth Trends [Internet]. 2017 [cited 2018 Jul 1]; Available from: https://www.drugabuse.gov/publications/drugfacts/monitoring-future-survey-high-school-youth-trends

45. NFLIS (National Forensic Laboratory Information System). 2015 Annual Report. 2015;1–32. Available from: http://www.cysticfibrosis.ca/uploads/Registry Report 2015/2015 Registry Annual Report EN.pdf

46. Dresen S, Ferreirós N, Pütz M, Westphal F, Zimmermann R, Auwärter V. Monitoring of herbal mixtures potentially containing synthetic cannabinoids as psychoactive compounds. Journal of Mass Spectrometry 2010;45(10):1186–1194.

47. Leggett JD, Aspley S, Beckett SR, D'Antona AM, Kendall DA, Kendall DA. Oleamide is a selective endogenous agonist of rat and human CB1 cannabinoid receptors. British Journal of Pharmacology 2004;2:253–262.

48. Schneir AB, Cullen J, Ly BT. “Spice” girls: Synthetic cannabinoid intoxication. Journal of Emergency Medicine 2011;40(3):296–299.

49. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Synthetic cannabinoids and “Spice” drug profile [Internet]. [cited 2017 Jan 1]; Available from: http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cannabinoids

50. Howlett AFR. Cannabinoid inhibition of adenylate cyclase pharmacology of the response in neuroblastoma cell membranes. Molecular Pharmacology 1984;26: 532–538.

51. Bow EW, Rimoldi JM. The Structure – Function Relationships of Classical Cannabinoids : CB1 / CB2 Modulation. Perspectives in Medicinal Chemistry 2016;1940:17–39.

52. Ghosh E, Kumari P, Kaiman D, Shukla A. Methodological advances: The unsung heroes of the GPCR structural revolution. Nature Reviews. Molecular Cell Biology 2015;16(2):69–81.

53. Devane WA, Dysarz FA, Johnson RM, Melvin S, Howlett C. Determination and characterization of a cannabinoid receptor in rat brain. Molecular Pharmacology 1988;34(5):605–613.

54. Demuth D, Molleman A. Cannabinoid signalling. Life Sciences 2006;76(6):549–563.

55. Munro S, Thomas K, Abu-Sharr M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993;365(6441):61–65.

56. Howlett AC, Barth F, Bonner TI. et al.. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological Reviews 2002;54(2):161–202.

57. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, et al. Cannabinoid receptor localization in brain. Proceedings of the National Academy of Sciences of the United States of America 1990;87:1932–1936.

58. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. European Journal of Biochemistry 1995; 232(1):54–61.

59. Maresz K, Carrier EJ, Ponomarev ED, Hilliard CJ, Dittel BN. Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. Journal of Neurochemistry 2005;95(2):437–445.

60. Bronstein AC, Spyker DA, Cantilena LA, Green JL, Rumack BH, Dart RC. 2010 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 28th Annual Report. Clinical Toxicology 2011;49: 910–941.

61. Castaneto MS, Wohlfarth A, Desrosiers NA, Hartman RL, Gorelick DA, Huestis MA. Synthetic cannabinoids pharmacokinetics and detection methods in biological matrices. Drug Metabolism Reviews [Internet] 2015;47(2):124–174. Available from: http://www.tandfonline.com/doi/full/10.3109/03602532.2015.1029635

62. Levine B. Principles of Forensic Toxicology 4th ed. Washington, D.C.: American Association for Clinical Chemistry, 2013.

63. Bergamaschi MM, Queiroz RH, Chagas MH et al. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. Neuropsychopharmacology 2011;36(6):1219–1226.

64. Spaderna M, Addy PH, D'Souza DC. Spicing things up: synthetic cannabinoids. Psychopharmacology 2013;228(4):525–540.

65. Barratt M, Cakic V, Lenton S. Patterns of synthetic cannabinoid use in Australia. Drug and Alcohol Review 2012;32(2):141–146.

66. Phikal DT. Marijuana for K2 withdrawal: An experience with spice. 2013; Available from https://erowid.org/experiences/exp.php?ID=94940

67. Rominger A, Cumming P, Xiong G, et al. Effects of acute detoxification of the herbal blend "Spice Gold" on dopamine D2/3 receptor availability: A [18F]fallypride PET study. European Neuropsychopharmacology 2013;23(11): 1606–1610.

68. Pant S, Dholaria BD, Kaur V, et al. Spicy seizure. The American Journal of Medical Sciences 2012;334(1):67–68.

69. Fantegrossi WE, Moran JH, Radominska-Pandya A, Prather PL. Distinct pharmacology and metabolism of K2 synthetic cannabinoids compared to Δ9-THC: Mechanism underlying greater toxicity? Life Sciences 2014;97(1):45–54.

70. Watanabe K, Yamaori S, Funahashi T et al. Cytochrome P450 enzymes involved in the metabolism of tetrahydrocannabinols and cannabinol by human hepatic microsomes. Life Sciences 2007;80(15):1415–1419.

71. Chimalakonda KC, Seely KA, Bratton SM, Brents LK, Moran CL, Endres GW, et al. Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: Identification of novel cannabinoid receptor ligands. Drug Metabolism and Disposition 2012;40(11):2174–2184.

72. Grotenhermen F. Pharmacokinetics and pharmacodynamics of cannabinoids. Clinical Pharmacokinetics 2003;42(4):327–360.

73. Pitt CG, Hendron RW, Hsia RS. The specificity of the Duquenois color test for marihuana and hashish. Journal of Forensic Sciences 1972;17(4):1–8.

74. Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology. Journal of Analytical Toxicology [Internet] 2013;37(7):452–474. https://doi.org/10.1093/jat/bkt054

75. Bayne S, Carlin M. Forensic Applications of High Performance Liquid Chromatography. CRC Press, 2010;

76. Bell S. Forensic Chemistry. 2nd ed. Glenview, IL: Education, Inc., 2013;

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