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ANALYSIS OF SYNTHETIC CANNABINOIDS IN URINE, PLASMA, AND EDIBLES UTILIZING MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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KAYLA M. BENVENUTO

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Approved by

First Reader

Sabra Botch-Jones, M.S., M.S., M.A., D-ABFT-FT
Assistant Professor, Program in Biomedical Forensic Sciences
Department of Anatomy & Neurobiology

Second Reader

Claude R. Mallet, Ph.D.
Senior Consulting Scientist Workflow Integration
Waters Corporation

Third Reader

Sean Kocur, M.S., Ph.D.
Forensic Toxicologist, R&D Manager
SCIEX

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ANALYSIS OF SYNTHETIC CANNABINOINDS IN URINE, PLASMA, AND EDIBLES UTLIZING MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

KAYLA M. BENVENUTO

ABSTRACT

Synthetic cannabinoids (SCs), present a multitude of problems in terms of maintaining up-to-date, reliable, specific, and sensitive methods of detection. Synthetic cannabinoids are novel psychoactive substances originally synthesized for medical use and research purposes. Abuse of these compounds, however, has demonstrated a variety of effects ranging from euphoria to aggressive behavior and loss of consciousness. The most dangerous reported result of synthetic cannabinoids use has been death. The number of synthetic cannabinoid compounds detected drastically increased from two to over 80 compounds within six years. The marketing of these compounds, similar naming, and described pharmacological interactions, create the dangerous and very false perception that SCs are similar to, or the same as, tetrahydrocannabinol in cannabis products.

This research focused on the development of a method to detect and quantify seven synthetic cannabinoids in urine, plasma, and gummy bears. The seven synthetic cannabinoids studied include XLR-11, AB-PINACA 5-pentanoic acid metabolite, UR-144 5-pentanoic acid metabolite, 5F-PB-22, AM-2201 4-hydroxypentyl metabolite, JWH-018, and JWH-018 5-hydroxypentyl metabolite. Sample preparation methods and a two dimensional liquid chromatography tandem mass spectrometry method were optimized and developed for analysis of the seven SCs in each matrix. The method was

successfully applied to 17 authentic urine case samples previously screened positive for synthetic cannabinoids and a calibration curve for each matrix was generated from spiked samples at varying concentrations. Utilizing two-dimensional (2D) chromatography for the analysis of synthetic cannabinoids allowed for a novel approach to be employed. With this method, 100% organic samples were analyzed with improved resolution and increased sensitivity.

The sample preparation method for the urine and plasma samples included a protein precipitation technique with acid followed by solid phase extraction (SPE) on a mixed-mode reversed phase strong anion exchange sorbent. The spiked gummy bear samples were prepared in 50% methanol in water, dissolved by heating, and extracted with SPE on the same sorbent used for the urine and plasma samples. A 200µL injection of the 100% MeOH extracts was injected into 2D-LC-MS/MS for analysis with a loading and diluting solvent consisting of water and 2% ammonium hydroxide and elution solvents containing water or methanol with 0.5% formic acid. These conditions were optimized with an automated method development protocol assessing various conditions such as mobile phase solvents, pH additives, and trap column chemistries. The final chromatography method utilized an ACQUITY ultra performance liquid chromatography (UPLC) ethylene bridged hybrid (BEH) C8 2.1 x 30mm, 10µm trap column and an ACQUITY UPLC high strength silica with tri-functional C18 bonding (HSS T3) analytical column 2.1 x 150mm, 1.7µm.

The urine calibration curve produced had a linear dynamic range (LDR) of 0.05-2.5ng/mL for UR-144 5-COOH and AB-PINACA 5-COOH and 0.05-5ng/mL for the

other five synthetic cannabinoids. R^2 values included 0.992 and 0.993 for UR-144 5-COOH and AB-PINACA 5-COOH, respectively and 0.995 or above for the other five compounds. Synthetic cannabinoids were detected at varying concentrations in all 17 urine case samples. Analysis of plasma and gummy bear samples was also successfully carried out. Plasma calibration curves had a LDR 0.05-10ng/mL with all R^2 values above 0.995. Gummy bear calibration curves produced a LDR of 0.05-10ng/mL or 0.05-2.5ng/mL with R^2 values over 0.995. All extraction recovery values were greater than 80% with the exception of 63% recovery for AB-PINACA 5-COOH in the gummy bear matrix. Suppression effects of 8%, 19%, and 6.6% were observed for urine, plasma, and gummy bears, respectively. Relatively low recovery values, reduced linear dynamic ranges, and suppression matrix effects for the carboxylic acid analytes assessed in this research suggested an alternative approach may be more successful for the analysis of these particular compound types in all three matrices. Overall, a sensitive, specific, and reliable method was developed with low limits of detection and quantification for efficient and rapid analysis of compounds at trace levels utilizing 2D-LC-MS/MS.

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LIST OF ABBREVIATIONS

1D	One Dimensional
2D	Two Dimensional
ACN	Acetonitrile
BEH	Ethylene Bridged Hybrid
C8	Silica Column with n-octylsilyl substituent
CE	Capillary Electrophoresis
CEDIA	Cloned Enzyme Donor Immunoassay
DART	Direct Analysis Real Time
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
EMIT	Enzyme Multiplied Immunoassay Technique
ESI	Electrospray Ionization
FA	Formic Acid
FID	Flame Ionization Detectors
GC	Gas Chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
H_3PO_4	Phosphoric Acid
HCl	Hydrochloric Acid
HEIA	Homogenous Enzyme Immunoassay
HLB	Hydrophilic Lipophilic Balance

HPLC	High Performance Liquid Chromatography
hr	Hour
HSS T3	High Strength Silica Column with tri-functional C18 bonding
I.D.	Internal Diameter
IPA	Isopropanol
kV	Kilovolts
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
M	Molar concentration (mol/L)
MA	Massachusetts
MALDI	Matrix-Assisted Laser Desorption/Ionization
MeOH	Methanol
mL	Milliliter
MO	Missouri
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
ng	Nanogram
NH_4OH	Ammonium Hydroxide

nL	Nanoliter
NPD	Nitrogen Phosphorus Detector
ppm	Parts per million
QuEChERS	Quick Easy Cheap Effective Rugged Safe
SIM	Selected-Ion Monitoring
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
TX	Texas
UPLC	Ultra Performance Liquid Chromatography
USA	United States of America
UV	Ultraviolet
W1	Wash 1
W2	Wash 2
µg	Microgram
µm	Micrometer

1. INTRODUCTION

1.1 Background

Synthetic cannabinoids are novel psychoactive compounds that have become increasingly popular with a dramatic expansion in terms of the number of compound types since they were first used illicitly. Typically, synthetic cannabinoids are a powder dissolved in a solvent and sprayed onto an herbal substance [1]. There have been a variety of street names used for these compounds including “Armageddon,” “aroma,” “Aztec gold,” “black mamba,” “cloud 9,” “demon,” “dream,” “Mad Hatter,” and “Mr. Nice Guy” [2]. These compounds are more commonly referred to as “Spice,” “K2,” and “SC” [1-3]. Spice compounds were originally synthesized for research purposes, as they are believed to have potential medical use [2]. They are studied medicinally for treatment of pain, “obesity, neurological diseases, emotional disturbances, and other psychiatric disorders including drug addiction” [4]. However, illicit use of SCs was described in Europe around 2004 and in the United States in 2008 [2, 5]. Since then, the popularity of synthetic cannabinoids has considerably increased. Additionally, there has been a significant growth in the types of compounds classified as synthetic cannabinoids. The compelling characteristics associated with their use such as ease of access, affordability, the potential for the substance to go undetected in standard drug testing methods, the ability to develop a stronger high in comparison to that associated with marijuana use, and the misconception that SCs are legal have undoubtedly played a role in the increased popularity and synthesis of synthetic cannabinoids [1, 2].

1.1.2 Types of Synthetic Cannabinoids, Classification, and Naming

There are many different compounds classified as synthetic cannabinoids, and with slight structural modifications to each, the list continues to grow. The cyclohexylphenol CP-47, 497 and the aminoalkylindole JWH-018 were among the first compounds to be synthesized [6]. In addition to JWH-018, other compounds that are grouped in the napthoylindole classification include JWH-015, JWH-019, and JWH-122. Phenylacetylindoles, benzoylindoles, adamantylindoles, and dibenzopyrans are also described classes of synthetic cannabinoids [5-7]. SCs such as RCS-4 and AM-694 are classified as benzoylindoles while HU-210 is considered a dibenzopyran, or a classical cannabinoid [6]. There is a lengthy chemical name associated with each synthetic cannabinoid, such as N-(1-adamantyl)-1-pentyl-1H-indole-3-carboxamide for the compound APICA and N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1H-indazole-3-carboxamide for AB-PINACA [8, 9]. However, the names more frequently used, like the ones previously listed, consist of a combination of different letters and numbers. Many times, the name corresponds to the individual or company that synthesized the compound or the location where it was first analyzed. Other times, the name may be termed in a certain way by illegal manufacturers and dealers to appeal to their users. The 'AM' in AM-2201 and the 'JWH' in the JWH series refers to the individuals who synthesized each compound, Alexandros Makriyannis and John W. Huffman, respectively. On the other hand, XLR-11 was named after liquid rocket fuel and 2NE1 after a South Korean girl band [8]. Sometimes the name differs from one compound to another because of the structural modifications made such as the fluorine addition that creates the difference

between PB-22 and 5F-PB-22. Simple changes to compounds create major issues in keeping up with the manufacturing of certified reference materials and methods of detection for all of the synthetic cannabinoids that are synthesized.

1.1.3 Manufacturing, Packaging, Sales, and Consumption

SCs are manufactured as powders and "contain modified molecular structures of illegal or controlled substances" [2]. They are typically manufactured in China and then shipped in bulk [8]. The powder is dissolved in a solvent such as methanol or acetone and then sprayed onto an herbal-like matter, or dried plant substance. In addition to the synthetic cannabinoids, additives including vitamin E and clenbuterol, flavors, and even other drugs such as cannabis, amphetamines, cocaine, and Kratom may be encountered [1, 2]. SCs have also been detected in other mixtures such as those containing stimulants or hallucinogens, ecstasy tablets, and liquids for e-cigarettes [8].

The plant matter with synthetic cannabinoids sprayed on is sold on the Internet or places such as head shops, gas stations, truck stops, and convenient stores in the form of incense, potpourri, and/or aromatherapy products [2, 5]. The false perception that these products are legal is often the result of the misleading labeling and marketing. Spice is commonly labeled 'not for human consumption,' 'herbal incense,' or 'for aromatherapy only' [2, 3, 6, 10]. They are also labeled in such a manner to avoid legal action or limitations on sales and distribution. Spice is typically packaged in shiny, metallic wrappers with bright colors and creative names. For example, some incense packets are named "Spice Gold" and "Yucatan Fire" [2, 8]. This type of packaging is utilized to gain the attention of young adults or first time users.

SC administration has been reported orally, rectally, and via vaporization with liquids utilized in electronic cigarette use. The primary method of administration, however, is smoking [11]. Unlike smoking marijuana, synthetic cannabinoids have a much stronger effect.

1.1.4 Scheduling

The scheduling of SCs has been difficult to keep up with since, with a slight structural modification, it is very easy for manufacturers to switch to the production of another synthetic cannabinoid once one structure becomes controlled [8]. The first major action taken to schedule SCs was in March 2011 when the United States Drug Enforcement Agency (DEA) listed JWH-018, JWH-073, JWH-200, CP-47, 497, and CP-47, 497 C8 homologue as Schedule I drugs [5]. Then, in May 2013, UR-144, XLR-11, and AKB-48 were also scheduled as Schedule I under the controlled substances act [5]. The Synthetic Drug Abuse Prevention Act of 2012 was signed by President Obama in July 2012 scheduling JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-200, JWH-203, JWH-250, JWH-398, AM694, AM2201, RCS-4, RCS-8, HU- 210, CP 47,497-C7, CP 47,497-C8 and their analogs as Schedule I controlled substances [1, 7]. On July 26, 2012 "the US DEA conducted "Operation Log Jam," seizing 18.4 million packets of synthetic cannabinoids, $36 million in cash, and arresting more than 90 people from 109 US cities" [1]. More recently, the DEA temporarily placed AB-CHMINACA, AB-PINACA, and THJ-2201 into Schedule I [9]. As a response to the continued legislation efforts, SC manufacturers are rapidly synthesizing new synthetic cannabinoid compounds

negatively impacts maintenance of up to date drug testing, the development of certified reference materials, legislative action, and the progress on research and treatment [7, 8].

1.2 Toxicology

1.2.1 Cannabinoid Receptors and Comparison to Cannabis

Synthetic cannabinoids elicit effects similar to those associated with the active ingredient in cannabis, tetrahydrocannabinol (THC). They also act on the same receptors in the body with agonistic effects. THC is the component of cannabis that contributes to the psychoactive effects often displayed when cannabis is used [8]. While SCs are commonly marketed and described as "synthetic marijuana" or as a substitute for cannabis, synthetic cannabinoids are very different from THC and the other natural cannabinoids present in the cannabis plant [1]. THC is a "low-efficacy partial agonist" and synthetic cannabinoids are "potent, full- and high-efficacy agonists"[1]. Furthermore, SCs have been described to be up to five times more potent than THC in marijuana, which enables stronger sympathomimetic and hallucinogenic effects [2].

In the body, THC and SCs act on two currently characterized cannabinoid receptors, CB1 and CB2. These receptors are "G-protein coupled receptors that were discovered in the 1990s and also have endogenous ligands" [12]. Cannabinoid-1 receptors are located in areas such as the heart, intestines, and liver and they are also largely present in the central nervous system. They are primarily responsible for the psychoactive effects that THC and SC compounds elicit. CB-2 receptors may possibly be located in the brain but they are mostly observed in the liver, lung, and kidney [13]. CB-2 receptors are responsible for analgesic effects. SC compounds have 100 times the binding

affinity to CB-1 and CB-2 receptors as compared to THC [12, 14]. As a result of the interactions with the cannabinoid receptors, SCs can present a multitude of effects.

1.2.2 Effects

Most of the information known about the effects associated with synthetic cannabinoid abuse is learned from medical case reports, users on online Internet forums, emergency departments, and Poison Control Centers. The onset and duration of symptoms can vary. They can be immediate or develop hours after use and they may last up to several hours [1]. Most importantly, use has been linked to "serious health consequences, including seizures, renal failure, and death" [2]. SCs are also weak monoamine oxidase inhibitors, which can lead to serotonin syndrome. Euphoria, relaxation, increased creativity, tingling, and calmness are a few acute symptoms that have been described to be associated with SC use. Additional symptoms include altered mental status, dry mouth, cough, tachycardia, injected conjunctivae, rhabdomyolysis, acute kidney injury, cardiac arrhythmias, myocardial infarction, addiction, aggressive behavior, agitation, chest pain, hypertension, loss of consciousness, nausea, and restlessness [2, 15]. Panic attacks, psychosis, and suicidal attempts have also been reported where "psychosis related to Spice use has received particular attention both in the media and medical literature" [1]. It has been discussed many times that the effects connected to psychosis are what ultimately forces the individual abusing SCs to get help, such as asking for medical attention.

In terms of chronic SC use, schizophrenia and possible memory loss are also concerns [2]. Another major area of concern is the effect SCs can have on driving

impairment. The sedating effects, fine motor skill impairment, blurry vision, pupillary changes, and light sensitivity could all play a role in affecting an individuals' safe driving ability [1, 15]. The problem with the effects SCs demonstrate is that there is very little information known about the toxicology that is behind these outcomes. Furthermore, as a result of the "variation and complexity of [the] herbal blends," it is difficult to gather information on the "cumulative effects" of SCs [6]. There can also be increased risks associated with SC use as a result of variations in the manufacturing processes. The SC solution may not be sprayed on to the herbal matter in an even distribution leading to some packages being more concentrated, and therefore much more potent, than others [8].

In regards to treatment, there has not yet been an antidote developed specifically for synthetic cannabinoid intoxication. Effects that are common to other types of drug use or medical conditions, such as hallucinations, agitation, and seizures can be treated with other drugs such as benzodiazepines [2]. Until there is more information about the toxicological and pharmacological characteristics of SCs, there may be limited treatment options.

1.2.3 Pharmacokinetics/Pharmacodynamics

Since synthetic cannabinoids are novel substances, there is still very little information known about the toxicity [8]. For instance, the LD_{50} is unknown for many SCs and the list of synthetic cannabinoid compounds continues to dramatically increase, making it challenging to maintain an understanding of the pharmacokinetic and pharmacodynamic properties of each compound. The liver is a primary location for SC

metabolism in addition to other secondary areas such as the kidneys [16]. Most studies have been conducted using *in vitro* experiments yielding some useful information regarding SC metabolism. Through these experiments, it was concluded that the JWH-018 metabolites consist of mono-, di-, and trihydroxylated forms in addition to N-dealkylated, carboxylated, and/or dehydrogenated products where the major product tends to be the monohydroxylated compounds [6]. Synthetic cannabinoid metabolites can be derived from multiple parent compounds. For example, it has been largely discussed that the metabolite JWH-018 5-hydroxypentyl can be the metabolite of either JWH-018 hydroxylation or AM-2201 defluorination [6, 16]. For SCs with a pentyl side chain, Diao and Huestis explained the major metabolic pathway to include hydroxylation followed by oxidation producing carboxylic acid and carbonyl metabolites [16]. They also described a similar pathway with fluoropentyl SCs after a defluorination step. For SCs with an ester linkage, ester hydrolysis appeared to be the major pathway producing carboxylic acid compounds [16]. Overall, the metabolites of the parent compounds are most prevalent in metabolic research that has been carried out thus far, emphasizing the importance and necessity of developing methods to detect all types of synthetic cannabinoids [16].

1.3 Literature Review on Synthetic Cannabinoid Analysis Techniques

1.3.1 Investigated Matrices and Sample Preparation

There are several detection techniques that have been used to screen for synthetic cannabinoids, but the methods for quantitative analysis are restricted. The quantitative and qualitative methods currently utilized become limited as new compounds are synthesized. Sample preparation of synthetic cannabinoids ultimately depends on the matrix. There have been several matrices studied thus far including urine, serum, hair, oral fluids, herbal blends, chemical powders, whole blood, plasma, mouse tissue, and sewage [5, 6]. Urine is a widely used matrix as it is less invasive to obtain in comparison to other matrices, enabling larger sample volumes to be collected for testing. It also allows for a longer window of detection and metabolites are commonly observed providing for additional verification. Preparation of urine typically involves a hydrolysis step with the use of a strong acid, base, or enzyme [6]. Synthetic cannabinoids in oral fluids have also been studied but such a matrix is less preferred over urine, perhaps because it possesses limiting qualities such as the slow rate of diffusion of SCs from the oral fluids into the bloodstream [6].

The sample preparation of herbal blends is fast and easy as long as the samples are representative since the synthetic cannabinoids may not be distributed evenly in concentration and volume throughout the herbal substrate [6]. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) have been reported sample preparation techniques of biological matrices demonstrating “nearly identical results” when comparing the two methods [6]. LLE has been described to be a useful extraction method

for SCs in biological matrices because they are highly hydrophobic compounds [17]. Yet, SPE has also been discussed as a primary method used in the analysis of biological matrices because it allows the best purification and concentration of target analytes while minimizing "matrix [effects] and [improving] the results of mass spectrometry (MS) measurements" [6]. It was suggested that SPE might have better results over LLE for some SCs, such as the carboxylic acid metabolites, which are an important analyte of interest when studying a urine matrix [6].

1.3.2 Urine, Plasma, and Edible Matrices

1.3.2.1 Urine

Urine is a common matrix in the analysis of many illicit substances. Typical sample preparation in SC urine analysis includes SPE or LLE techniques [6, 17, 18]. One method prepared authentic urine specimens with 10µL of urine, 10µL of internal standard stock solution, 10µL of methanol, 50µL of 0.4 M ammonium acetate buffer, and 2000 units of beta-glucuronidase [19]. This solution was vortexed, incubated, cooled, precipitated, vortexed for a second time, and centrifuged resulting in a total preparation time greater than two hours. Furthermore, the study was limited as only 5µL of the sample was injected "to reduce possibility of MS contamination" [19]. Another study used a salting out technique in combination with LLE. The samples were hydrolyzed with beta-glucuronidase, vortexed, centrifuged, and the organic layer was diluted with formic acid and water before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis [20]. While this method allowed a quick and simple preparation, the authors discussed the desire to develop a lower limit of detection (LOD), which may have been

enhanced with injection of the 100% organic sample obtained from the LLE rather than diluting the sample with an aqueous component [20]. An SPE method used included the addition of internal standard, 800µL of water, 2M sodium acetate buffer, and beta-glucuronidase to 20µL of urine [21]. This was followed with a one hour incubation step, which was then proceeded with cooling to room temperature, centrifugation, and automated SPE. Before LC-MS/MS analysis, evaporation to dryness and reconstitution were required again contributing to a total sample preparation time of over two hours. The limit of quantitation (LOQ) values developed were common with most other studies at approximately 0.25ng/mL or 1ng/mL [21].

1.3.2.2 Plasma and Blood

Blood samples, or plasma generated from blood samples, are a useful matrix in SC analysis in regards to detection of the parent analytes. Plasma is also a cleaner sample in comparison to whole blood. Zaitsu *et al.* analyzed synthetic cannabinoids in plasma collected from centrifuging blood samples obtained from an investigative case [22]. LLE and protein precipitation were used to prepare the plasma with an internal standard, overnight incubation and enzymatic hydrolysis, vortex and centrifugation steps, evaporation, and reconstitution. Though there have been limited studies on the analysis of SCs in blood, serum, and plasma, LLE seems to be the most popular extraction method chosen for these matrices [6, 17].

1.3.2.3 Edibles

The analysis of synthetic cannabinoids in edibles, particularly gummy bear candy, has not been investigated. However, there have been studies performed on edibles to

analyze other compounds of interest such as dyes and the analysis of natural cannabinoids. As a result of the dense, sticky, and colorful nature of gummy bear candies, analysis of this matrix can be relatively complex. A study assessing unsulfonated dye in sweets, including strawberry gummy bears, heated the sample and implemented a simple filtration step through a 0.45µm membrane filter before analysis with high performance liquid chromatography (HPLC) [23]. In another study researching natural cannabinoids, sample preparation for gummy candy consisted of cutting the sample into fine pieces, homogenization, and the addition of water [24]. This mixture was then hydrated with a shaker for approximately one hour. After, an acetonitrile solvent and an internal standard mixture were added and the sample was shaken in a homogenizer for one minute. Quick Easy Cheap Effective Rugged Safe (QuEChERS) extraction salts were added, the sample was shaken again, centrifuged, and the acetonitrile layer was diluted via serial dilutions. The samples were then analyzed with LC-MS/MS [24].

1.3.3 Analysis Techniques

Several techniques have been utilized in synthetic cannabinoid analysis. Immunoassays such as enzyme-linked immunosorbent assays (ELISA) and enzyme immunoassays (EIA) have been used screening methods of detection in urine [6, 25]. Thin layer chromatography (TLC) is a separation technique that has been used on herbal SC mixtures. Znaleziona *et al.* discussed spectral methods such as nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy as useful techniques in the analysis of SCs because they are helpful with the "identification of 'unidentified peaks'", which can be applied in the understanding of the various isomers of the different compounds [6].

However, it was also mentioned that IR is not an ideal method to use on complex mixtures because of the extensive isolation steps required beforehand. Nano-liquid chromatography, capillary electrophoresis (CE) with ultraviolet (UV) detection and ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and direct analysis in real time (DART) are other methods that have been studied in the analysis of SCs [5, 6]. MALDI and DART were found to be particularly useful in "forensic laboratories in the sense of cost benefit, time savings, and broad applicability" but these methods are relatively new and further research is required before they can be successfully applied in the analysis of synthetic cannabinoids [6].

Gas chromatography/mass spectrometry (GC/MS) has been a predominant instrument studied in SC detection [5, 6, 18]. While mass spectrometry is the method of detection most used, flame ionization detectors (FID) and nitrogen phosphorus detectors (NPD) have also been coupled with GC for SC analysis. The issues with GC analysis include the thermal degradation of some synthetic cannabinoids and some SCs requiring derivitization for a more efficient analysis, therefore contributing to an increased overall sample preparation and analysis time [6]. The most utilized qualitative and quantitative techniques are liquid chromatography and mass spectrometry.

1.3.3.1 Liquid Chromatography for Synthetic Cannabinoid Analysis

Liquid chromatography has played a major role in the method development of detecting new and existing synthetic cannabinoids. It has been used in both preliminary and confirmation methods [6, 7, 19, 21]. Most methods apply C8 or C18 columns with particle sizes less than 2µm, a 10-150µm internal diameter (I.D.), and a gradient elution.

These parameters enable quicker flow rates, faster analysis, and increased sensitivity [6]. The MS parameters include selected-ion monitoring (SIM) and multiple reaction monitoring (MRM) modes, however this requires prior information to be known about the compounds. Scheidweiler and Huestis discussed mass spectrometry to be useful as a screening technique since new SC compounds "may not cross-react in antibody-based techniques" while MS also has the ability to incorporate "new analytes as rapidly as reference standards become available" [7]. This is a particularly important point of emphasis as new synthetic cannabinoids, and therefore certified reference materials, are constantly being synthesized and a quick method of detection, or the ability to enter new reference information, is necessary as a result. Scheidweiler and Huestis published a method that screens for SCs and several metabolites in urine using LC-MS/MS [7]. Knittel *et al.* studied the correlation between parent SCs in blood and their corresponding metabolites in urine with LC-MS/MS in addition to successfully identifying and quantifying the studied compounds [26]. Jang *et al.* developed a LC-MS/MS method to identify 37 SC metabolites in urine with a focus on the separation of positional isomers [21]. There have been a significant number of studies involving the LC-MS/MS analysis of SCs, but further method development is continuously desired as new compounds are synthesized.

1.3.3.2 Liquid Chromatography Parameters

The parameters of reviewed LC-MS/MS studies vary depending on the matrix and synthetic cannabinoid evaluated. Some studies focused on one or two SCs while others analyzed over 30 compounds [21, 25]. Many studies assessed the original SC

compounds, such as JWH-018, while a limited number of research focused on newer compounds. One study found a linear dynamic range of 0.5-10ng/mL using LC-MS/MS analysis of human urine samples and Jang *et al.* determined a range of 2.5-75ng/mL [19, 25]. LOQ values of 0.1ng/mL and 2.5ng/mL were common in many studies and a LOD of 0.1ng/mL for some compounds and 0.025 for other SCs was observed [17, 25, 27]. Recovery values varied and ranged from 50-100% [19, 27]. Of all the LC-MS/MS methods reviewed, however, multidimensional liquid chromatography analysis of synthetic cannabinoids was not observed.

1.4 The Importance of Method Development for SC Analysis

1.4.1 Statistics and SC Emergence

As of May 2016, it was reported that synthetic cannabinoids are "the largest group of new psychoactive substances monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)" [8]. There is a serious issue with the ability of SCs to produce mass poisonings. For instance, in 2015 the synthetic cannabinoid ADB-FUBINACA was linked to a major outbreak of poisonings in the United States [8]. Even with actions taken to schedule SCs, abuse continues to be described in surveys taken throughout the country [7]. In 2009, there were only two types of synthetic cannabinoids reported, but in 2015 the number of new SCs reported dramatically increased to 84 compounds [28]. Furthermore, there were 22 synthetic cannabinoids, such as EG-018 and MDMB-FUBINACA, reported for the first time in 2015 clearly demonstrating the rapid emergence of new compounds [28]. In addition to new compounds continuously and quickly developing, popularity of other forms such as a SC resin, similar to cannabis

resin, has increased [8]. If the resins are more concentrated than the typical amounts of SCs sprayed on herbal substrates, the issues associated with mass poisonings may be further complicated.

1.4.2 Analysis

There are numerous reasons associated with the importance of developing reliable methods for SC detection including the number of compounds, increased use, the chemical diversity, speed of their emergence, and popularity within many age groups. The primary concern with synthetic cannabinoids is lethality. Discoveries such as "processing and packaging facilities and large quantities of synthetic cannabinoids in Netherlands and Belgium suggests the involvement of organized crime in the distribution process," which has the potential to exacerbate issues [8]. The development of efficient sample preparation methods is also necessary for reasons such as matrix effects and the ability of other components in a sample to affect the successful detection of SCs [6]. Many reports have described increased seizures, manufacturing, and sales of synthetic cannabinoids [6, 28].

Researchers continue to synthesize and search for compounds to use medicinally, enabling an easy transition of these compounds to the illicit market [5]. It was also emphasized that SC metabolites will not always produce positive results in typical screening analyses [16]. This and the continuous emergence of new compounds contribute to the difficulty in maintaining reliable and up-to-date detection methods. Additionally, the methods that are developed are not all "fully validated and used for quantification purposes" [6]. There is a need for the development of selective, sensitive,

high throughput, cost-effective, and less time consuming quantitive and qualitative analytical methods to successfully detect, monitor, and respond to the issues associated with synthetic cannabinoid use [6, 8].

1.5 Instrumentation Theory

1.5.1 Enzyme Immunoassay

Immunoassay techniques are useful screening methods for several reasons. The required materials are readily available for most assays and compounds of interest. For example, synthetic cannabinoid testing materials can be purchased from a variety of locations including Randox Laboratories, National Medical Services, Immunalysis Corporation, Cayman Chemical, and Neogen Corporation [4]. Immunoassay tests are quick, inexpensive, have the option for automation, and require minimal sample preparation [4]. ELISA is a common immunoassay method utilized while EIA methods involve a slightly different technique. EIA can also be described as an enzyme multiplied immunoassay technique (EMIT), cloned enzyme donor immunoassay (CEDIA), or homogenous enzyme immunoassay (HEIA). EIA techniques use homogenous mixtures, competitive antibody binding and drug-drug interactions to analyze samples [14, 29]. The competitive description is utilized to represent the fact that there is competition for the antibody sites between unlabeled and labeled antigen. In comparison to ELISA testing, there are no separation steps required in EIA [29]. Enzyme activity is measured spectrophotometrically and absorbance readings are used to produce results. EIA testing does not have any washing or incubation steps like ELISA and the materials are provided in a format that is ready for use upon arrival with several advantages such as “low matrix

effects, reduced sample preparation and high throughput" opportunities [14]. While there are many advantages to immunoassay techniques, several non-synthetic cannabinoid substances can produce false positive or inconsistent results for a SC test. The "ever-changing availability" of SCs and the difficulty in the evaluation of cross reactivity studies on these novel compounds make it extremely difficult to maintain up to date, specific, sensitive, developed, and validated synthetic cannabinoid immunoassay methods [4, 14]

1.5.2 Single Dimension (1D) Liquid Chromatography

In general, liquid chromatography involves a stationary phase, usually a solid such as silica, and a liquid mobile phase, which carries a sample through the stationary phase. The components of a sample typically separate based on their affinity to either the stationary or the mobile phase. Many LC applications involve the use of reverse-phased chromatography where a polar mobile phase and a nonpolar stationary phase, such as C18, are implemented [30]. There are many different types of liquid chromatography such as HPLC and ultra performance liquid chromatography (UPLC) [31]. There are also various separation mechanisms such as liquid-solid partition, which is the most common [32]. UPLC can be described as a type of liquid chromatography that utilizes a solid phase with smaller particle sizes and maintains higher pressures in comparison to HPLC [32]. In UPLC, separations can be performed "5-10 times faster than conventional HPLC by employing sub-2μm diameter [stationary phase] particles" enabling better resolution efficiency [33]. Liquid chromatography coupled to mass spectrometry is especially beneficial in the quantitation and detection of drugs at trace levels.

The general layout of a HPLC system includes a sample manager and injection port, a high-pressure pump, a solvent reservoir, a column, a detector, and a computer [32]. The sample is injected in the sample manager where it meets the liquid mobile phase that is being pumped through the column via the high-pressure pump. Then, depending on the affinity of the components in a sample to the mobile or stationary phase, the components are separated as they move through the column at different rates. The detector collects information such as the time the various analytes elute off the column and other details that play a role in identification such as mass and charge. This information is then processed and visualized, typically on a computer. A liquid chromatography unit can be connected to various types of detectors, but the most common instrument utilized is the mass spectrometer.

1.5.3 Mass Spectrometry

Mass spectrometry is a popular, sensitive method of detection utilized in many types of analyses. After a sample goes through the LC component, it is converted to a "gas-phase ion" for analysis in the mass spectrometer [34]. MS instruments consist of an ion source, a mass analyzer, an ion detector, and a computer. The ion source converts the liquid into the gas phase [35]. Electrospray ionization (ESI), like the method used in this research, is one example of an ion source where the sample is ionized and formulated into an aerosol-like spray from a high voltage [34]. The ionized sample is then accelerated through the mass analyzer with an electric and/or magnetic field where the path of each ion is redirected according to their mass and charge [35]. A quadrupole mass analyzer, the technique utilized in this research, implements an electric field. Triple quadrupole

analyzers have four rods with radiofrequency and direct currents enabling ions with a specific mass to charge ratio to reach the detector such as those maintained during multiple reaction monitoring [36]. The first quadrupole may be set on static mode, which allows the passage of certain ions (the precursor ion). The ion then reaches the second quadrupole, or collision cell, where Argon gas is used for fragmentation. The third quadrupole may also be set on static mode to select particular ions of interest such as the product ions created from the precursor ion in the first quadrupole. At the ion detector, a vacuum is applied eliminating any unwanted collisions with the ions of the analyte of interest. Ion data is organized based on mass-to-charge ratio and relative abundance, which is converted to a mass spectrum [34, 35].

1.5.4 Two-Dimensional (2D) Liquid Chromatography

A two-dimensional liquid chromatography format can be easily implemented from a 1D LC instrument. The main difference between 1D and 2D is the addition of one or more columns, valves, and/or pumps. While two columns are generally used to execute the two-dimensional aspect, a variety of techniques and concepts can be incorporated to perform multidimensional chromatography. In comprehensive chromatography, for instance, the second column may be described as "a very chemically selective detector for the separation on the first column," which emphasizes the highly efficient resolution capacity multidimensional chromatography can provide [37]. Furthermore, the concept of 'heart cutting' chromatography can be applied where only the "interesting portion" of the eluate from the first dimension is transferred to the second [37].

Other 2D chromatography techniques may implement AT-column dilution and trap/elute concepts, which focus on trapping analytes of interest on the first dimension, or trap column, to then perform a more efficient separation of those analytes on the second dimension, or analytical column. This was the technique applied in this research. The additional pump may be used to provide the AT-column dilution effect pumping an aqueous component into a mixture with the organic sample from the injection port [38]. This concept enables the injection of 100% organic samples in addition to aqueous samples where a dilution takes place at the joining of the loading stream and the dilution stream. Upon the mixing of each stream, a dilution of approximately 5% can be achieved [38]. This enables the production of more efficient results for compounds that are not as soluble or stable in aqueous solvents as may be in 100% organic solvents.

The trap column may contain particle packing materials of 10µm in size or greater, which allows the injection of large volumes and focuses the target analyte. Through optimization of loading flow rates, chemistry, and additives, the target analyte can be trapped with maximum peak trapping and minimal breakthrough [38]. After trapping the analyte of interest, a backflush elution is carried out transferring the desired eluate to the analytical column. The analytical column contains much smaller particle sizes (2µm) for a more efficient resolution. The chromatography performed on the second dimension is then transferred to the detector. The trap/elute concept provides excellent separation and specificity with a more efficient workflow in comparison to the limitations observed with traditional one dimensional chromatography methods.

1.5.5 1D vs. 2D Chromatography

One of the greatest advantages of 2D chromatography is, perhaps, the ability to inject 100% organic samples reducing overall sample preparation time significantly. The AT-Column dilution concept enables the injection of 100% organic samples thus eliminating the reconstitution and evaporation steps necessary with most SPE preparation methods as previously discussed in the reviews of other synthetic cannabinoid analysis methods. The time required to prepare samples for LC analysis and any evaporative loss are significantly reduced. In this manner, the large number of samples forensic scientists have to analyze on a daily basis could be more promptly accommodated. The hydrophobic properties of SCs may also play a role in regards to the recovery values of samples between 100% organic extracts versus aqueous, reconstituted extracts injected in the LC-MS/MS [17]. Some compounds may prefer to be dissolved in 100% organic solution without the presence of any aqueous components.

Larger sized sample injections can also be utilized with 2D-LC-MS/MS. The ability of various columns, additives, and solvents to be evaluated with 2D analysis provides the opportunity to perform a micro-extraction protocol where analysts can "evaluate several elution parameters in a short-time period" such as the completion of chromatography optimization in 18 hours [39]. With the extremely high resolving power provided by 2D technology, a more efficient analysis of complex samples can be achieved with minimal matrix effects. The combination of both dimensions is useful in developing sensitive, specific, and robust methods to detect SCs and these factors are particularly beneficial with the continuous synthesis of new SC compounds.

1.6 Research Objective

The objective of this research was to develop a reliable, sensitive, and selective multidimensional chromatography method to successfully detect and quantify seven synthetic cannabinoids in urine, plasma, and edible samples. Rapid sample preparation methods were explored to efficiently carry out this objective utilizing a 2D-LC-MS/MS instrumentation technique.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Synthetic Cannabinoids Evaluated

This research focused on seven synthetic cannabinoids: XLR-11, AB-PINACA 5-pentanoic acid metabolite (AB-PINACA 5-COOH), UR-144 5-pentanoic acid metabolite (UR-144 5-COOH), 5F-PB-22, AM-2201 4-hydroxypentyl metabolite (AM-2201 4-OHpentyl met), JWH-018, and JWH-018 5-hydroxypentyl metabolite (JWH-018 5-OHpentyl met) (Figure 1). The compounds were chosen for analysis based on a list developed of the top detected SCs and their common metabolites [12, 21, 25, 27, 40-42].

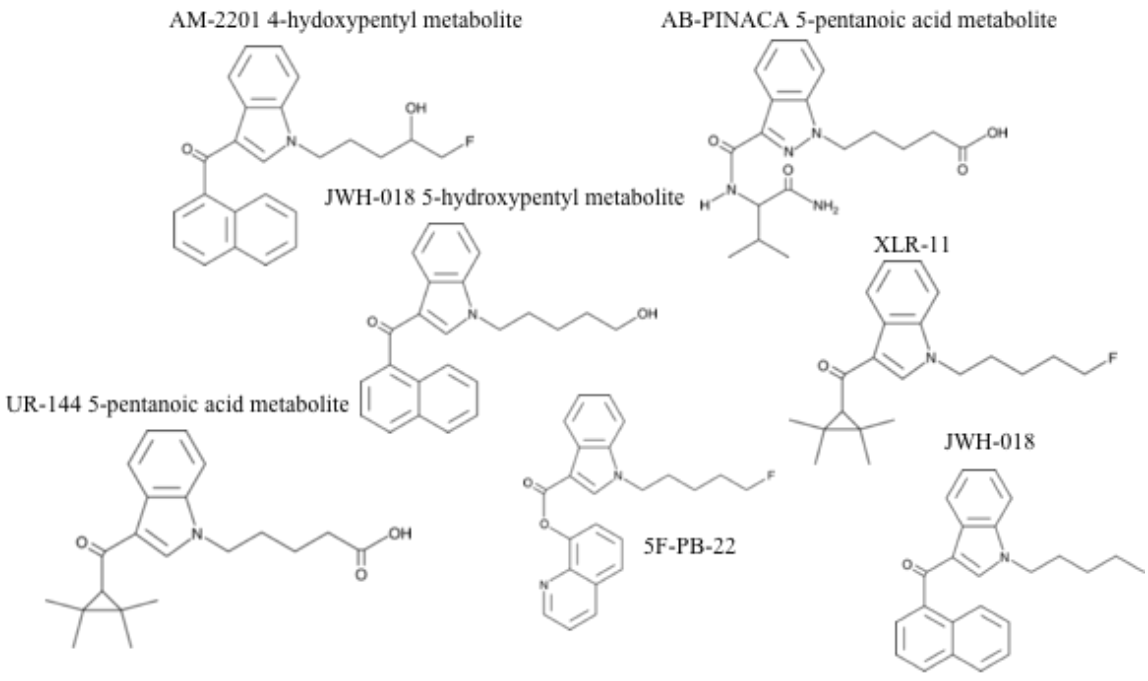

Figure 1: Structures of Synthetic Cannabinoids Analyzed [43]

2.1.2 Standards and Reagents

Synthetic cannabinoid standards and internal standards were obtained from three sources. The standards received from Lipomed, Incorporated (Cambridge, MA, USA) included UR-144-N-pentanoic acid metabolite 1mg/mL isopropanol (IPA), 5F-PB-22 1mg/mL acetonitrile (ACN), JWH-018 N-(5-hydroxypentyl) 1mg/mL methanol (MeOH), JWH-018 D-11 1mg/mL MeOH, and JWH-018 1mg/mL MeOH. AM-2201 4-hydroxypentyl metabolite and XLR-11 were from Sigma-Aldrich (St. Louis, MO, USA) and both concentrations were 100µg/mL MeOH. AB-PINACA 5-pentanoic acid metabolite was obtained from Cerilliant Corporation (Round Rock, TX, USA) in a MeOH solution of 100µg/mL. The solvents used were Optima grade and consisted of MeOH, ACN, acetone, ammonium hydroxide (NH_4OH), hydrochloric acid (HCl), formic acid (FA), and phosphoric acid (H_3PO_4). All solvents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The MilliQ grade water used in this study was obtained from EMD MilliporeSigma (Darmstadt, Germany).

2.1.3 Human Urine Case Samples

A total of 17 urine samples that were screened and tested positive for synthetic cannabinoids with an EIA test were collected from Clinigen, Incorporated (Woburn, MA, USA). These samples were prepared and analyzed with the optimized extraction and 2D-LC-MS/MS methods developed in this research. Negative urine samples were obtained from a non-drug using volunteer.

2.1.4 Instrumentation and Software

An ACQUITY UPLC® (Waters Corporation, Milford, MA, USA) was utilized in 2D configuration. The 2D format was prepared with three pumps (Figure 2). The first pump, otherwise termed the loader/dilutor, was used to create AT-column dilution. The AT-column dilution was produced with two streams – stream A loaded the samples from the injection loop into a 50µL mixer while stream B pumped the dilution solvent into the mixer at a high flow rate. The second pump was utilized for elution purposes with an aqueous and organic stream. The third pump performed a reconditioning step, which washed the system with a 1:1:1 acetonitrile/methanol/acetone mixture for two minutes and followed with a re-equilibration to bring the system back to its initial loading conditions. The detector used to analyze the eluates from the chromatography portion was a tandem MS, Xevo TQD (Waters Corporation, Milford, MA, USA) with positive electrospray ionization (ESI). Three replicate injections were performed on each sample with the average information obtained from the three replicates utilized to complete data analysis.

MassLynx© version 4.1 and TargetLynx© version 4.1 (both Waters Corporation, Milford, MA, USA) were used to carry out analyses on the instrumentation, visualize results, and perform data analysis. MassLynx© was used to visualize mass spectrums and chromatograms. TargetLynx© was utilized to perform quantitative analysis, such as the generation of calibration curves.

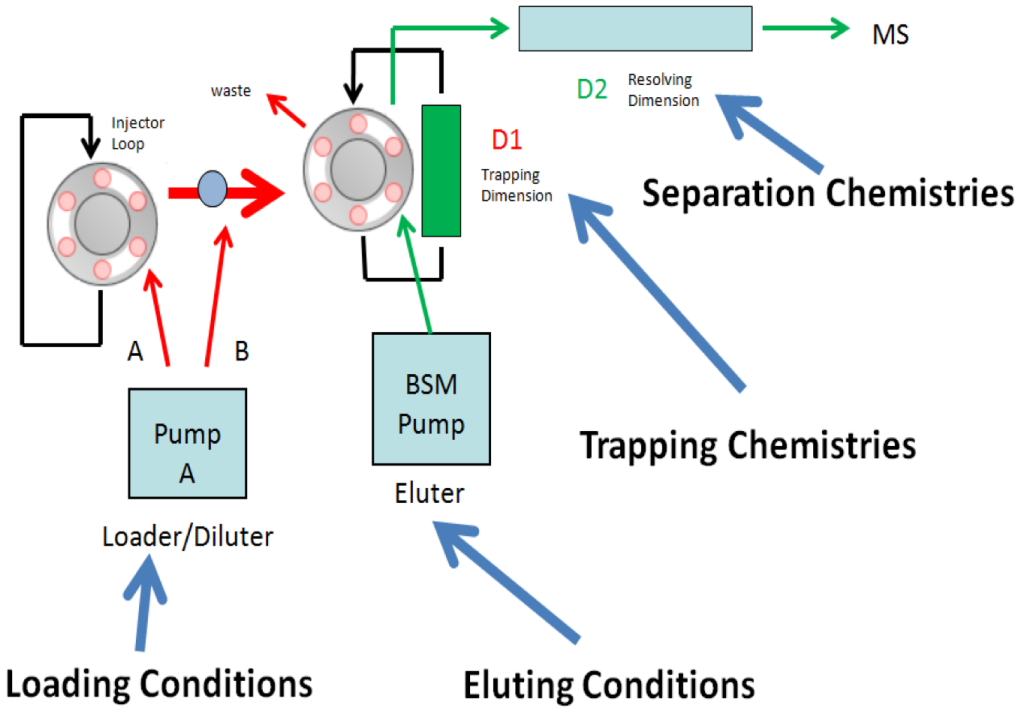

Figure 2: 2D-LC Fluidic Pathway [38]

2.2 Methods

2.2.1 Multiple Reaction Monitoring Optimization

A 10µg/mL (10ppm) solution of each compound was prepared in 50/50 water: MeOH. The solutions were then attached to the MS for direct infusion one by one. For each compound, a precursor ion was determined in MS1 scan mode and two product ions were determined in MS/MS mode. The most intense product ion was selected for quantification and the second most intense product ion was used as a qualifier. Collision energy (CE) and cone voltage were also optimized for each compound. As the CE increased, the precursor ion became fragmented and the product ions were visualized in the mass spectrum produced. The value at which the signal was the most intense for each

transition was chosen for each parameter. All optimized values are displayed in the multiple reaction monitoring (MRM) table below (Table 1). Constant MS parameters include capillary voltage 3.0kV, 150°C source temperature, 550°C desolvation temperature, and desolvation gas and cone gas flow rates of 1100 L/hr and 50 L/hr, respectively.

Table 1: MRM Compound Optimization Table. Transitions were determined for all standards and the internal standard.

Compound	Ion Mode	Precursor ion	Cone Voltage	Product ions	CE
AM-2201 4-hydroxypentyl metabolite	ESI+	376.2	30	155.0	25
				127.0	30
JWH-018	ESI+	342.2	30	155.0	30
				127.0	30
JWH-018 5-hydroxypentyl metabolite	ESI+	358.2	35	155.0	30
				127.0	30
XLR-11	ESI+	330.2	40	125.1	25
				97.0	30
5F-PB-22	ESI+	377.2	20	232.1	10
				144.0	30
UR-144 5-pentanoic acid metabolite	ESI+	342.2	30	125.0	20
				83.0	30
AB-PINACA 5-pentanoic acid metabolite	ESI+	361.20	25	344.2	10
				316.2	15
JWH-018 D11	ESI+	353.2	30	155.0	25
				127.0	30

2.2.2 Chromatography Method Development

Two stock solution mixtures of all SCs were prepared for chromatography method development. Mix A consisted of UR-144 N-pentanoic acid metabolite, 5F-PB-

22, JWH-018 5-hydroxypentyl metabolite, and JWH-018 in 100% MeOH to form a final concentration of 10µg/mL. Mix B was prepared with AB-PINACA 5-pentanoic acid metabolite, AM-2201 4-hydroxypentyl metabolite, and XLR-11 in 100% MeOH to form a final concentration of 10µg/mL. Both mixtures were combined and a serial dilution was performed to prepare stock solutions of lower concentration for analysis. Three different 1ng/mL solutions in MeOH, ACN, and MilliQ water were assessed on different trap and analytical columns with different loading and elution conditions throughout this portion of the research. With each change made to the previously listed conditions, the following remained constant: the sample injection volume was set at 100µL and the samples were loaded with water at a flow rate of 0.1mL/min and diluted with water at a 1.9mL/min flow rate creating a 5% total dilution. After the loading and diluting steps, the samples were loaded onto the trap column at a flow rate of 2.0mL/min. From the trap column, the target analytes were re-focused onto the analytical column and eluted at 0.5mL/min flow rate with a linear gradient elution 5% organic solvent to 95% totaling a 10-minute run time. Some of these conditions were further evaluated at a later date, after optimal loading pH, elution solvent, and elution pH were established.

Three analytical columns and two trap columns were assessed. All columns utilized were from Waters Corporation, Milford, MA, USA. The first analytical column was an ACQUITY UPLC BEH (ethylene bridged hybrid) C18, 2.1 x 50mm, 1.7µm. The second column evaluated was an ACQUITY UPLC Phenyl, 2.1 x 50mm, 1.7µm. Originally, it was determined that the BEH C18 column was the optimal analytical column in comparison to the phenyl column. However, after further evaluation and

method development, the final column chosen with the best signal intensity and peak shape was an ACQUITY UPLC HSS T3 (high strength silica with tri-functional C18 bonding), 2.1 x 150mm, 1.7µm.

The two trap columns assessed were an ACQUITY UPLC HLB (hydrophilic-lipophilic balance), 2.1 x 30mm, 20 µm and an ACQUITY UPLC BEH C8 (n-octylsilyl-silica), 2.1 x 30mm, 10µm. After data analysis, it was determined that the C8 column would provide the best results for this method. This observation was strengthened by the fact that HLB columns are strongly hydrophilic, therefore undesirable for retaining hydrophobic synthetic cannabinoids.

The 1ng/mL samples in water, MeOH, and ACN were run over night with combinations of different trap columns and additives in an automated process completing chromatography method optimization in 12 hours. Each sample was injected three times and the third injection was utilized for analysis during chromatography evaluation. An example of one set of permutations is presented below in Figure 3 with water as a loading solvent, MeOH as an elution solvent in the top portion of the figure and ACN as an elution solvent in the bottom portion. This chromatography optimization was performed with a BEH C18 analytical column. Formic acid was used to create pH 3 conditions and NH_4OH was used to create conditions of pH 10. The same set of conditions was run a second time using MeOH as an elution solvent and a phenyl analytical column. It was determined that MeOH was a better elution solvent than ACN (Figure 4). In comparison to MeOH, ACN signal intensities were lower and peak distortion such as tailing and shouldering was prominent.

The 2D instrumentation and automated technology enabled the chromatography evaluation, over 16 permutations, to be completed in a short amount of time. Overall, it was determined that a C8 trap column, pH 10 loading conditions, and a pH 3 MeOH elution solvent were the optimal chromatography conditions for synthetic cannabinoid analysis. The results of the permutations displayed in Figure 3 are depicted in Appendix A (Tables A and B).

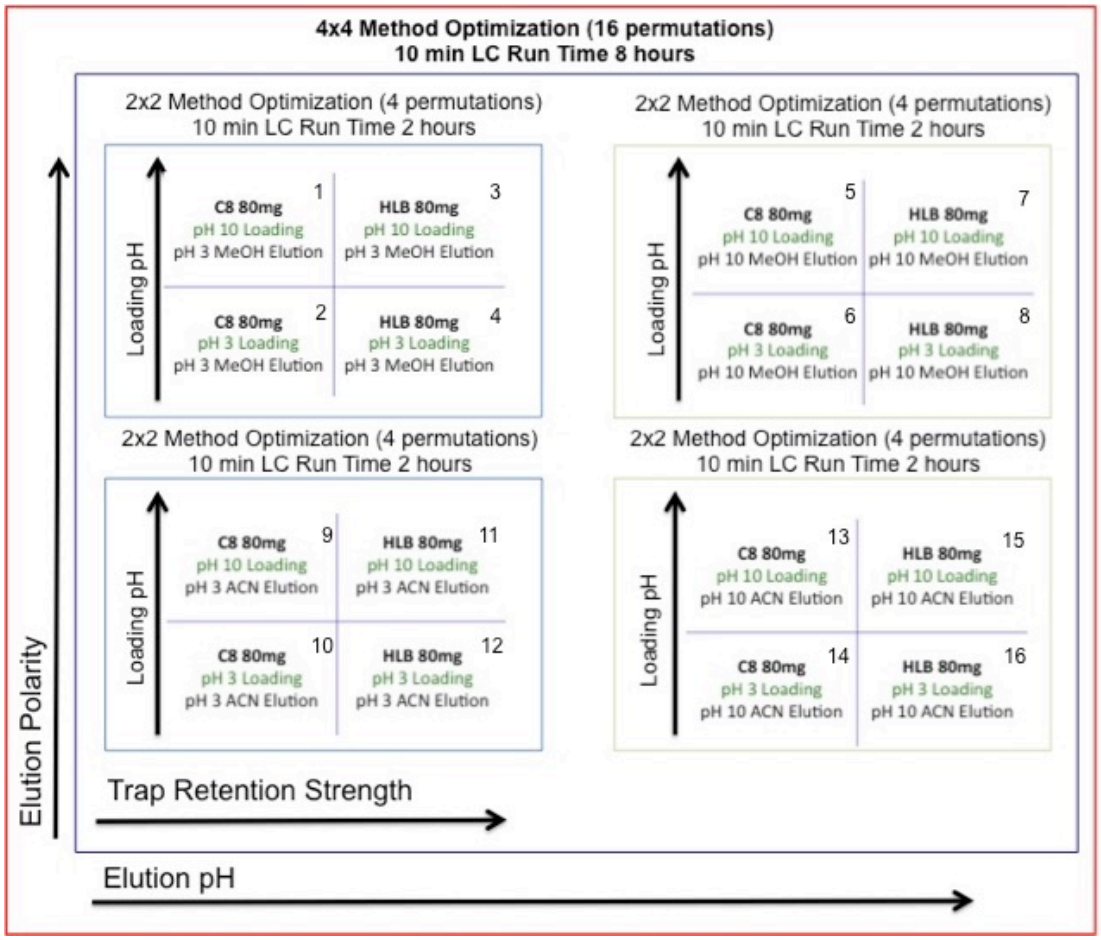

Figure 3: 4x4 Method Optimization Scheme

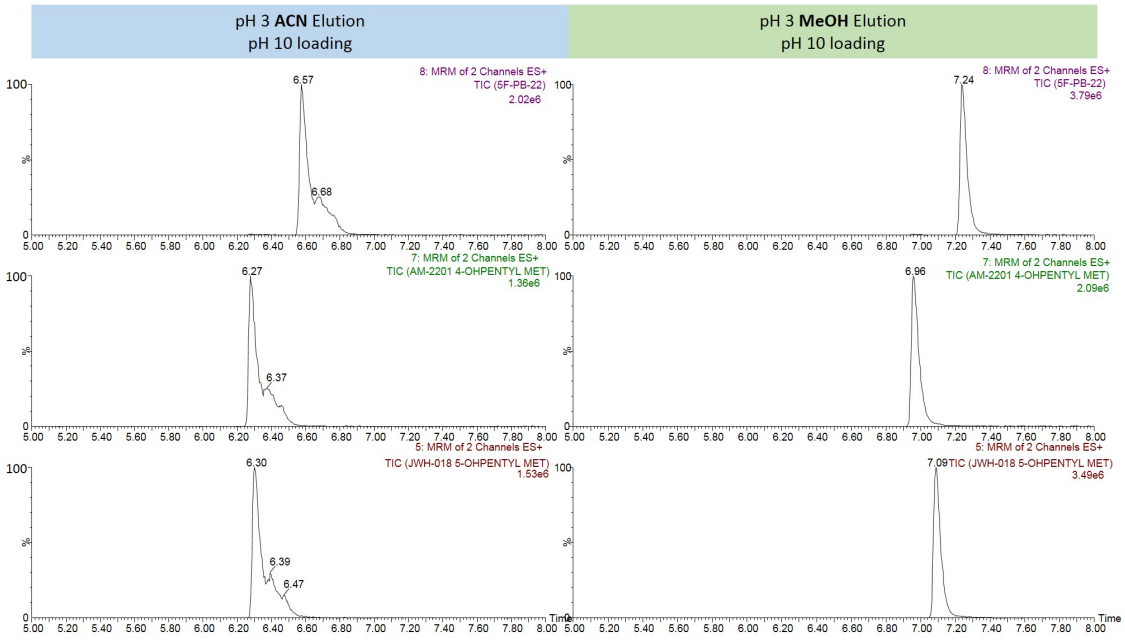

Figure 4: Elution Solvent Comparison – ACN vs. MeOH

The solvents and mobile phase additives chosen for the final method can be visualized in the XLR-11 chromatograms in Figures 5 and 6. In both figures, the conditions that remained the same were the BEH C18 analytical column, BEH C8 trap column, and MeOH elution solvent. It is clear upon initial visualization that water is the least appropriate solvent to prepare standard synthetic cannabinoid solutions in. Between MeOH and ACN, standards prepared with 100% MeOH had slightly higher intensities than those prepared with ACN. Furthermore, MeOH was the best overall standard solvent for all compounds analyzed in this research (Appendix A). Figure 5 displays constant pH 3 loading with pH 3 vs. pH 10 elution conditions. The pH 3 MeOH elution was a whole magnitude higher in intensity than that produced with pH 10 MeOH elution. Figure 6 displays a constant pH 10 load with a comparison of pH 3 and pH 10 elution conditions.

Again, the pH 3 eluting condition was a magnitude higher in intensity, with a better peak shape than pH 10 elution. Figures 5 and 6 can also be compared to evaluate the difference between pH 3 loading and pH 10 loading. Specifically, by observing the chromatograms that display MeOH standard solution with a pH 3 elution, it can be concluded that loading conditions at pH 10 were slightly higher in intensity compared to pH 3 loading.

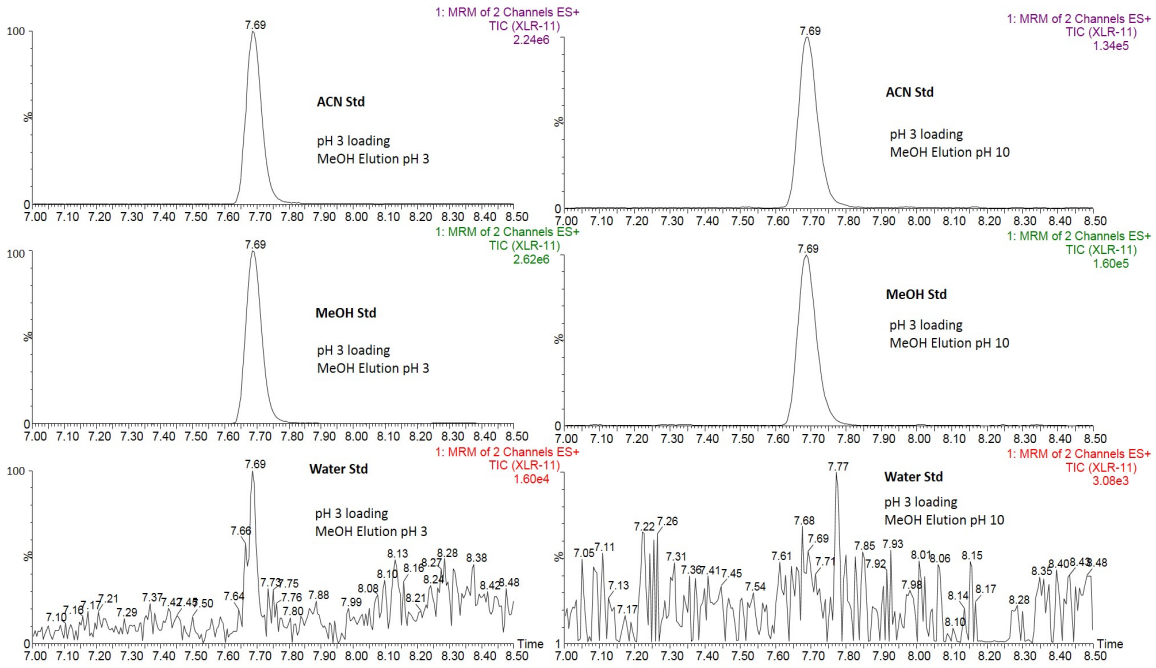

Figure 5: Chromatography Evaluation A. Constant pH 3 loading – pH 3 vs. 10 MeOH elution.

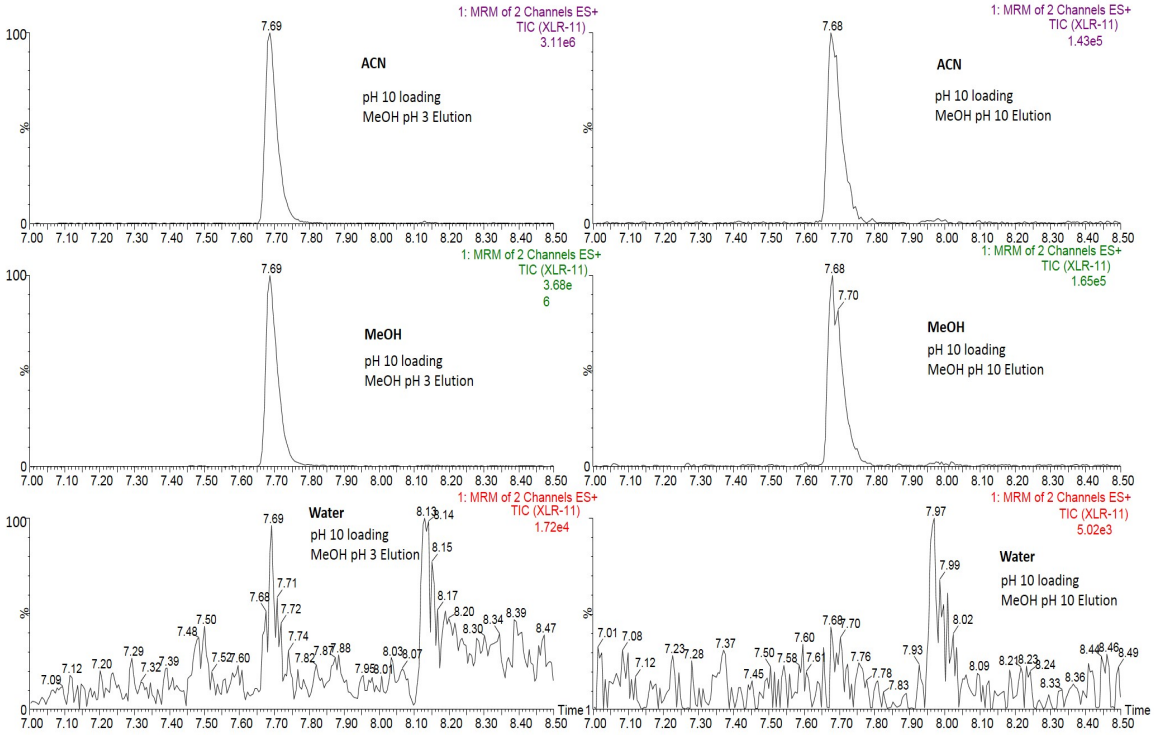

Figure 6: Chromatography Evaluation B. Constant pH 10 loading - pH 3 vs. pH 10 MeOH elution.

Several columns were explored throughout the method development process. The optimal trap column chosen for this method was a C8. Figure 7 depicts different SCs on different trap columns under the optimal loading and eluting conditions previously discussed. In comparing the C8 trap column to the HLB trap column, it is clear that HLB produced results with overall weaker signal intensities, significant tailing and peak distortion. Three analytical columns were tested under various conditions. Figure 8 depicts a comparison between all analytical columns assessed at the optimal loading and elution conditions with a C8 trap column. The BEH C18 column was initially chosen over a phenyl column due to its slightly higher intensities and better peak shape including less tailing overall for the SCs analyzed. After troubleshooting during extraction

evaluation, it was ultimately determined that an HSS T3 analytical column would be the best choice for analyzing SCs. In comparison to BEH C18, the HSS T3 column had stronger signals, tighter bandwidths, and significantly reduced peak distortion. The combination of a slightly nonpolar C8 trap column, nonpolar HSS T3 analytical column, and a polar mobile phase were used to develop a more robust, reversed-phase liquid chromatography method in the analysis of synthetic cannabinoids.

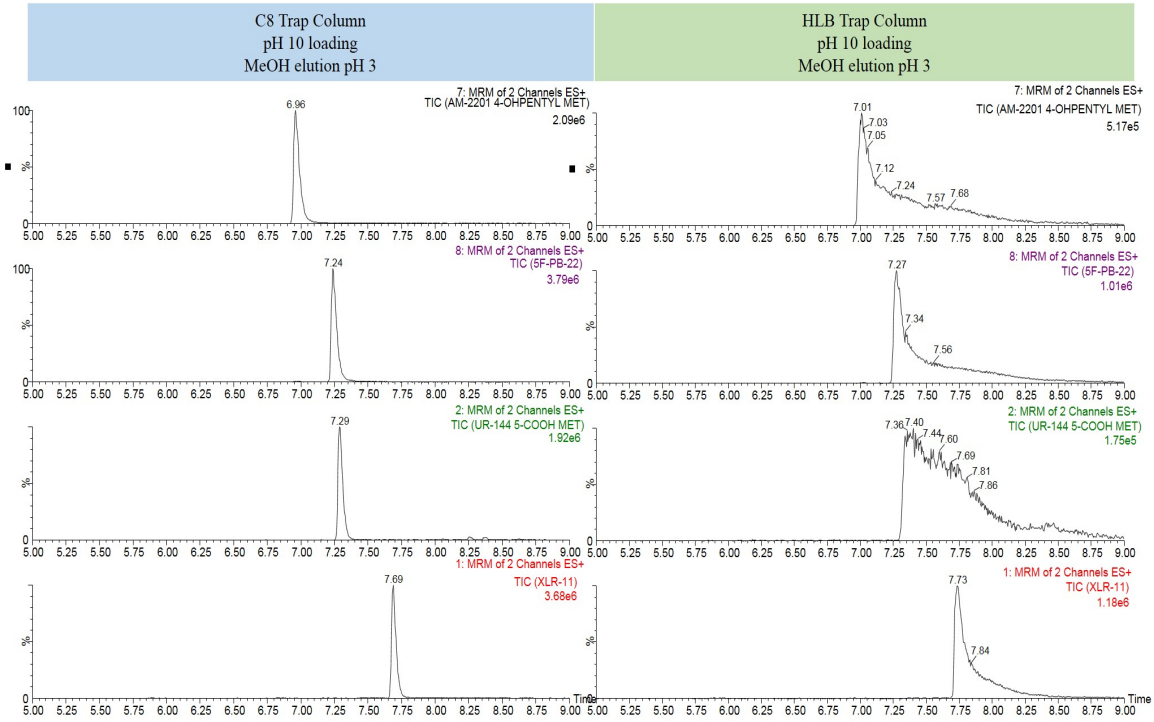

Figure 7: Trap Column Comparison – BEH C8 vs. HLB.

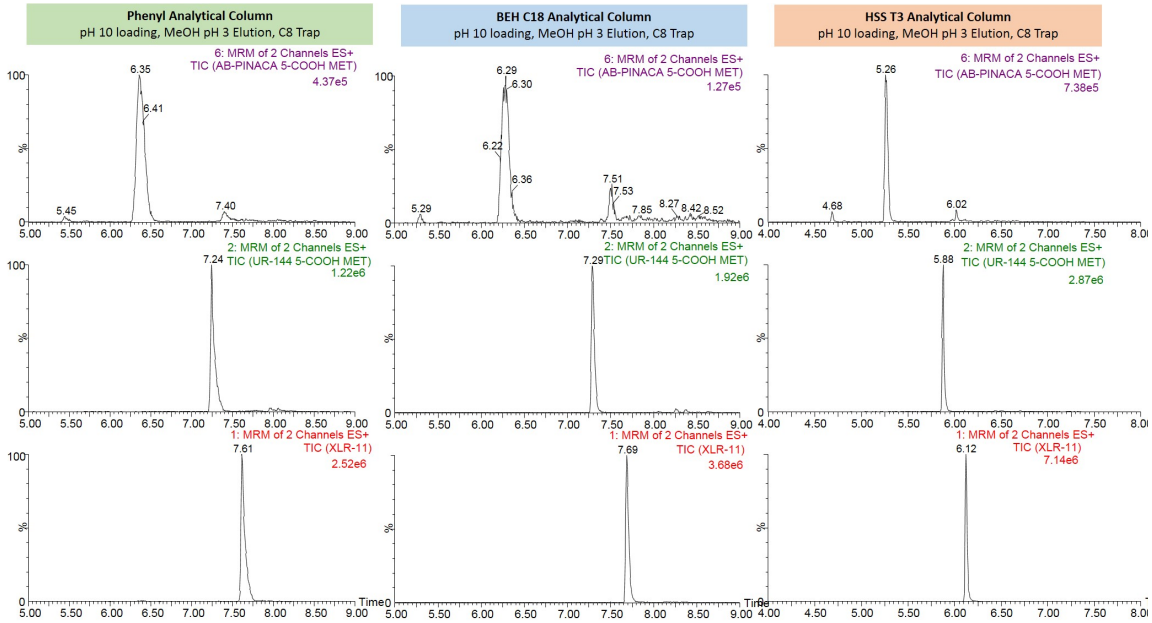

Figure 8: Analytical Column Comparison – Phenyl vs. BEH C18 HSS T3

As previously discussed, a comparison of the C18 analytical column to a HSS T3 column was performed for troubleshooting purposes (Figure 8). Before changing the analytical column type, the mobile phases were changed out with fresh solutions and the analytical and trap column were replaced with new columns. Furthermore, new standard solutions were made in MeOH and analyzed with the improved conditions. However, there were still undesirable results such as retention time drift and significant tailing on peaks of interest. At that point, the BEH C18 analytical column was replaced with the HSS T3. HSS T3 columns have a higher strength silica particle stationary phase with trifunctional bonding of C18 enabling a better retention of hydrophobic analytes like synthetic cannabinoids. The column temperature, gradient time frame, and flow rates were also optimized at this point. The initial expectation of HSS T3 column use would

involve higher retention times of the analytes in comparison to those associated with a C18 and phenyl analytical column as HSS T3 contains a high retention stationary phase. That was the case in this research; however, higher retention times are not visualized in Figure 8 as previously described because the overall method run time was altered between evaluation of the C18 column and the HSS T3 column.

2.2.2.1 Carryover Evaluation

During the extraction evaluation process, an issue developed where signals were absent or significantly low on extracted samples but were easily visualized on the unextracted standard samples. Carryover became a point of interest and was evaluated for further method optimization. Figure 9 demonstrates a comparison of results with and without the reconditioning and re-equilibration steps. There are three water blanks following a 10 ng/mL spike under each system. The chromatograms on the left hand side clearly portray carryover from the 10 ng/mL spike sample to the blanks. The addition of reconditioning and re-equilibration steps successfully eliminated any significant carryover as displayed in the chromatograms on the right hand side of Figure 9.

Therefore, a third pump was added to the LC system to add a wash and reconditioning step after each run. Line A was comprised of water and 2% NH_4OH and line B was prepared with a solution of ACN/MeOH/Acetone. Also, a trap and sample manager wash solution comprised of ACN/MeOH/Acetone was prepared. At 7.00 minutes during the run, line A switched to Line B at a flow rate of 2mL/min for two minutes. At 9.00 minutes, line B switched back to line A, which switched the organic wash out with a high pH water solution as was determined to be required for loading

during chromatography method development. The sample injection volume was also adjusted from 100µL to 200µL. The final conditions developed and optimized for this method, providing the best signal intensity, resolution, and peak shape, are summarized in Table 2.

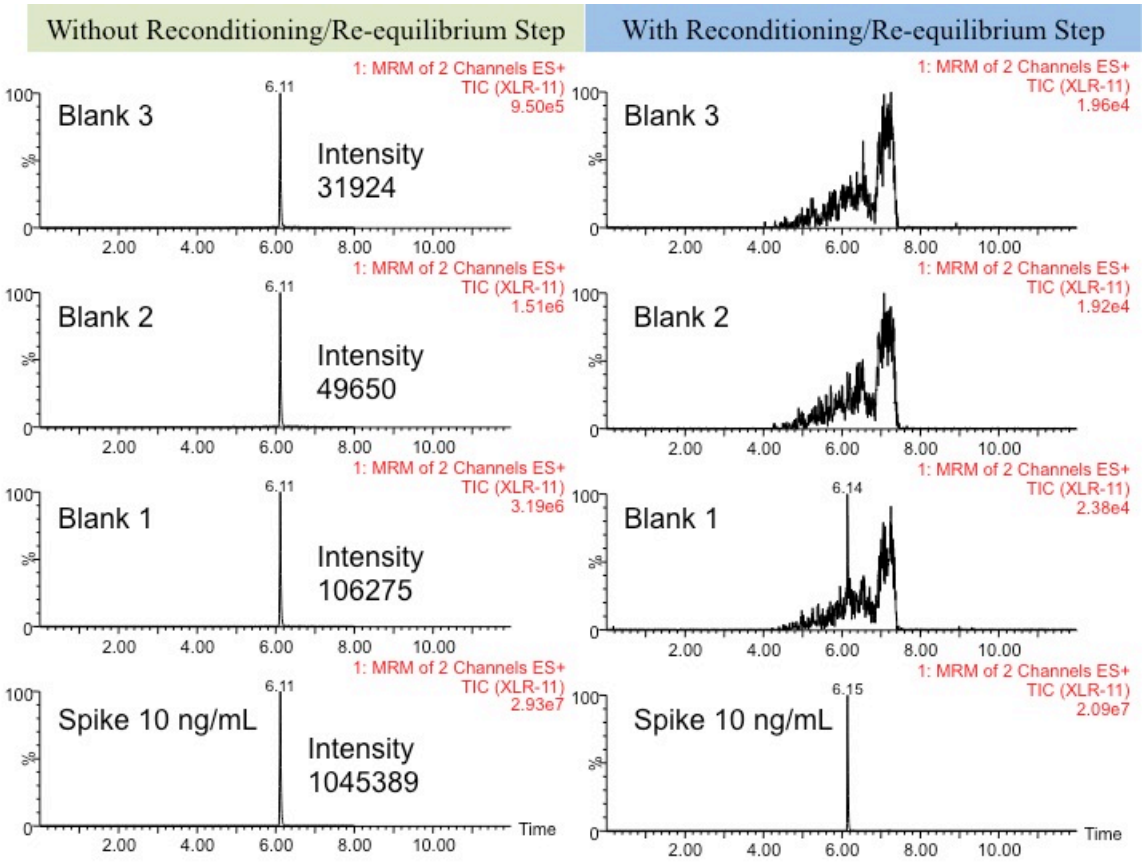

Figure 9: Carryover Results for XLR-11

Table 2: Final Chromatography Conditions

UPLC Conditions	AQUITY UPLC with "Trap and Elute" and AT-Column Dilution conditions
Injection Volume	200µL
Loading Conditions	MilliQ water + 2% NH_4OH (pH 10)
Loading Flow Rate	2 mL/min
AT-column dilution	5%
Trap Column	ACQUITY UPLC C8, 2.1 x 30mm, 10µm
Analytical Column	ACQUITY UPLC HSS T3, 2.1 x 150mm, 1.7µm
Analytical Column Temp	70°C
Mobile phase A	Water + 0.5% Formic Acid
Mobile phase B	MeOH + 0.5% Formic Acid
Elution Gradient	3 minute linear gradient 5% to 95% Mobile Phase B
Elution flow rate	0.600mL/min
Wash/Recondition Step	2 minute MeOH/ACN/Acetone wash with 2mL/min flow rate
Total Run Time	12 minutes

2.2.3 Solid Phase Extraction Method Development

Considering the matrices explored, SPE was the method of choice in this research. Typically, knowledge of the pKa value of compounds is useful in determining which sorbent to start with in SPE evaluation but in the case of SCs, pKa values were not known. While chromatography method optimization showed signs of SCs displaying some acidic properties, several SPE sorbents were explored. The SPE cartridges evaluated included 150mg 6cc Oasis® MCX, Oasis® MAX, and Oasis® HLB (Waters

Corporation, Milford, MA, USA). Oasis cartridges contain HLB copolymer based sorbents meaning both water and lipid soluble components can be captured. MCX and MAX cartridges are two types of Oasis cartridges. They are mixed-mode in the sense that they have a reverse phase portion and a strong ion exchange portion. MCX has a strong cation exchanger, which is good for performing SPE on basic compounds while MAX has a strong anion exchanger, good for retaining acidic compounds. HLB cartridges contain strong, hydrophilic, reversed phase sorbents and are useful in SPE on all types of compounds.

Depending on the acidic, neutral, or basic behavior of the analytes of interest, MAX and MCX sorbents can be very useful extraction techniques. The reverse phase component consists of silica bonded with C18. The sulfite moiety on the cation exchanger of MCX provides a pKa of approximately 1, which enables ionized basic compounds to be retained. The ammonium moiety on the anion exchanger in MAX sorbents creates a pKa of approximately 14, capturing ionized acidic compounds of interest. During SPE on MAX cartridges, a wash containing water and ammonium hydroxide removed weak acidic interferences while ionizing acidic compounds of interest on the reverse phase portion transferring them to the anion exchanger. A second wash of methanol and ammonium hydroxide neutralized basic and neutral compounds causing them to elute off of the reverse phased portion. Finally, a mixture of methanol and formic acid neutralized the acidic compounds of interest transferring them from the ion exchanger to the reverse phase portion for elution. MCX SPE is performed in a similar manner using acidic mixtures during wash steps and basic mixtures during elution.

Two different solutions at a concentration of 1ng/mL for all SCs were prepared for the first extraction optimization. One set contained MeOH as a solvent and the other set utilized ACN as a solvent. These solutions were used as unextracted standards, which were placed directly into an LC vial for analysis. They were also used as the sample to be extracted. In every experiment, unextracted standards were utilized for extraction evaluation and recovery comparison purposes. A conditioning step with approximately 2mL MeOH followed by approximately 2mL MilliQ water was performed in each SPE. In each wash and elution step, 2mL of solution were used. The following table represents the wash and elution steps carried out in the MCX and MAX applications (Table 3).

Table 3: Steps for MAX and MCX Solid Phase Extraction

Cartridge	**MCX**	**MAX**
Wash 1	0.1 N HCl	MilliQ water + 5% NH4OH
Wash 2 (for MeOH loaded solutions)	MeOH + 5% FA	MeOH + 5% NH4OH
Elution (for MeOH loaded solutions)	MeOH + 5% NH4OH	MeOH + 5% FA

Evaluation of the MCX and MAX extraction methods concluded the MAX sorbent to provide a more efficient extraction in isolating the compound of interest in comparison to that of MCX. The initial extraction performed assessed the difference between the sample loading at pH 3, pH 7, and pH 10. The loading pH is essential in ensuring the analyte of interest is ionized so that it will be retained and efficiently extracted from the sample as a result of its interactions with the sorbent. The loaded sample contained 2mL of a spiked standard 1ng/mL solution in MeOH (or ACN) with its

respective pH additive. The load step, wash 1 (W1), wash 2 (W2), and elution step were collected and analyzed for each method. Figure 10 displays a comparison of MCX and MAX collected eluates of the metabolites AB-PINACA 5-COOH and UR-144 5-COOH at pH 3 MeOH loading. The MAX application to pH 3 loaded samples in MeOH had 10X signal intensities compared to that produced from MCX in addition to significantly less noise and better Gaussian peak shapes. It was also concluded that MeOH was the best solvent to utilize in comparison to ACN.

Further evaluation of the MCX collections determined there to be a high percentage of some compounds of interest eluted in W2, suggesting acidic behavior (Table 4). In the evaluation of the MAX collections, it appeared that half of the compounds of interest were binding to the ion exchanger portion while the other half were binding to the reversed phase component. This can be observed in the split recovery percentages displayed between the W2 and the elution step for MAX results in Table 4. This observation described potential zwitterion properties of SCs. The data in the table was calculated by taking the sum of the average of the three replicate injections for load A, W1, W2, and elution. A percent recovery was then calculated for each step by taking the average of the three injections and dividing that value by the sum**.** Overall, zero breakthrough was displayed in the MAX collections. The MAX cartridge with low pH loading conditions was concluded to provide the best synthetic cannabinoid extraction.

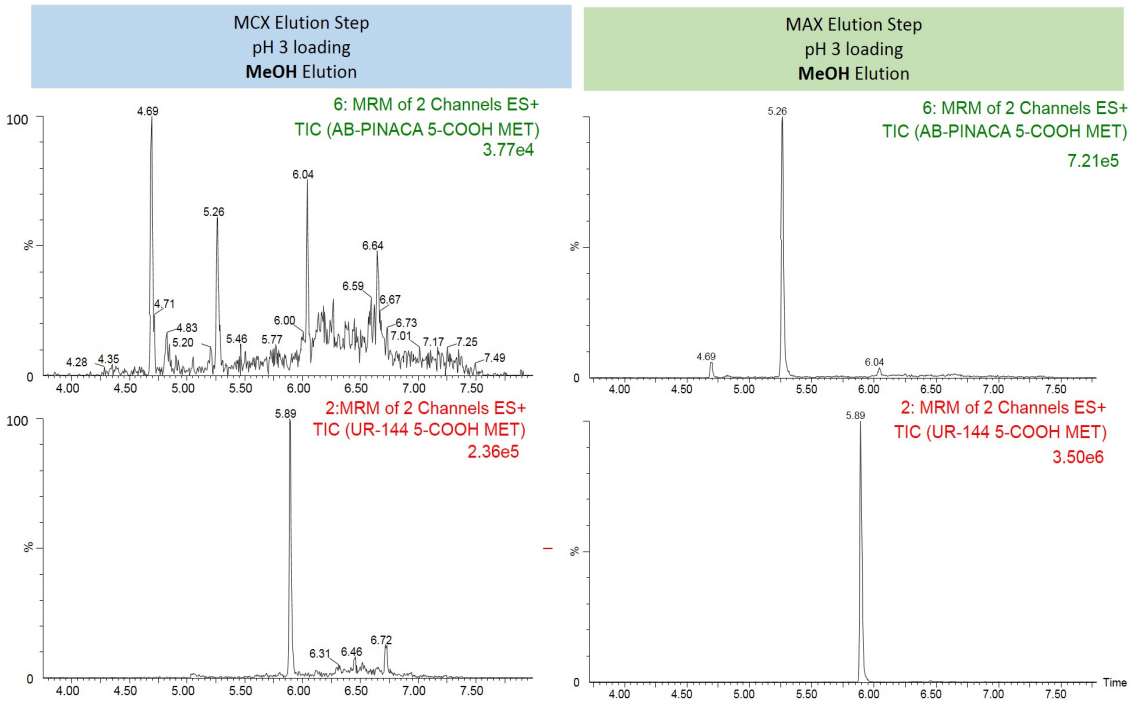

Figure 10: A Comparison of MAX and MCX - Solid Phase Extraction Optimization

Table 4: MAX vs. MCX – MeOH Extraction Recoveries.

XLR-11			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.11	Load A	0.39
W1	0.16	W1	0.52
W2	51.44	W2	30.05
Elution MeOH + 5%FA	48.28	Elution MeOH + 5%NH4OH	69.04
UR-144 5-COOH			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.02	Load A	0.07
W1	0.02	W1	0.22
W2	0.32	W2	36.52
Elution MeOH + 5%FA	99.65	Elution MeOH + 5%NH4OH	63.18
JWH-018			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	2.13	Load A	1.36
W1	1.15	W1	0.90
W2	30.51	W2	41.98
Elution MeOH + 5%FA	66.21	Elution MeOH + 5%NH4OH	55.76
JWH-018 5-hydroxypentyl			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.02	Load A	0.03
W1	0.02	W1	0.10
W2	55.81	W2	65.89
Elution MeOH + 5%FA	44.16	Elution MeOH + 5%NH4OH	33.98
AB-PINACA 5-COOH			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.48	Load A	1.28
W1	0.60	W1	1.22
W2	0.16	W2	86.59
Elution MeOH + 5%FA	98.76	Elution MeOH + 5%NH4OH	10.91
AM-2201 4-hydroxypentyl			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.03	Load A	0.02
W1	0.02	W1	0.09
W2	57.63	W2	66.65
Elution MeOH + 5%FA	42.32	Elution MeOH + 5%NH4OH	33.24
5F-PB-22			
MAX pH 3 load	**Recovery (%)**	**MCX pH 3 load**	**Recovery (%)**
Load A	0.06	Load A	0.05
W1	0.04	W1	0.37
W2	39.54	W2	12.67
Elution MeOH + 5%FA	60.36	Elution MeOH + 5%NH4OH	86.91

The observation of potential zwitterion characteristics of the compound of interest suggested further extraction optimization would be necessary. The loading pH was optimized as previously discussed, however, the additional parameter of loading solution organic strength was assessed with varying pH values for further extraction evaluation. The optimization of a combination of parameters, such as loading pH and organic strength, is useful in obtaining maximum recovery. Therefore, an extraction was performed on an HLB cartridge using elution solvents with varying percentages of MeOH. The HLB protocol carried out consisted of the conditioning and loading steps previously discussed and an elution step with different solutions of MeOH. Ten different MeOH solutions were used – 10, 20, 30, 40, 50, 60, 70, 80, 90, an 100% MeOH in water loaded on ten cartridges yielding ten separate, collected eluates. This experiment was performed a second time with the MeOH elution solutions at pH 3 using 0.5% formic acid and a third time with the MeOH elution solutions at pH 10 using 0.5% NH_4OH. The pH 10 elution portrayed zero breakthrough up until 80-90% MeOH while the pH 7 and pH 3 elution demonstrated breakthrough at lower percentages of MeOH.

Table 5 displays the carboxylic acid SCs and their highest recovery percentages spread over a variety of pH 3 elution solutions. The recovery percentages of the other five SCs at pH 3 elution were spread evenly across all MeOH elution solutions. Table 6 displays the percentage recoveries of the other five synthetic cannabinoids analyzed. The highest recoveries were observed at a range of 80-100% MeOH. The recovery percentages of the carboxylic acid SCs at pH 10 elution were spread evenly across all MeOH elution solutions. The split recovery percentages from the MAX evaluation and

the spread recovery values across different elution solutions of varying MeOH percentages and pH values demonstrated the importance of developing a more efficient method of extraction in terms of recovery.

Table 5: HLB Extraction - Elution pH 3

PH	Elution Solution	UR-144 5-COOH Metabolite Recovery (%)	AB-PINACA 5-COOH Metabolite Recovery (%)
3	Elution 10% MeOH + FA	0.97	0.47
	Elution 20% MeOH + FA	0.92	0.33
	Elution 30% MeOH + FA	0.86	0.17
	Elution 40% MeOH + FA	0.87	0.78
	Elution 50% MeOH + FA	0.56	4.33
	Elution 60% MeOH + FA	0.31	46.32
	Elution 70% MeOH + FA	0.29	41.24
	Elution 80% MeOH + FA	25.54	3.92
	Elution 90% MeOH + FA	56.85	0.84
	Elution 100% MeOH + FA	12.84	1.61

Table 6: HLB Extraction - Elution pH 10

PH	Elution Solution	XLR-11 Recovery (%)	JWH-018 Recovery (%)	JWH-018 5-Ohpentyl metabolite Recovery (%)	AM-2201 4-Ohpentyl Metabolite Recovery (%)	5F-PB-22 Recovery (%)
10	Elution 10% MeOH + NH4OH	0.43	5.40	0.23	0.17	0.39
	Elution 20% MeOH + NH4OH	0.29	4.30	0.25	0.23	0.39
	Elution 30% MeOH + NH4OH	0.35	4.05	0.30	0.22	0.53
	Elution 40% MeOH + NH4OH	0.44	3.91	0.43	0.30	0.50
	Elution 50% MeOH + NH4OH	0.60	4.63	0.66	0.47	0.84
	Elution 60% MeOH + NH4OH	0.68	5.73	0.79	0.54	1.09
	Elution 70% MeOH + NH4OH	0.83	7.73	0.78	0.54	1.33
	Elution 80% MeOH + NH4OH	1.18	8.59	1.89	2.43	1.37
	Elution 90% MeOH + NH4OH	38.14	9.06	32.87	38.35	19.14
	Elution 100% MeOH + NH4OH	57.07	46.61	61.80	56.75	74.42

As a result of the HLB evaluation with different MeOH elution solutions, the original MAX method previously described (Table 3) was altered to include a different W2 solution and elution solvent. The final MAX method is summarized in table 7. Wash 1 was utilized to wash off any weak interferences and ionize the compounds of interest off the reversed phase portion and onto the ion exchanger. Wash 2 removed any

additional interferences while keeping the compounds of interest on the ion exchanger. A wash containing 70% methanol in water was ultimately used because in an experiment where 80% was used, approximately 5% of the compounds were unable to be recovered in the elution. Elution 1 was used to elute any compounds of interest that may have been stuck to the reversed phase portion of the sorbent. Elution 2 neutralized the remaining, acidic SCs off of the ion exchanger causing those compounds to transfer to the reversed phase component and elute off of the cartridge. Two elution steps were utilized to ensure the most efficient extraction of the zwitterion analytes was completed. A comparison of the original MAX method and the final MAX method was performed and all loading, wash, and elution steps were collected and analyzed.

Table 7: Final MAX Solid Phase Extraction Method Protocol

Cartridge	**MAX**	
Condition 1	~2mL MeOH	
Condition 2	~2mL MilliQ Water	
Load	100mL solution (100mL MilliQ water at pH 3 + 2mL centrifuged sample)	
Wash 1	2mL MilliQ water + 5% NH4OH	
Wash 2	2mL 70% MeOH in water + 5% NH4OH	
Elution 1	1mL 100% MeOH in water + 5% NH4OH	Pool elution 1 & 2 together collecting a total of 2mL sample for analysis
Elution 2	1mL 100% MeOH in water + 5% FA	

Figure 11 displays a comparison between the original and final MAX methods for XLR-11, which provides an overall description of the results for all analytes studied in this research. Three samples at a concentration of 0.1, 1, and 10ng/mL MeOH were prepared for loading where 2mL of each solution were placed into a plastic centrifuge tube with 2mL of MilliQ water. Each sample was then placed into its own tube containing 100mL of MilliQ water and 2mL formic acid (to create pH 3 loading conditions). Three more samples were prepared the same way with 2mL of urine added to each centrifuge tube instead of water, enabling a protein crash to take place.

The final 100mL solutions were then loaded on to the SPE cartridges for extraction. The chromatograms displayed in figure 11 represent those produced from the samples prepared with water added. The final MAX method produced a higher recovery in the elution than that portrayed in the elution from the original MAX method. It can also be observed that some of the compound of interest is lost in the wash step when using the original method, as the wash step has higher signal intensity than the elution. The signal intensity of the original method shows loss of compound when comparing the intensity of the unextracted standard to the intensity of the extract. The signal intensity of the extract obtained from the MAX final method displayed almost 100% recovery in comparison to that obtained in the unextracted standard, confirming the efficiency of utilizing the MAX final method over the MAX original method in the extraction of synthetic cannabinoids.

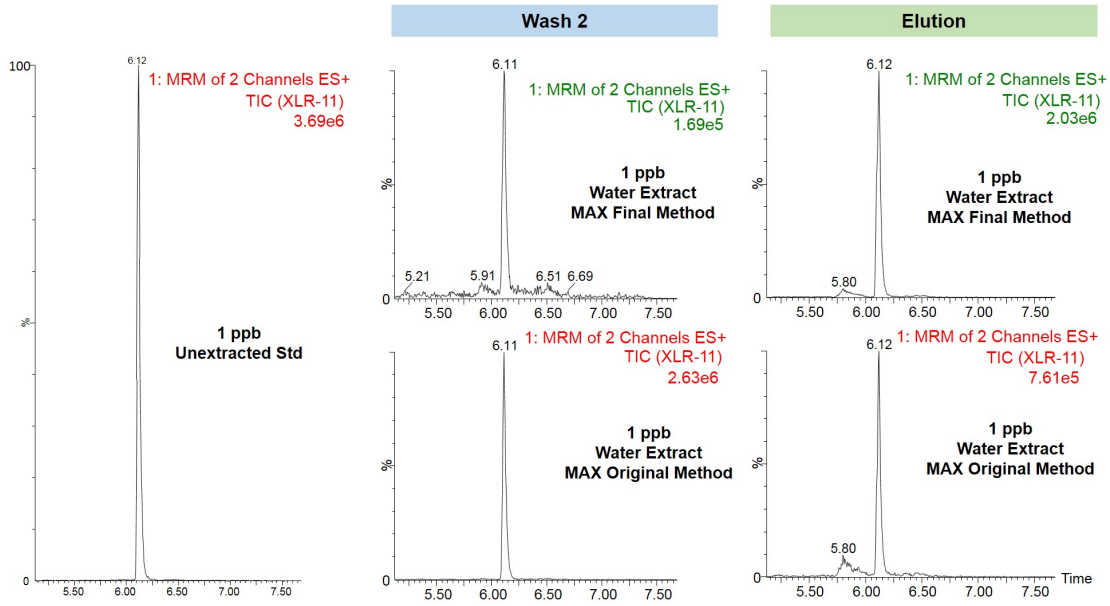

Figure 11: Comparison of MAX Original and MAX Final Methods – XLR-11

2.2.3.1 Urine Sample Preparation Optimization

When comparing the MAX final and MAX original methods during extraction optimization, it was observed that matrix effects and the way the samples were prepared before SPE may have been affecting overall recoveries. For example, Figure 12 displays a signal intensity reduction by almost 10X from the unextracted standard to the urine extract for AM-2201 4-hydroxypentyl metabolite. The high recovery observed from the unextracted standard to the water extract but poor recovery between the water and urine extracts suggested binding issues. Other instances showed the compound of interest in the unextracted standard, but missing in the extracts.

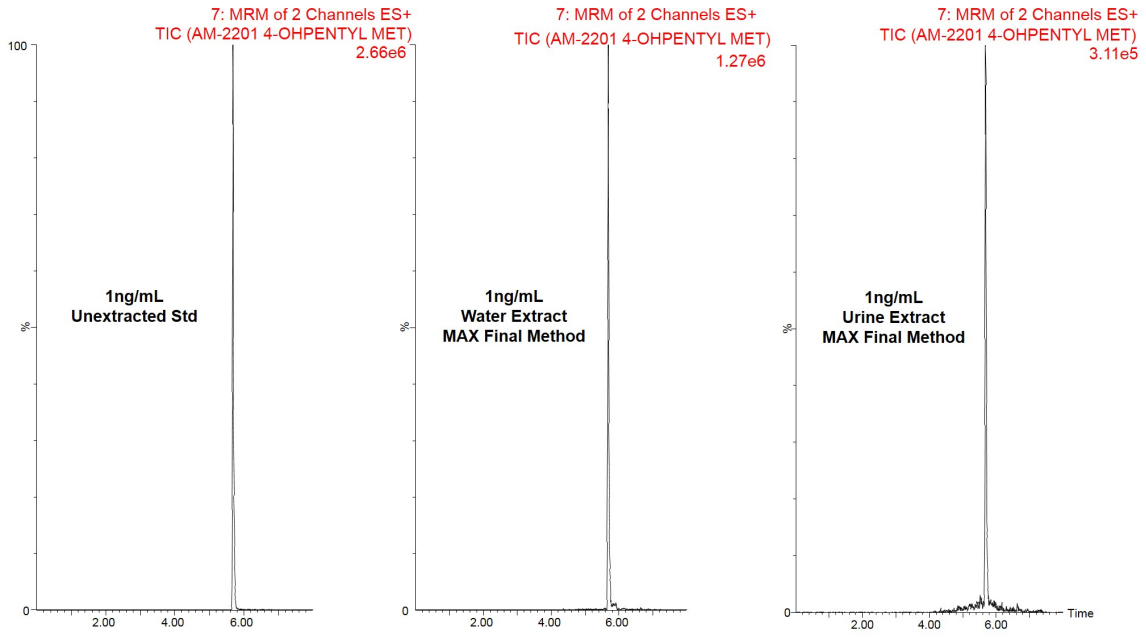

Figure 12: Depiction of Potential Binding Issues Resulting from Protein Crash Preparation

In order to eliminate any binding issues, the protein precipitation method was altered to include a glass centrifuge tube instead of a plastic centrifuge tube and 50µL of phosphoric acid. All protein crashed samples were centrifuged for five minutes at 3900rpm. The supernatant was then poured off into 100mL MilliQ water (spiked with 2mL formic acid to create pH 3 conditions) before loading onto the SPE cartridge for analysis.

The final method utilized in urine sample preparation consisted of a protein crash, dilution, and SPE. The protein crash was performed by adding 2mL of MeOH, 50µL phosphoric acid, and 2mL of urine to a 20mL glass centrifuge tube. After capping and shaking manually, the samples were centrifuged. The supernatant was then poured off into 100mL of MilliQ water to create a dilution of less than 5% and 2mL of formic acid

were added to create pH 3 loading conditions. The 100mL samples were then loaded onto an MAX 6cc 150mg SPE cartridge with a 100mL attachment on it for solid phase extraction (see Figure 13). A negative pressure pump was used to help load the samples on the column (~10-15psi). The MAX final method was then performed, collecting and transferring a 2mL final eluate to an LC vial. Pressure on the wash and elution steps was maintained ~5psi. A volume of 20µL internal standard JWH-(018) d_{11} was added to each unextracted and extracted sample before 2D-LC-MS/MS analysis.

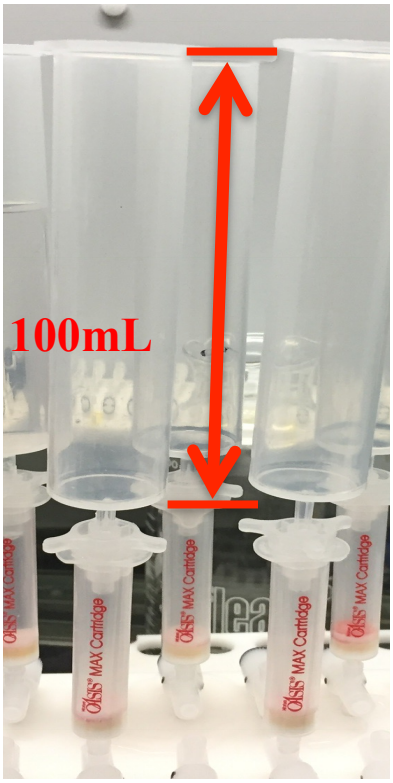

Figure 13: 100mL attachment for SPE cartridge

2.2.3.2 Plasma Sample Preparation

The plasma samples were prepared in a similar manner as the urine samples except a slightly higher proportion of MeOH was utilized. A protein crash was first

performed by adding 3mL MeOH and 50µL phosphoric acid to 2mL of plasma in a 20mL glass tube. The tubes were capped, shaken, and centrifuged. The supernatant was transferred to 100mL water at pH 3 and processed with the same protocol previously described for the urine samples.

2.2.3.3 Edible Sample Preparation Optimization

The edible matrix explored was gummy bear candy (red, green, orange, clear and yellow). The complexity of the gummy matrix in comparison to urine and plasma required a different type of sample preparation before SPE. The gummy bears were cut into fourths, creating ~0.5g pieces. Two-fourths (to make ~1g) of a sample were added to four 20mL glass vials with 3mL of 70%, 80%, 90%, and 100% MeOH in water. Eight more samples were created in the same manner, four samples contained ACN in water and four contained acetone in water. This experiment was repeated two more times, once with 4mL and the next with 5mL total solution. From there, observations were made regarding sample changes at room temperature, after sonication, and after heating. The objective was to completely dissolve the gummy bear sample into solution to ultimately carry out SPE in the shortest amount of time possible.

There were several differences observed based on the various conditions of each sample. There were no significant changes in the room temperature samples in regards to dissolution. The MeOH based samples showed fading color on the gummy bears but there was not any color observed in the solution. After ten minutes of sonication, color and cloudiness were observed in the 70% MeOH, ACN, and acetone solutions but color was not observed in the samples with higher percentages of organic solvent (Figure 14).

Additionally, the gummy bears in the 70% organic solutions were very sticky after sonication. Heating seemed to provide more desirable results overall. After 15 minutes of heating the solutions from ~60-80°C, the gummy bears in the 70% MeOH and ACN organic solutions were completely dissolved (Figure 14). Since the presence of water in the solutions seemed to play a major role in gummy bear dissolution, solutions of 100% water and 50/50 water/organic were prepared and evaluated. The gummy bears in all 5mL solutions were completely dissolved after 15 minutes of heating with the acetone solvent taking the longest to complete dissolution (Figure 14).

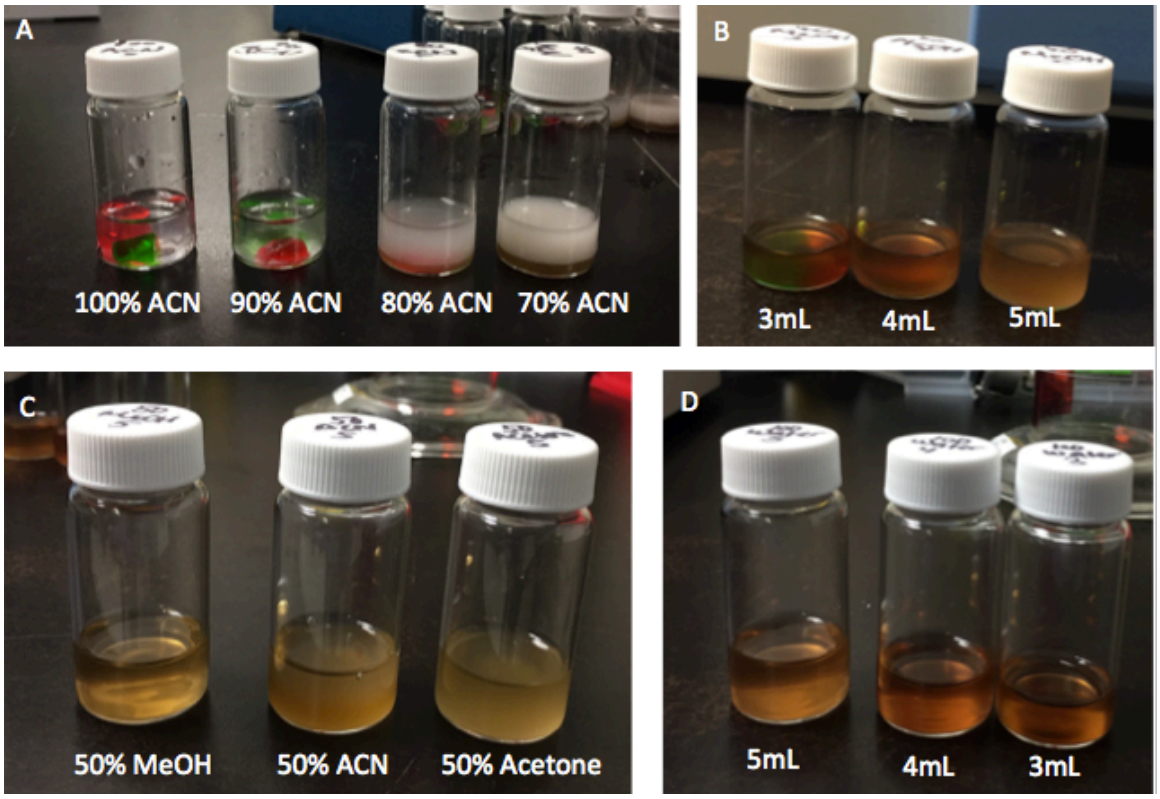

Figure 14. Gummy Bear Sample Preparation Observations. (A) ACN solutions (5mL) after 10 minutes of sonication. (B) 70% MeOH solutions after 15 minutes of heating. (C) 50% organic solutions (5mL) after 15 minutes of heating. (D) 100% water solutions after 15 minutes of heating.

Further evaluation was completed comparing the heating method to homogenization with a Precellys Evolution homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Solutions of 100% MeOH, 100% Acetone, 100% ACN, 100% water, 50% ACN in water, and 50% MeOH in water were prepared. The 100% organic solutions were only assessed with homogenization as they had already been evaluated with heating and resulted in an undesirable dissolution. After homogenization with four ball ceramic ball bearings in a 15mL tube for three 90-second cycles at 6000rpm, dissolution in the 100% organic solutions was not observed. Figure 15 depicts the results of various samples after homogenization and heating. The samples with 50% organic (ACN and MeOH) and 100% water in both methods appeared to produce an efficient breakdown of the complex gummy matrix. An interesting observation was that in all experiments ACN solutions formed two layers. Both layers, however, were evaluated as one in future extractions.

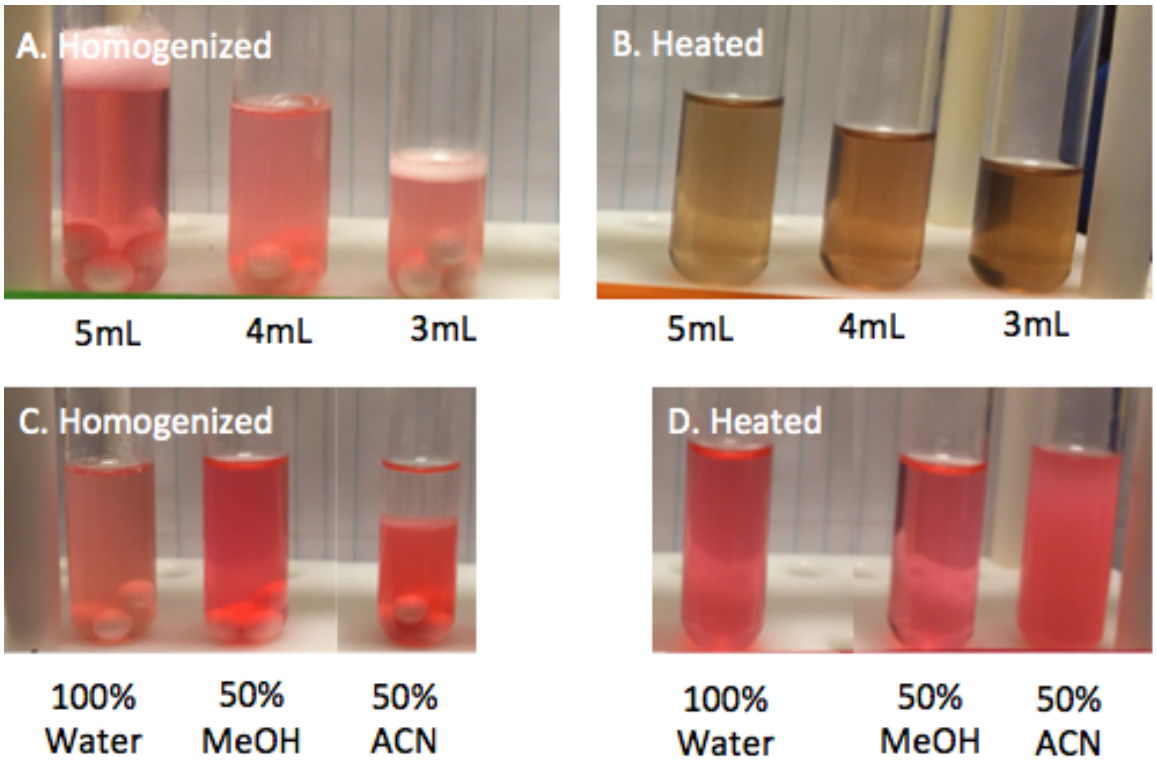

Figure 15. Comparison of Homogenization and Heating. (A) 100% water samples at 3mL, 4mL, and 5mL processed via homogenization. (B) 100% water samples at 3mL, 4mL, and 5mL processed via heating. The brown color is the result of red and green colored gummy bears whereas red gummy bears were the only candy used in A,C, and D. (C) 100% water, 50% MeOH, and 50% ACN samples processed via homogenization. (D) 100% water, 50% MeOH, and 50% ACN samples processed via heating. After settling, the heated 50% ACN samples formed two layers.

SPE was performed to compare the extraction rates of the 100% water, 50% MeOH, and 50% ACN homogenized and heated solutions. After the dissolution method was completed, the samples were centrifuged at 3900rpm for five minutes. The supernatant was poured off into 100mL of water, which was then loaded onto the conditioned MAX SPE cartridges. The solutions containing 100% water took a significantly longer amount of time to load through the column (more than 20 minutes), while 50% ACN and MeOH samples only took 10-15 minutes. It was additionally noted that the color was retained on the column through all loading, washing, and elution steps

producing an overall clear solution in the eluate.

The 50% MeOH and 50% ACN samples were prepared for 2D-LC-MS/MS analysis to determine the best sample preparation method. Approximately 1g of sample was dissolved in 5mL of 50% MeOH or 50% ACN in water. The samples were then dissolved by heating for 15 minutes at 60-80°C or homogenized in three 90-second cycles. After dissolution, the samples were centrifuged for five minutes at 3900rpm and the supernatant was poured off into 100mL of MilliQ water with 2% formic acid. SPE was performed with the MAX final method and the eluates were transferred to a LC vial, spiked with 20µL of JWH-018 d_{11} internal standard, and analyzed with 2D-LC-MS/MS.

Analysis of the 2D-LC-MS/MS results ultimately concluded the method dissolving 1g of gummy bear sample in 5mL of 50% MeOH in water by heating to be the best sample preparation method. Figure 16 displays a comparison of the homogenized and heated samples in 50% MeOH using the unextracted standard samples as a reference. Overall, the heated samples had much better Gaussian peak shapes and higher intensities. The homogenized samples produced wider peaks, more noise, and in the case of XLR-11 for instance, a signal reduction of almost 10X compared to that of the unextracted standard. When comparing the samples in 50% ACN, heating provided slightly better results than homogenization, however both methods resulted in signal reduction and intense background noise (Figure 17). Figure 18 presents a comparison between the heated samples in 50% MeOH and the heated samples in 50% ACN. Utilizing MeOH as a solvent with the heated dissolution technique provided better Gaussian peak shapes, more intense signals, and very little background noise in comparison to the other three

methods evaluated. Some signal intensity differences were more significant than others, such as 5F-PB-22 (Figure 18).

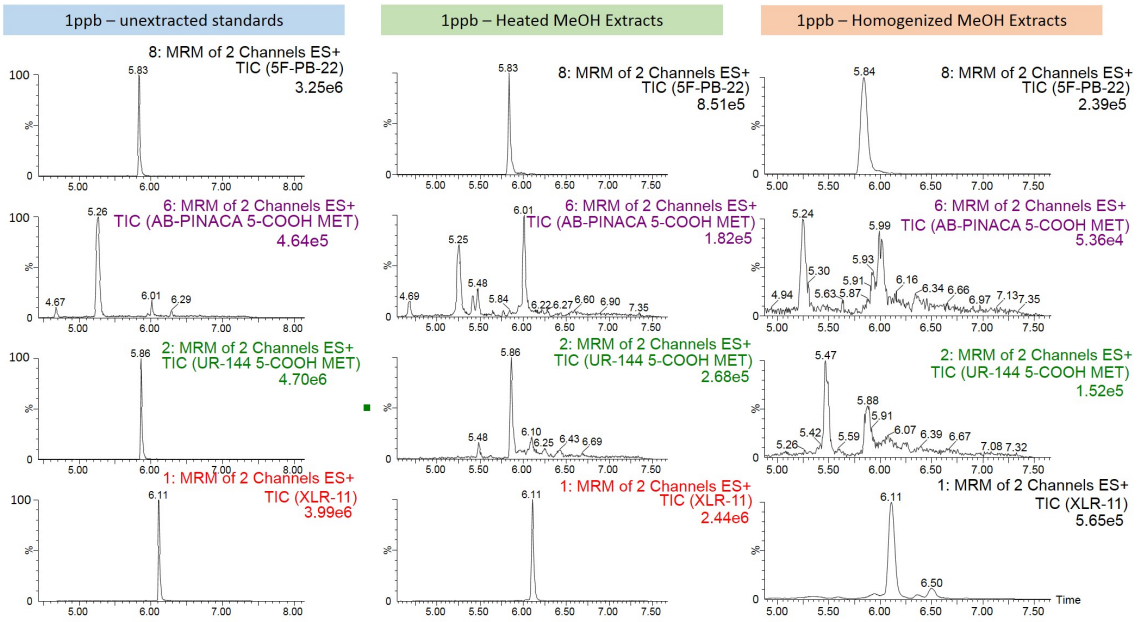

Figure 16: Edible Sample Preparation Optimization – Heated vs. Homogenized 50% MeOH in Water Samples

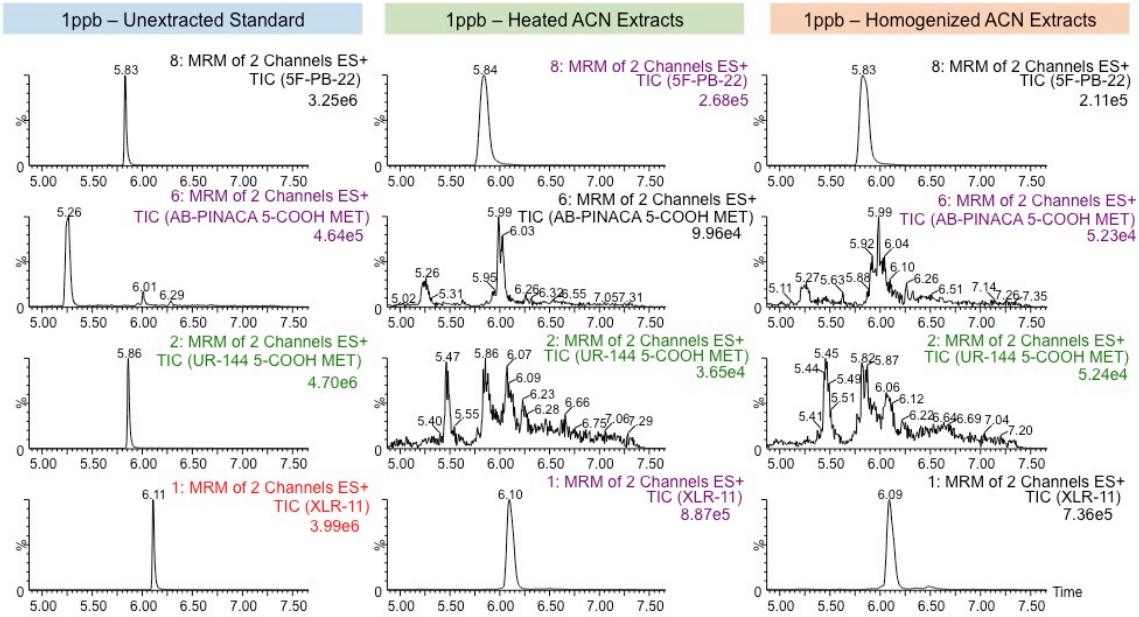

Figure 17: Edible Sample Preparation Optimization – Heated vs. Homogenized 50% ACN in Water Samples

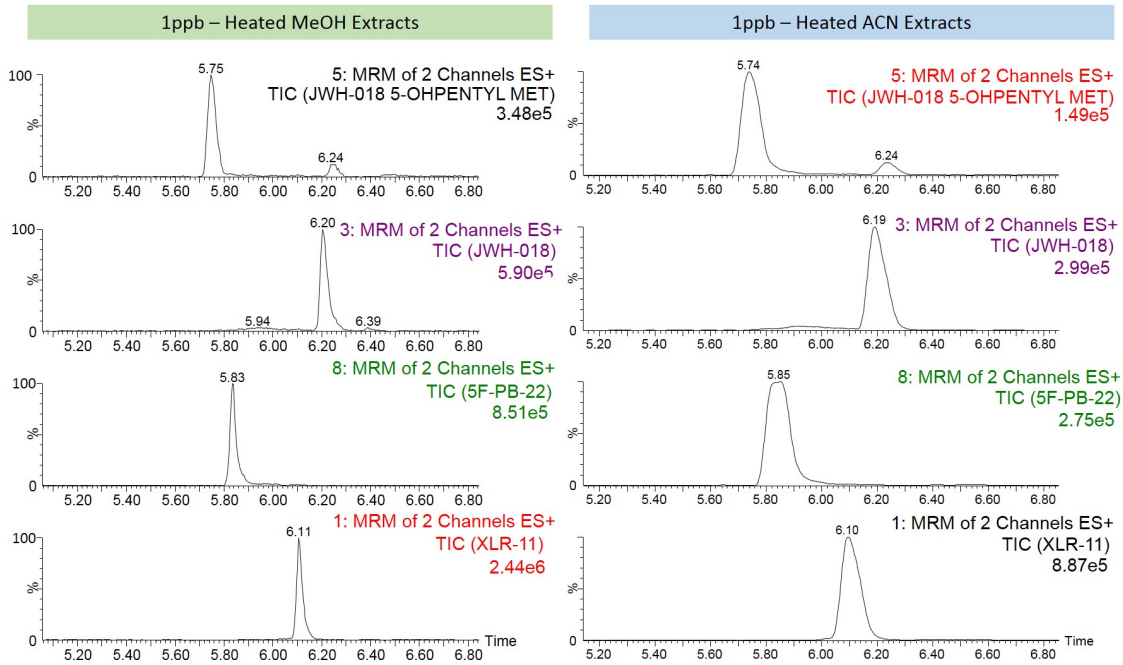

Figure 18: Edible Sample Preparation Optimization – Heated 50% MeOH vs. Heated 50% ACN Samples

2.2.4 Calibration Curve Generation

2.2.4.1 Urine and Plasma

For quantitation purposes, a calibration curve was produced for all matrices in addition to producing a water extract and an unextracted standard curve on the same day each matrix was extracted. Eight calibrators, or spiking solutions, with different concentrations were prepared for each curve – 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10ng/mL. Stock solutions (A, B, and C) at concentrations of 1000, 100, and 10ng/mL, respectively, in MeOH containing all seven synthetic cannabinoids analyzed in this study were utilized in the preparation of the calibrators. Table 8 describes the amounts of each solution used to prepare the calibrators. Spiking solutions utilized for the water, urine, and plasma extracted curves contained 100% MeOH as a solvent. Spiking solutions utilized as unextracted standards contained half MeOH + 0.5% formic acid and half

MeOH + 0.5% NH_4OH to represent the same conditions the extracted samples were collected under in (see MAX final method protocol Table 7).

The urine calibration curve was generated by preparing eight solutions with the final concentrations listed in Table 8. Each solution contained 2mL of negative urine, 2mL of the respective calibrator solution, and 50µL of phosphoric acid. The plasma calibration curve was generated in the same manner, however 3mL of the respective calibrator solution was used. The protein precipitation and MAX final method previously described were then performed to yield samples for 2D-LC-MS/MS analysis. The collected eluates were spiked with 20µL JWH-018 d_{11} internal standard after extraction.

Table 8: Preparation of Calibrator Solutions

Final Concentration (ng/mL)	Stock Solution	Volume Stock Solution (µL)	Volume MeOH (mL)
10	A	100	9.9
5		50	9.95
2.5		25	9.975
1	B	100	9.9
0.5		50	9.95
0.25		25	9.975
0.1	C	100	9.9
0.05		50	9.95

2.2.4.2 Edibles

The calibrators for the gummy bear curves were made differently than the plasma and urine curves as the preparation methods differed. Approximately 1g of gummy bear

sample was added to eight 20mL glass vials. In order to create the optimal 50% organic 5mL solution, 2.5mL of water and 2.5mL organic solution were added to the 20mL vial. The organic solution added consisted of 2mL of the respective calibrator solution (prepared with 100% MeOH as described in Table 8) and 500µL of MeOH. The calibrators were then heated for 15 minutes at 60-80°C, transferred to 15mL glass centrifuge tubes and centrifuged for five minutes at 3900rpm. The supernatant was poured off into 100mL of MilliQ water with 2% formic acid. SPE was performed with the MAX final method and the eluates were transferred to a LC vial, spiked with 20µL of JWH-018 d_{11} internal standard, and analyzed with 2D-LC-MS/MS.

3. RESULTS AND DISCUSSION

3.1 Calibration Models

3.1.1 Urine

Three curves were generated in the analysis of synthetic cannabinoids in urine. An unextracted standard curve, a water extract curve, and a urine extract curve were prepared utilizing the eight calibrator solutions previously described in their respective matrix (Table 8). A recovery sample prepared at 1ng/mL and a blank were also analyzed with the water and urine curves. A calibration curve equation was calculated for each compound utilizing the TargetLynx software and a ratio of the internal standard and calibrator area counts. A calibration curve with linear and quadratic fit, 1/x and $1/x^2$, was assessed for each analyte in each matrix to determine the best fit curve for each compound.

Table 9 portrays the linear dynamic range, LOD, LOQ, recovery and R^2 values of the urine curve for each compound. R^2 values of 0.995 or greater were calculated for all compounds besides the two carboxylic acids, UR-144 5-COOH and AB-PINACA 5-COOH. Figures 19-21 depict the urine calibration curves produced for XLR-11, JWH-018, and AB-PINACA 5-COOH. XLR-11 and JWH-018 had a linear dynamic range of 0.05-5ng/mL while AB-PINACA 5-COOH linear dynamic range was 0.05-2.5ng/mL. LOD values were 0.005ng/mL and lower and LOQ values ranged from 0.0005ng/mL to 0.1ng/mL. The unextracted standard and water extract calibration curves run on the same day as the urine curve are displayed in Appendix B for XLR-11.

Table 9: Urine Calibration Curve Results

Compound	Polynomial Type	Weight	Linear Dynamic Range	LOD (ng/mL)	LOQ (ng/mL)	R2	Recovery (%)
XLR-11	Quadratic	1/X	0.05-5ng/mL	<0.005	0.005	0.997	98
UR-144 5-COOH	Quadratric	1/X	0.05-2.5ng/mL	0.05	0.1	0.992	81
JWH-018	Quadratic	1/X	0.05-5ng/mL	<0.005	0.005	0.995	97
JWH-018 5-hydroxypentyl	Linear	1/X	0.05-5ng/mL	<0.005	0.005	0.995	94
AB-PINACA 5-COOH	Quadratric	1/X	0.05-2.5ng/mL	0.05	0.1	0.993	104
AM-2201 4-hydroxypentyl	Quadratric	1/X	0.05-5ng/mL	<0.005	0.005	0.997	99
5F-PB-22	Quadratric	1/X	0.05-5ng/mL	0.00005	0.0005	0.996	106

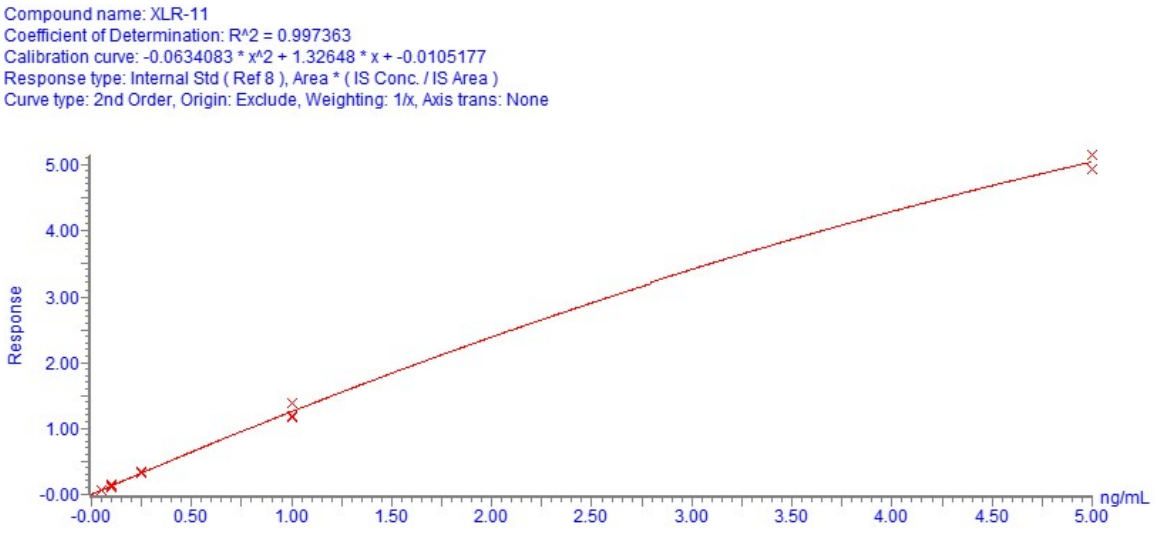

Figure 19: XLR-11 Urine Calibration Curve

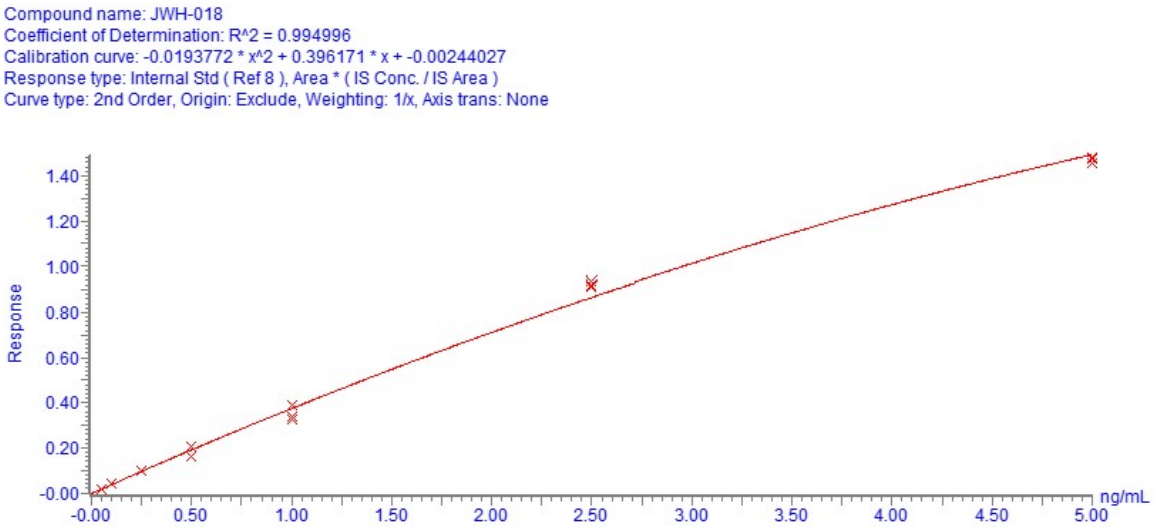

Figure 20: JWH-018 Urine Calibration Curve

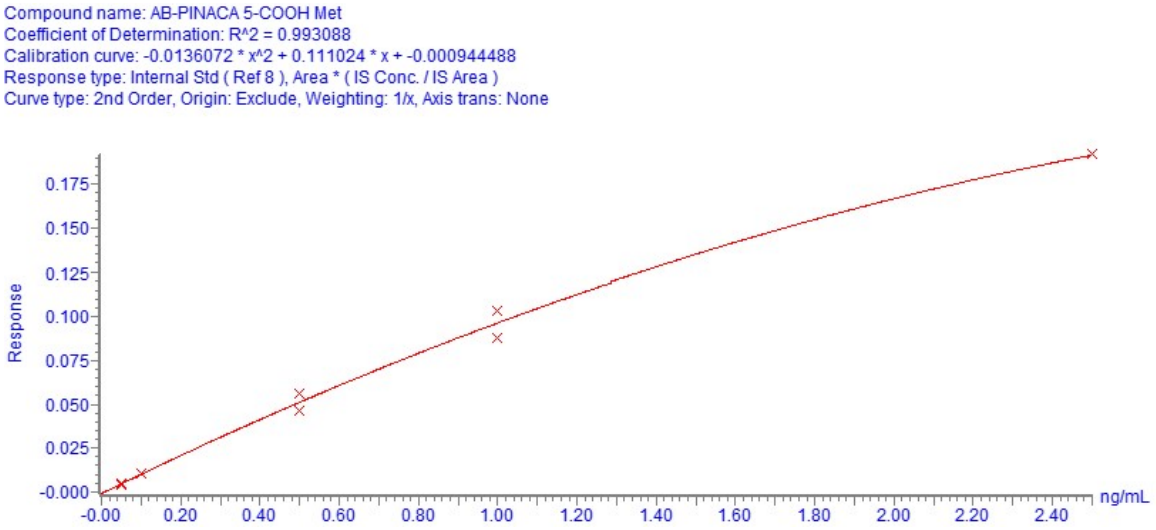

Figure 21: AB-PINACA 5-COOH Urine Calibration Curve

3.1.2 Plasma

Three curves were generated in the analysis of synthetic cannabinoids in plasma. An unextracted standard curve, a water extract curve, and a plasma extract curve were prepared with a recovery and blank sample and a calibration curve equation was calculated for each curve in the same manner as in the analysis of the urine curves. Table

10 displays the linear dynamic range, LOD, LOQ, recovery and R^2 values of the plasma curve for each compound. Excellent R^2 values of 0.995 or greater were calculated for all compounds. Figures 22-24 depict the plasma calibration curves produced for UR-144 5-COOH, AM-2201 4-hydroxypentyl, and 5F-PB-22. All compounds had a linear dynamic range of 0.05-10ng/mL. LOD values were 0.05ng/mL or less and LOQ values ranged from 0.005ng/mL to 0.1ng/mL. The unextracted standard and water extract calibration curves run on the same day as the plasma curve are displayed in Appendix B for XLR-11.

Table 10: Plasma Calibration Curve Results

Compound	Polynomial Type	Weight	Linear Dynamic Range	LOD (ng/mL)	LOQ (ng/mL)	R2	Recovery (%)
XLR-11	Quadratic	1/X	0.05-10ng/mL	<0.005	0.005	0.996	95
UR-144 5-COOH	Quadratric	1/X	0.05-10ng/mL	0.05	0.1	0.995	96
JWH-018	Quadratic	1/X	0.05-10ng/mL	<0.005	0.005	0.996	89
JWH-018 5-hydroxypentyl	Linear	1/X	0.05-10ng/mL	0.05	0.1	0.995	89
AB-PINACA 5-COOH	Quadratric	1/X	0.05-10ng/mL	0.05	0.1	0.995	85
AM-2201 4-hydroxypentyl	Quadratric	1/X	0.05-10ng/mL	<0.05	0.05	0.996	88
5F-PB-22	Quadratric	1/X	0.05-10ng/mL	<0.005	0.005	0.998	102

Compound name: UR-144 5-COOH Met
Coefficient of Determination: R^2 = 0.995329
Calibration curve: -0.0230684 * x^2 + 0.50442 * x + -0.0177438
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

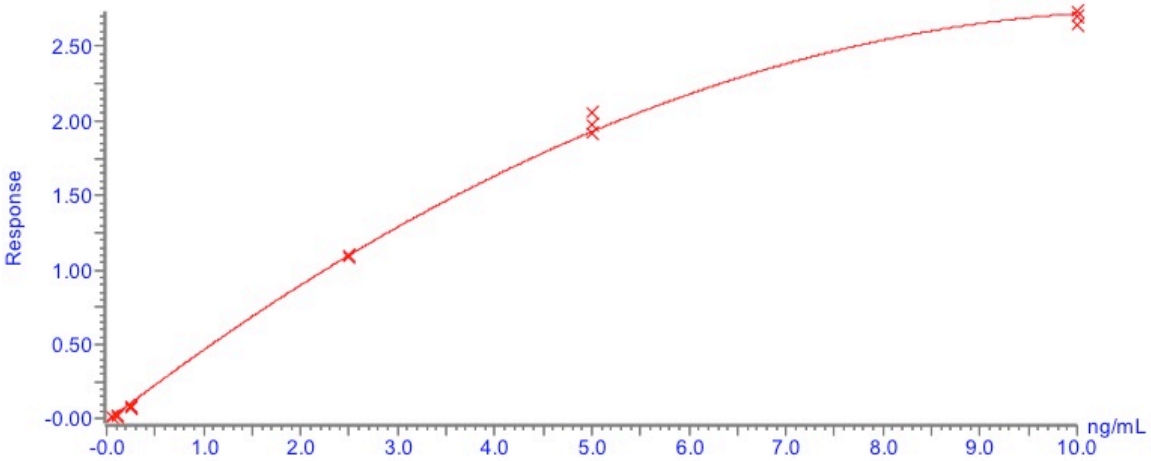

Figure 22: UR-144 5-COOH Plasma Calibration Curve

Compound name: AM-2201 4-OHPentyl Met
Coefficient of Determination: R^2 = 0.996071
Calibration curve: -0.00513089 * x^2 + 0.67503 * x + -0.00453416
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

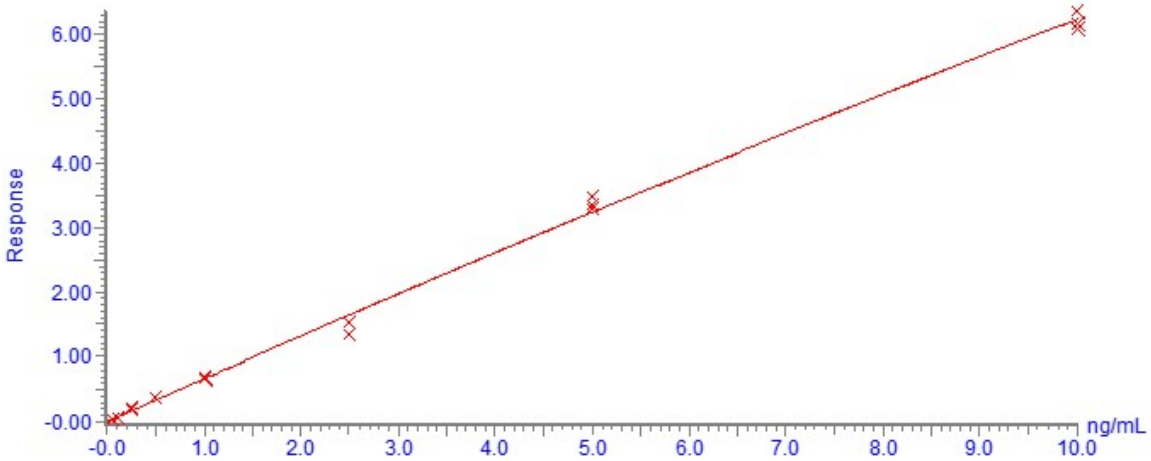

Figure 23: AM-2201 4-hydroxypentyl metabolite Plasma Calibration Curve

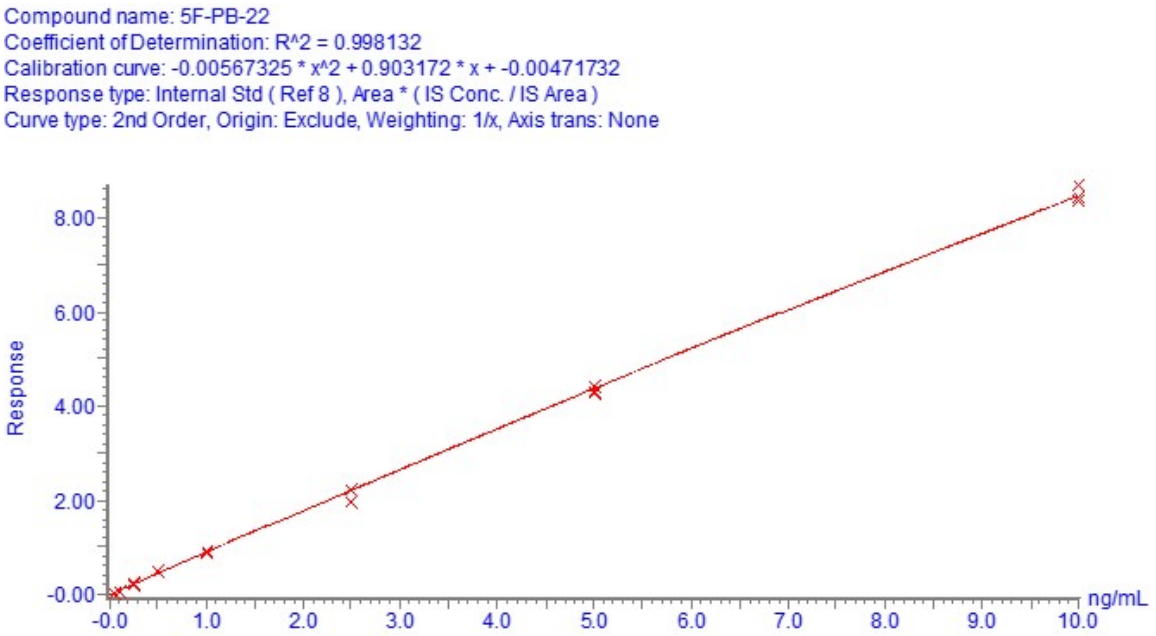

Figure 24: 5F-PB-22 Plasma Calibration Curve

3.1.3 Edibles

Three curves were generated in the analysis of synthetic cannabinoids in gummy bears. An unextracted standard curve, a water extract curve, and a gummy bear extract curve were prepared with a recovery and blank sample in addition to calculating a calibration curve equation for each curve in the same manner as in the analysis of the urine curve. Table 11 displays the linear dynamic range, LOD, LOQ, recovery and R^2 values of the gummy bear curve for each compound. Excellent R^2 values of 0.995 or greater were calculated for all compounds. Figures 24-26 depict the gummy bear calibration curves produced for XLR-11, JWH-018, and AB-PINACA 5-COOH. All compounds had a linear dynamic range of 0.05-10ng/mL except UR-144 5-COOH and AB-PINACA 5-COOH, which had a range of 0.02-2.5ng/mL. The LOD value for UR-144 5-COOH was 0.1ng/mL while the LOD values of the rest of the analytes studied in

this research were 0.05ng/mL or lower. The LOQ values ranged from 0.005ng/mL to 0.5ng/mL. The unextracted standard and water extract calibration curves run on the same day as the gummy bear curve are displayed in Appendix B for XLR-11.

Table 11: Gummy Bear Calibration Curve Results

Compound	Polynomial Type	Weight	Linear Dynamic Range	LOD (ng/mL)	LOQ (ng/mL)	R2	Recovery (%)
XLR-11	Quadratic	1/X	0.05-10ng/mL	<0.05	0.05	0.996	113
UR-144 5-COOH	Quadratric	1/X	0.05-2.5ng/mL	0.1	0.5	0.995	94
JWH-018	Quadratic	1/X	0.05-10ng/mL	<0.005	0.005	0.995	105
JWH-018 5-hydroxypentyl	Quadratic	1/X	0.05-10ng/mL	<0.005	0.005	0.995	106
AB-PINACA 5-COOH	Quadratric	1/X	0.05-2.5ng/mL	0.05	0.5	0.997	63
AM-2201 4-hydroxypentyl	Quadratric	1/X	0.05-10ng/mL	0.05	0.5	0.997	104
5F-PB-22	Quadratric	1/X	0.05-10ng/mL	<0.005	0.005	0.996	95

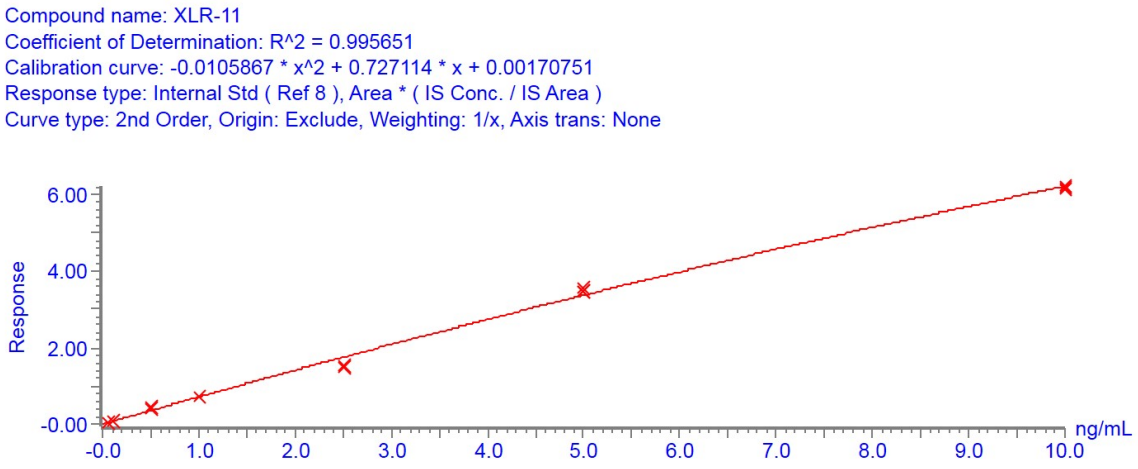

Figure 25: XLR-11 Gummy Bear Calibration Curve

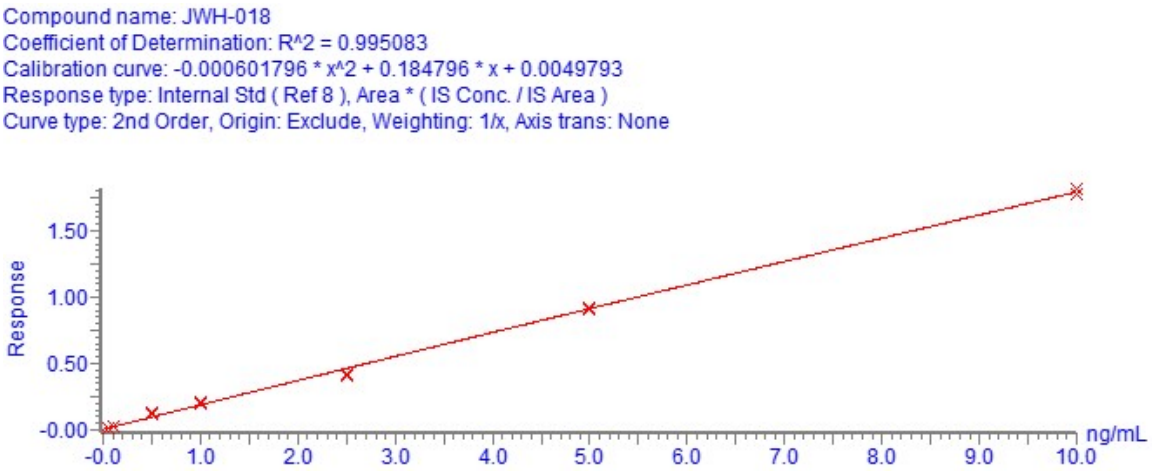

Figure 26: JWH-018 Gummy Bear Calibration Curve

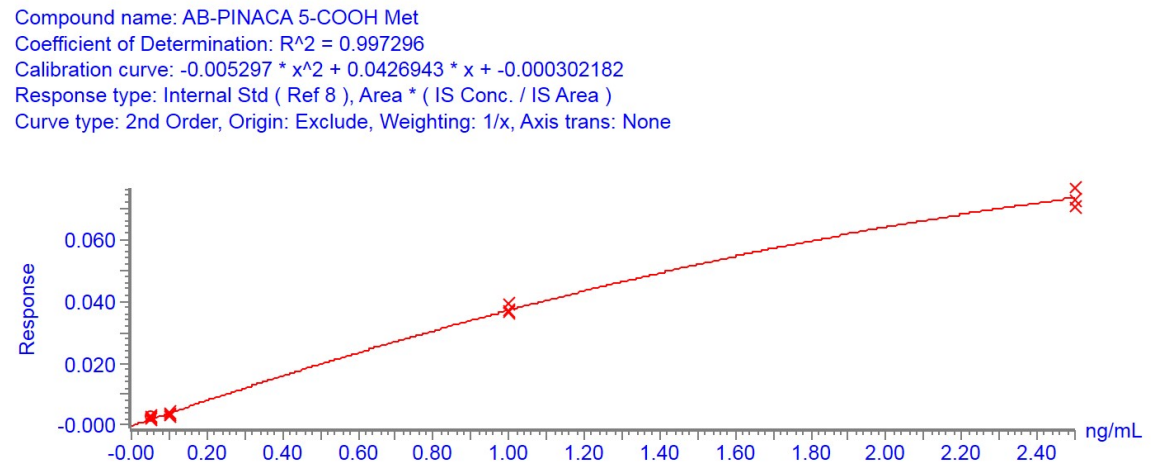

Figure 27: AB-PINACA 5-COOH Gummy Bear Calibration Curve

3.2 Matrix Effects and Recovery Calculations

Matrix effects were assessed and recovery calculations were performed for all three matrices. Extraction recovery was calculated using a comparison of the area counts and ion ratios for the unextracted neat standards and the matrix match extracted standards. The ion ratio was determined by dividing the analyte area count by the internal standard area count. A recovery value and matrix effects for the water curve run on the

same day as each studied matrix were assessed in addition to the recovery value and matrix effects from the urine, plasma, and gummy bear curves. The water recovery value and matrix effects were evaluated to determine any effects on the analyte recovery outside of the matrix such as water or column contaminants. Matrix suppression effects were observed in all three matrices and in the water extracts. The suppression was calculated as a percentage utilizing the area counts of the internal standard and calibrator by comparing the unextracted standard to the extracted standard. The calculated matrix effects from the water extracts was 7% suppression. Overall, the suppression effects were low and the recovery values for most of the analytes in all matrices were excellent, demonstrating the strength of the sample preparation and chromatography methods developed and optimized in this research.

3.2.1 Comparison of Unextracted and Extracted Standards

A comparison was conducted between the unextracted standard and the water, urine, plasma, and gummy bear extracted standards to assess variations in signal intensities, background noise, matrix effects, recoveries, and peak shapes. Figure 28 demonstrates an example of UR-144 5-COOH at 1.0ng/mL as an unextracted standard and in the four matrices previously listed. As expected, there was a signal reduction between the unextracted standard and the extracts, with some matrices displaying larger intensity variations than others. For example, there was only a slight reduction in intensity between the unextracted standard and the urine extract, but a signal reduction greater than 10X between the unextracted standard and the gummy bear extract. The gummy bear matrix presented more background noise and larger signal reduction in

comparison to all other matrices assessed. The water extract displayed surprisingly high background noise intensity. The background noise varied between the different SCs evaluated, for instance, XLR-11 demonstrated less background noise from matrix to matrix in comparison to UR-144 5-COOH.

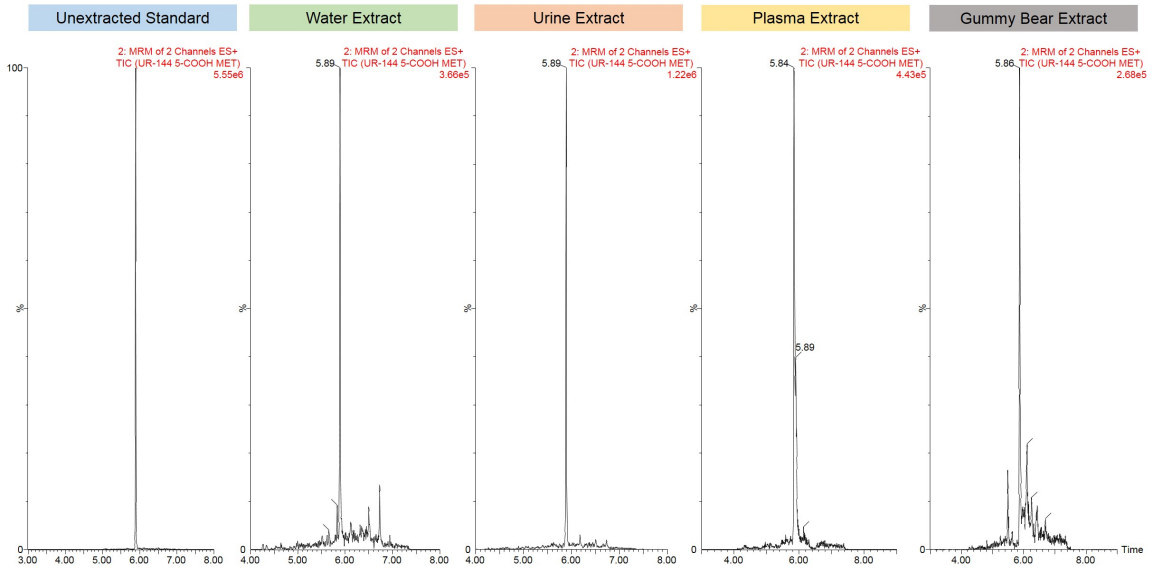

Figure 28: Chromatogram Comparison of UR-144 5-COOH Unextracted Standard and Extracts

3.2.2 Urine

A matrix match standard was used to calculated matrix effects and recoveries. The urine matrix produced an 8% suppression effect. Figure 29 depicts an example of the recovery and matrix effect calculations for UR-144 5-COOH and XLR-11. The first column represents the unextracted standards and displays the intensity of the internal standard, which was used in the calculations to determine matrix effects. The second column displays the water extracts for the internal standard and each compound and the third column represents the urine extracts. There is great recovery, approximately 80%

and above, between both the water and urine extracts. Furthermore, slight background noise can be observed in the chromatograms of the XLR-11 and UR-144 5-COOH urine extracts. A signal reduction was demonstrated when comparing the UR-144 5-COOH unextracted standard, water extract, and urine extract. The calculated recoveries of all analytes in urine are displayed in Table 9 with values including 97% recovery for JWH-018 and 106% recovery for 5F-PB-22.

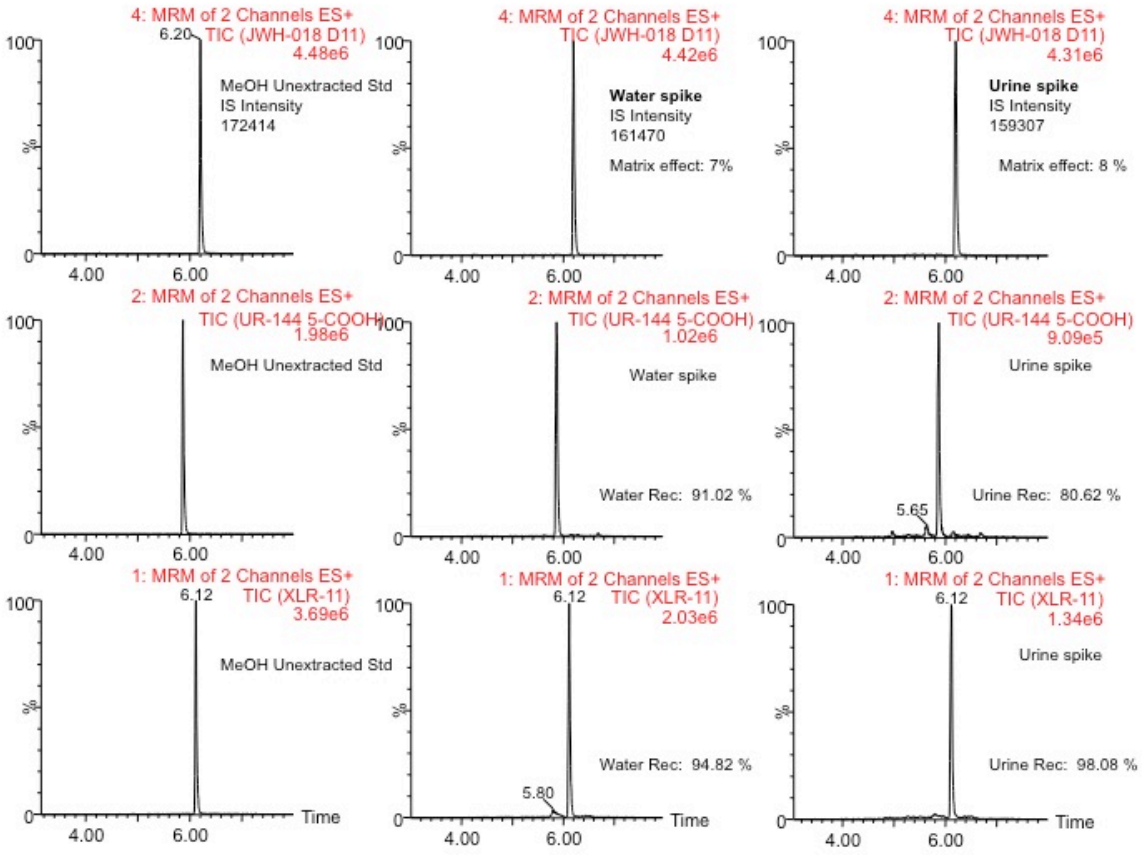

Figure 29: Matrix Effects and Recovery for XLR-11 and UR-144 5-COOH Urine Extracts

3.2.3 Plasma

The plasma recovery and matrix effects were calculated in the same manner as the urine extracts. Plasma extracts displayed 18.9% suppression, which were the highest matrix effects of all three matrices analyzed. The recovery values for all seven synthetic cannabinoids analyzed in plasma for this research are portrayed in Table 10 with 85% recovery for AB-PINACA 5-COOH and 95% recovery for XLR-11.

3.2.4 Edibles

Matrix effects and recovery values were also determined for the gummy bear extracts in MeOH. A calculated suppression of 6.6% was determined for the gummy extracts and recovery values were also calculated as depicted in Table 11. Recovery values for some analytes were excellent, such as 113% recovery for XLR-11 in the gummy bear matrix while other values were on the lower side of recovery such as 63% for AB-PINACA 5-COOH. Results like the relatively low recoveries, higher background noise, and smaller linear dynamic range for the carboxylic acid analytes ultimately suggested a different approach in sample preparation and 2D-LC-MS/MS method evaluation may be necessary to consider in further evaluation of synthetic cannabinoids in all matrices.

3.3 Case Sample Analysis and Quantitation

All 17 urine specimens were prepared and analyzed with the optimized sample preparation and chromatography methods. Table 12 presents the results and quantified values for all specimens assessed. All samples were positive for synthetic cannabinoids. An interesting result was that JWH-018 5-hydroxypentyl metabolite was not detected in

any of the samples analyzed. Most quantifiable samples contained UR-144 5-COOH or AB-PINACA 5-COOH. This observation emphasizes the importance of creating a separate sample preparation and chromatography method for a more efficient and successful analysis of the carboxylic acid synthetic cannabinoids in urine. A range of quantified values were calculated including 0.013ng/mL 5F-PB-22 detected in case 9 and high values of 115.76ng/mL and 73.35ng/mL AB-PINACA 5-COOH in cases 1 and 5, respectively. AM-2201 4-hydroxypentyl and 5F-PB-22 were detected in several case samples but the values were unable to be quantified, as they were less than the LOQ value for that particular compound. It was interesting to note that JWH-018, AB-PINACA 5-COOH, AM-2201 4-hydroxypentyl, and 5F-PB-22 were detected in several samples as majority of these samples were screened positive with an immunoassay kit that is described to detect XLR-11, UR-144 and their major metabolites [44]. This further highlights the importance of developing a method for new and existing SCs as well as demonstrates the issue of screening methods missing the detection of illicit substances in biological samples.

Table 12: Urine Case Sample Results and Quantitation

Case Number	XLR-11	UR-144 5-COOH	JWH-018	JWH-018 5-hydroxypentyl	AB-PINACA 5-COOH	AM-2201 4-hydroxypentyl	5F-PB-22
1	0.037ng/mL	<LOQ	ND	ND	115.76ng/mL	ND	0.009ng/mL
2	0.019ng/mL	0.47ng/mL	ND	ND	ND	0.0080ng/mL	ND
3	ND	<LOQ	ND	ND	30.42ng/mL	ND	ND
4	0.011ng/mL	ND	ND	ND	ND	ND	<LOQ
5	0.021ng/mL	<LOQ	ND	ND	73.35ng/mL	ND	ND
6	0.041ng/mL	ND	0.029ng/mL	ND	ND	<LOQ	0.0012ng/mL
7	ND	<LOQ	0.019ng/mL	ND	ND	ND	0.0023ng/mL
8	0.020ng/mL	<LOQ	0.038ng/mL	ND	ND	ND	0.0026ng/mL
9	0.030ng/mL	0.23ng/mL	0.037ng/mL	ND	0.12ng/mL	0.012ng/mL	0.013ng/mL
10	0.012ng/mL	0.16ng/mL	0.0087ng/mL	ND	<LOQ	ND	<LOQ
11	ND	ND	ND	ND	0.29ng/mL	ND	ND
12	0.012ng/mL	1.9ng/mL	ND	ND	29.15ng/mL	ND	<LOQ
13	ND	0.27ng/mL	ND	ND	3.53ng/mL	<LOQ	ND
14	0.010ng/mL	1.7ng/mL	ND	ND	0.17ng/mL	ND	ND
15	ND	<LOQ	ND	ND	ND	ND	ND
16	0.011ng/mL	0.34ng/mL	0.011ng/mL	ND	45.13ng/mL	ND	<LOQ
17	0.014ng/mL	1.2ng/mL	0.017ng/mL	ND	0.75ng/mL	ND	<LOQ

<LOQ: peak detected but not quantifiable
ND: Not detected

Figures 30-32 represent chromatograms for various case sample results. Figure 30 displays example chromatograms of analytes that were not detected in case 3 in comparison to the chromatograms of the 1ng/mL spiked standards for those respective compounds. High background noise baselines can be observed for AM-2201 4-hydroxypentyl, JWH-018, and XLR-11 demonstrating the absence of each analyte. Figure 31 displays the chromatograms and concentrations for all six SCs detected in Case 9 with a comparison to the unextracted standards. AB-PINACA 5-COOH was detected at a concentration of 0.12ng/mL and its resulting chromatogram depicts a peak at the same retention time with a very similar intensity as that displayed in the chromatogram for the 0.10ng/mL unextracted standard. Figure 32 represents a great example of the ability of the sample preparation and chromatography methods to detect the carboxylic acid

analytes studied in this research. AB-PINACA 5-COOH and UR-144 5-COOH were both detected in case sample 17 at a concentration of 0.75ng/mL and 1.2ng/mL, respectively. The 1.0ng/mL unextracted standard chromatogram of both analytes have a similar retention time and intensity in comparison to that detected and displayed in the resulting chromatograms for case 17. The efficiency of the sample preparation and extraction methods is also demonstrated in Figure 32 with the resulting case sample chromatograms presenting excellent peak intensities, low noise, and Gaussian peak shape. Other case sample chromatogram results can be found in Appendix B.

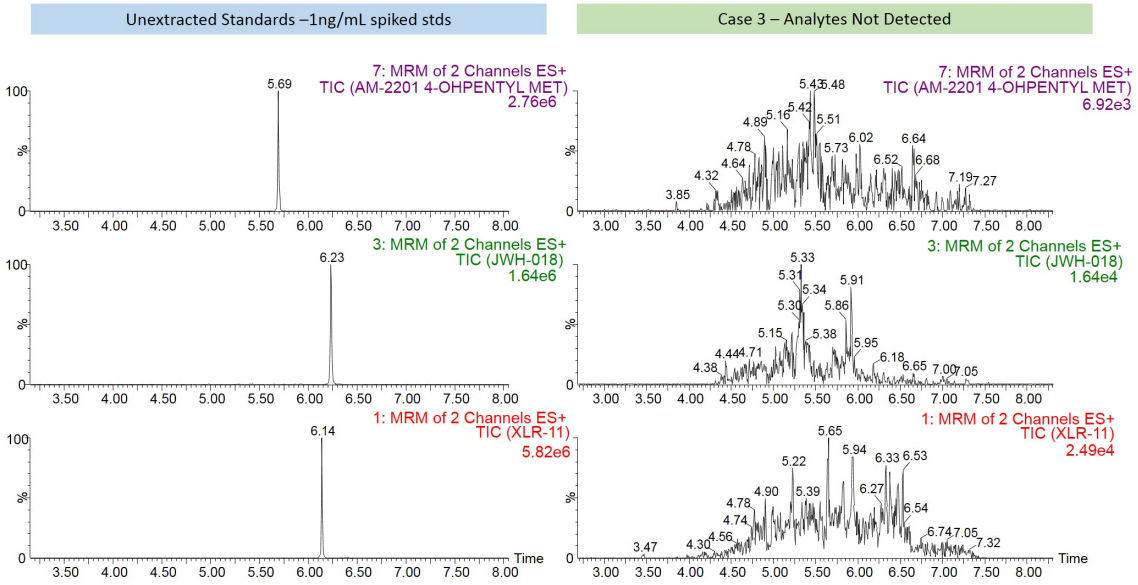

Figure 30: Case 3 Results – Chromatogram Examples of Analytes Not Detected

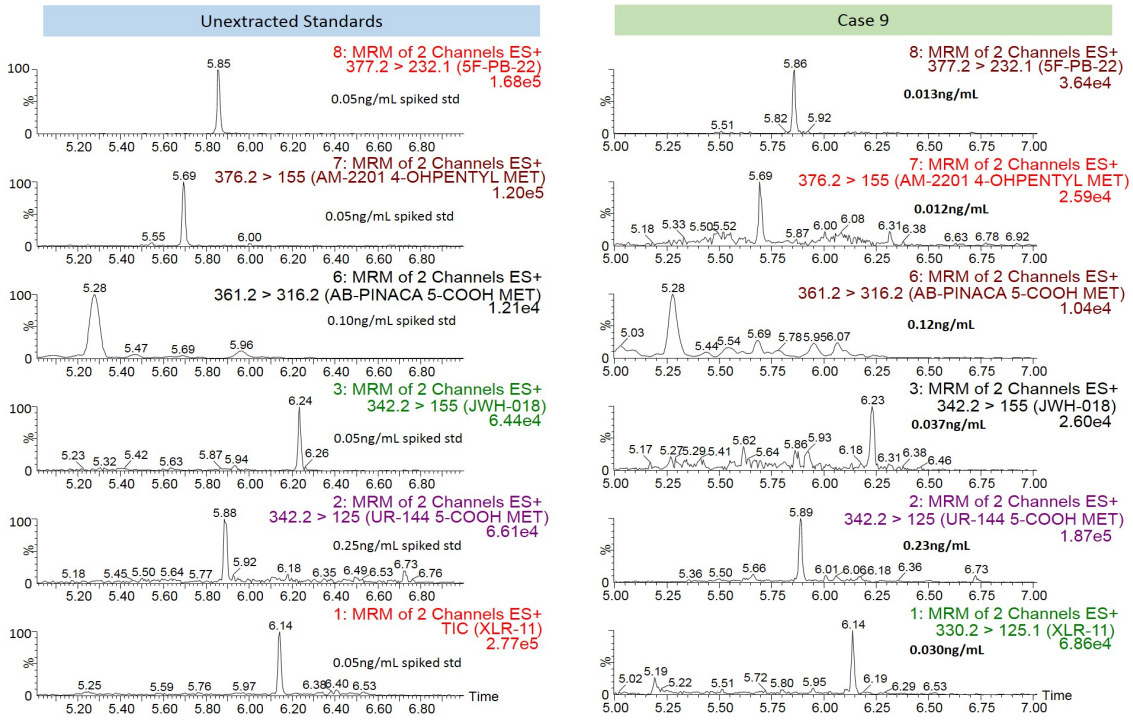

Figure 31: Case 9 Results - Chromatograms of All Analytes Detected

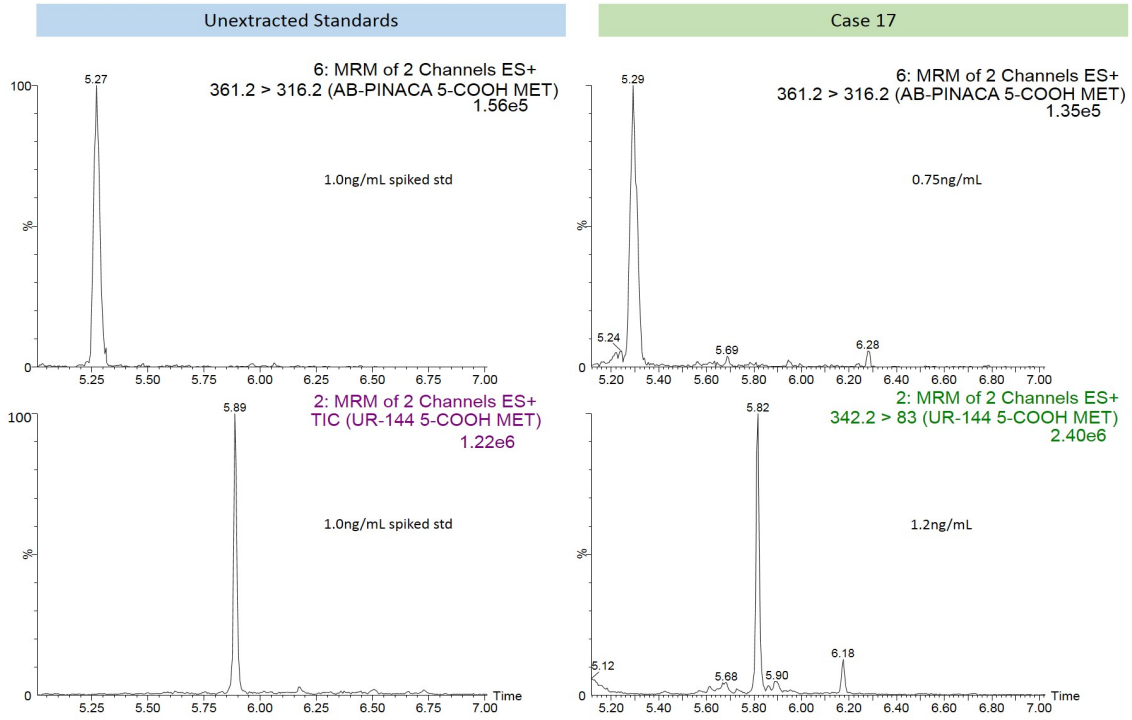

Figure 32: Case 17 Results – Chromatograms of Carboxylic Acid Analytes Detected

4. CONCLUSIONS

A 2D-LC-MS/MS method with an optimized sample preparation protocol was successfully developed and applied to analyze, detect, and quantify seven synthetic cannabinoids in urine, plasma, and edible samples. Furthermore, the overall sample preparation time required for each matrix assessed in this research was less than one hour including protein precipitation, heating, and SPE steps. Urine samples totaled 15 minutes while sample preparation for the plasma and edible samples was completed in approximately 30 minutes.

A urine calibration curve was developed for each analyte with R^2 values of 0.992 and 0.993 for UR-144 5-COOH and AB-PINACA 5-COOH, respectively and a linear dynamic range of 0.05ng/mL to 2.5ng/mL. The other five synthetic cannabinoids demonstrated R^2 values of 0.995 or above with a linear dynamic range of 0.05ng/mL to 5ng/mL. A suppression effect of only 8% was observed and recovery values for each analyte were calculated to be greater than 80%. Synthetic cannabinoids were detected in all 17 urine case samples examined with a variety of detected compounds and values. XLR-11 was detected at 0.03ng/mL, AB-PINACA 5-COOH at 0.12ng/mL, and UR-144 5-COOH at 1.9ng/mL in case 9. Analysis of plasma and gummy bear samples was also successfully carried out. Plasma curves had a linear dynamic range of 0.05-10ng/mL with all R^2 values above 0.995, recovery values of 85% or greater, and suppression effects of approximately 19%. Gummy bear curves yielded linear dynamic ranges of 0.05-10ng/mL and 0.05-2.5ng/mL with R^2 values over 0.995, 6.6% suppression effects, and recovery values ranging from 63-113%. Relatively low recovery values, reduced linear dynamic

ranges, and matrix effects for the carboxylic acid analytes assessed in this research suggested an alternative sample preparation and chromatography approach may provide more successful results for these particular compound types in all three matrices.

Ultimately, this research displays the ease of implementing two-dimensional technology to a variety of applications, such as forensic casework as demonstrated with the urine case samples analyzed with this method. A faster sample preparation method with high extraction efficiency was also developed, eliminating the need for the time consuming evaporation and reconstitution steps required in most SPE protocols. Method adjustments such as those accommodating specific compounds, like the carboxylic acid SCs in combination with this method will provide the ability to detect synthetic cannabinoids with specific results and significantly lower LOD and LOQ values.

5. FUTURE CONSIDERATIONS

There are several future considerations to assess in regards to developing the most reliable, sensitive, and specific method in the detection of synthetic cannabinoids. An investigation of sample preparation that utilizes various acids and bases and compares glass versus plastic sample vials may prove to be useful in correcting binding effects or matrix effects. For example, the relatively high suppression effects observed in the plasma analysis portion of this research may be able to be reduced. Furthermore, the addition of an acid or base in the sample preparation of the gummy bear samples may yield an overall more efficient extraction and therefore better recoveries, such as with the AB-PINACA 5-COOH analyte. Acetonitrile may also be an effective solvent to use as a mobile phase with this method for some compounds in comparison to methanol. Further consideration by comparing the two, perhaps with the addition of more synthetic cannabinoid analytes to the list analyzed, may provide insight as to the best mobile phase solvent for each synthetic cannabinoid.

An alternative approach to the chromatography and/or sample preparation methods to target improved extraction and analysis of the carboxylic acids assessed in this research may allow more useful results. For instance, the addition of a derivitization step prior to any sample preparation may produce a more suitable form of the carboxylic acid based compounds for UPLC analysis. The conversion of the hydroxyl group to an amine on THC proved to be a relatively useful method in the analysis of cannabinoids in blood in a study performed by Lacroix and Saussereau [45]. With the preparation of a derivative compound, the original carboxylic acid based compounds may be more

efficiently extracted in the sample preparation method and more easily analyzed, especially in regards to ionization efficiency, with the chromatography method developed in this research.

A method developed to detect and quantify more than seven synthetic cannabinoids would provide many benefits as demonstrated by the analysis of the urine case samples in this research. The screening method indicated the presence of some SCs while the 2D-LC-MS/MS method detected the presence of more synthetic cannabinoids than anticipated. The performance of stability studies may provide further insight in various aspects of this research. For instance, while the case samples were screened positive and successfully tested positive in this method, more compounds may have been quantifiable and/or detected if the case samples were tested earlier on. There was a time difference of at least six months between the dates the case samples were screened positive and the date they were analyzed with the developed and optimized 2D-LC-MS/MS method. The analysis of negatively screened urine case samples may also provide information on the reliability of this method and elements such as carryover may be further investigated. Lastly, the analysis of actual plasma and gummy bear case samples would provide the best overall assessment of the sample preparation and chromatography methods optimized and developed in this research.

APPENDIX A: Chromatography Evaluation

Table A: Chromatography Evaluation Methods 1-8

Method	1	2	3	4	5	6	7	8
Trap	C8	C8	HLB	HLB	C8	C8	HLB	HLB
Loading	pH10	pH3	pH10	pH3	pH10	pH3	pH10	pH3
	80mg	80mg	80mg	80mg	80mg	80mg	80mg	80mg
Eluting	lo pH MeOH	lo pH MeOH	lo pH MeOH	lo pH MeOH	hi pH MeOH	hi pH MeOH	hi pH MeOH	hi pH MeOH
Analytical Column	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18
XLR-11 (Water)								
XLR-11 (MeOH)								
XLR-11 (ACN)								
UR-144 5-COOH metabolite (Water)								
UR-144 5-COOH metabolite (MeOH)								
UR-144 5-COOH metabolite (ACN)								
JWH-018 (Water)								
JWH-018 (MeOH)								
JWH-018 (ACN)								
JWH-018 5-OHpentyl metabolite (Water)								
JWH-018 5-OHpentyl metabolite (MeOH)								
JWH-018 5-OHpentyl metabolite (ACN)								
AB-PINACA 5-COOH metabolite (Water)								
AB-PINACA 5-COOH metabolite (MeOH)								
AB-PINACA 5-COOH metabolite (ACN)								
AM-2201 4-OHpentyl metabolite (Water)								
AM-2201 4-OHpentyl metabolite (MeOH)								
AM-2201 4-OHpentyl metabolite (ACN)								
5F-PB-22 (Water)								
5F-PB-22 (MeOH)								
5F-PB-22 (ACN)								

Key

	No signal
	Signal detected with distortion such as high noise, shouldering, or tailing
	High signal detected, gaussian peak shape

Table B: Chromatography Evaluation Methods 9-16

Method	9	10	11	12	13	14	15	16
Trap	C8	C8	HLB	HLB	C8	C8	HLB	HLB
Loading	pH10	pH3	pH 10	pH3	pH10	pH3	pH10	pH3
	80mg	80mg	80mg	80mg	80mg	80mg	80mg	80mg
Eluting	lo pH ACN	lo pH ACN	lo pH ACN	lo pH ACN	hi pH ACN	hi pH ACN	hi pH ACN	hi pH ACN
Analytical Column	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18	BEH C18
XLR-11 (Water)								
XLR-11 (MeOH)								
XLR-11 (ACN)								
UR-144 5-COOH metabolite (Water)								
UR-144 5-COOH metabolite (MeOH)								
UR-144 5-COOH metabolite (ACN)								
JWH-018 (Water)								
JWH-018 (MeOH)								
JWH-018 (ACN)								
JWH-018 5-OHpentyl metabolite (Water)								
JWH-018 5-OHpentyl metabolite (MeOH)								
JWH-018 5-OHpentyl metabolite (ACN)								
AB-PINACA 5-COOH metabolite (Water)								
AB-PINACA 5-COOH metabolite (MeOH)								
AB-PINACA 5-COOH metabolite (ACN)								
AM-2201 4-OHpentyl metabolite (Water)								
AM-2201 4-OHpentyl metabolite (MeOH)								
AM-2201 4-OHpentyl metabolite (ACN)								
5F-PB-22 (Water)								
5F-PB-22 (MeOH)								
5F-PB-22 (ACN)								

Key

	No signal
	Signal detected with distortion such as high noise, shouldering, or tailing
	High signal detected, gaussian peak shape

APPENDIX B: Calibration Curves and Chromatogram Results

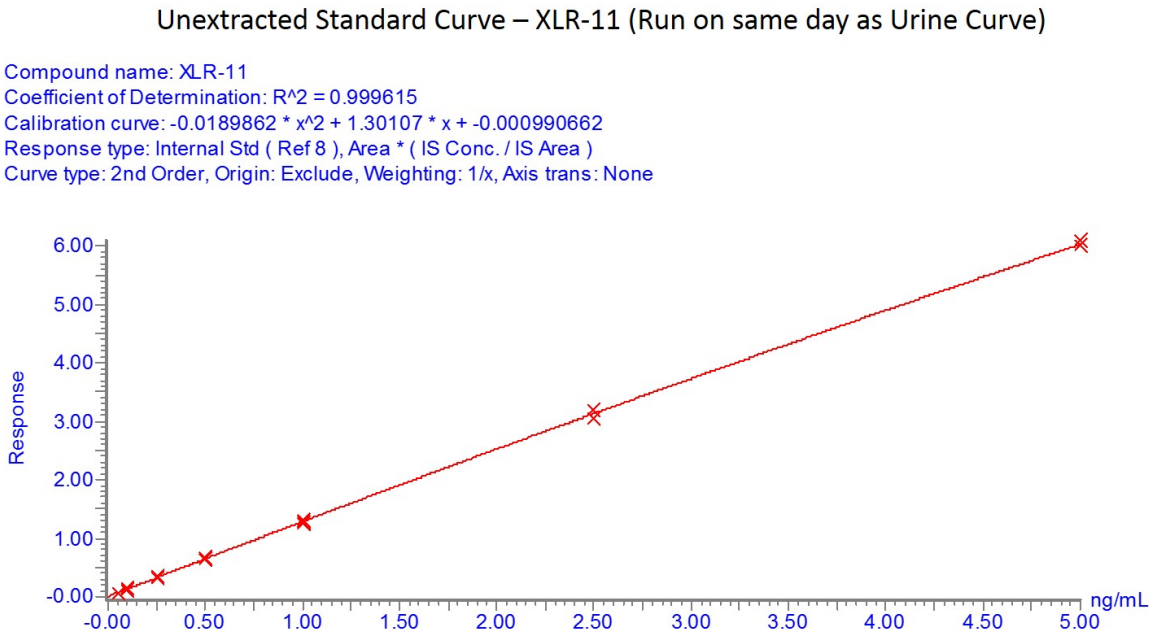

Figure A: XLR-11 Unextracted Standard Calibration Curve (Urine Extraction)

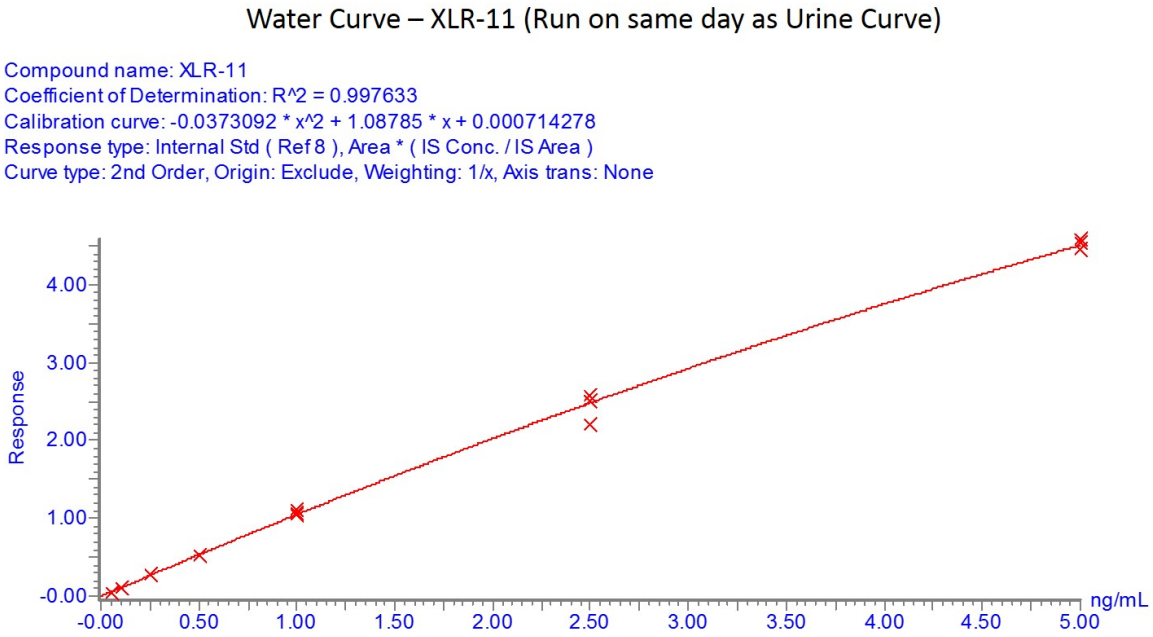

Figure B: XLR-11 Water Extract Calibration Curve (Urine Extraction)

Unextracted Standard Curve – XLR-11 (Run on same day as Plasma Curve)

Compound name: XLR-11
Coefficient of Determination: R^2 = 0.996336
Calibration curve: -0.00801673 * x^2 + 1.14064 * x + -0.0183437
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

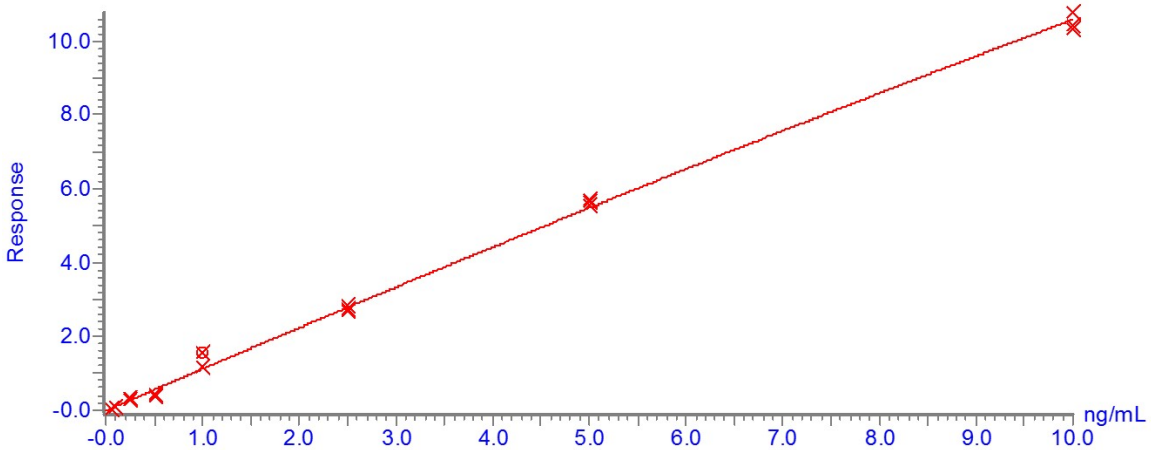

Figure C: XLR-11 Unextracted Standard Calibration Curve (Plasma Extraction)

Water Curve – XLR-11 (Run on same day as Plasma Curve)

Compound name: XLR-11
Coefficient of Determination: R^2 = 0.995065
Calibration curve: 0.0367335 * x^2 + 0.950524 * x + 0.012097
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

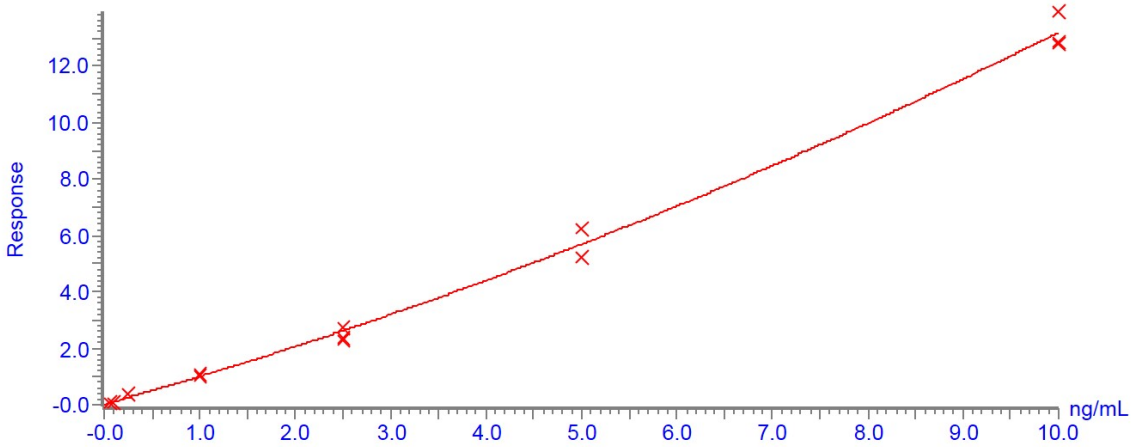

Figure D: XLR-11 Water Extract Calibration Curve (Plasma Extraction)

Plasma Curve – XLR-11

Compound name: XLR-11
Coefficient of Determination: R^2 = 0.996252
Calibration curve: 0.00426409 * x^2 + 0.94369 * x + 0.0117983
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

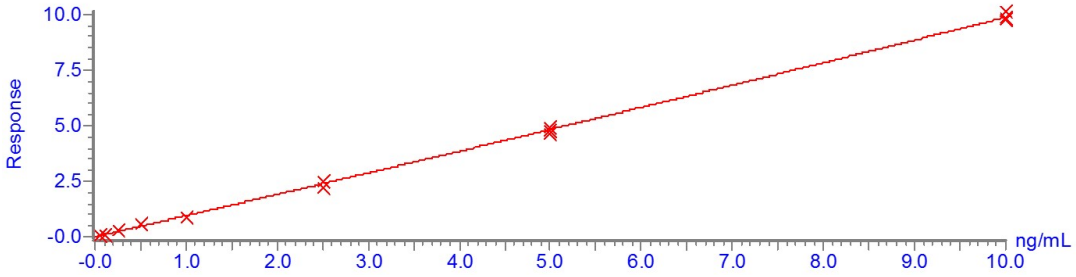

Figure E: XLR-11 Plasma Extraction Calibration Curve

Unextracted Standard Curve – XLR-11 (Run on same day as MeOH Heated Curve)

Compound name: XLR-11
Coefficient of Determination: R^2 = 0.996037
Calibration curve: -0.0103874 * x^2 + 1.11965 * x + 0.0118618
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

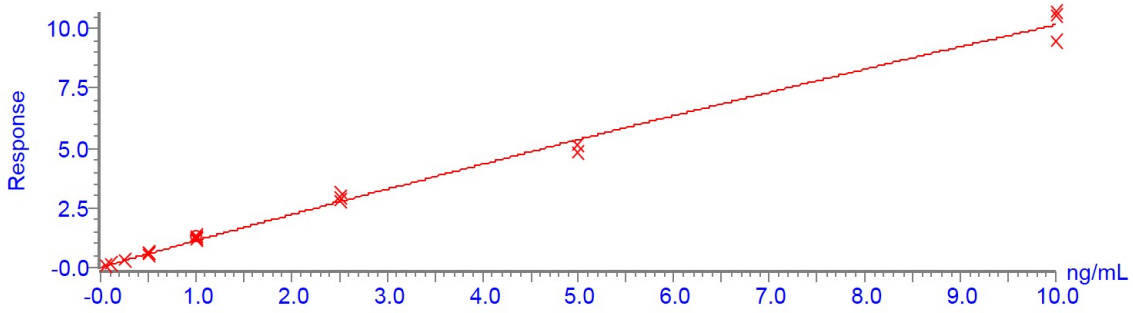

Figure F: XLR-11 Unextracted Standard Curve (Gummy Bear Extraction)

Water Curve – XLR-11 (Run on same day as MeOH Heated Curve)

Compound name: XLR-11
Coefficient of Determination: R^2 = 0.996822
Calibration curve: -0.013578 * x^2 + 1.01963 * x + 0.00233675
Response type: Internal Std (Ref 8), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None

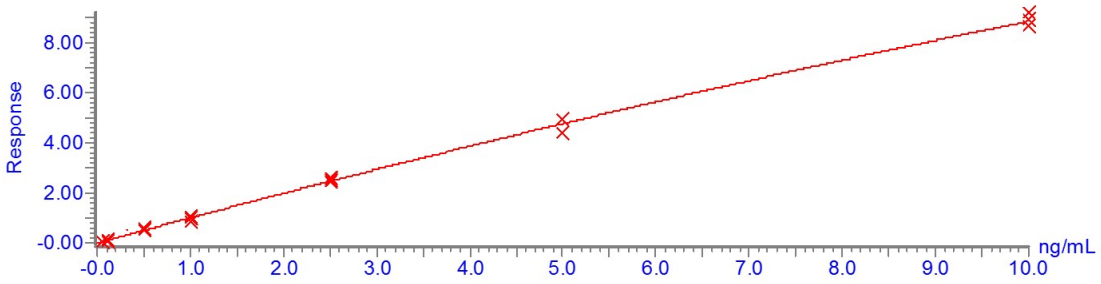

Figure G: XLR-11 Water Extract Calibration Curve (Gummy Bear Extraction)

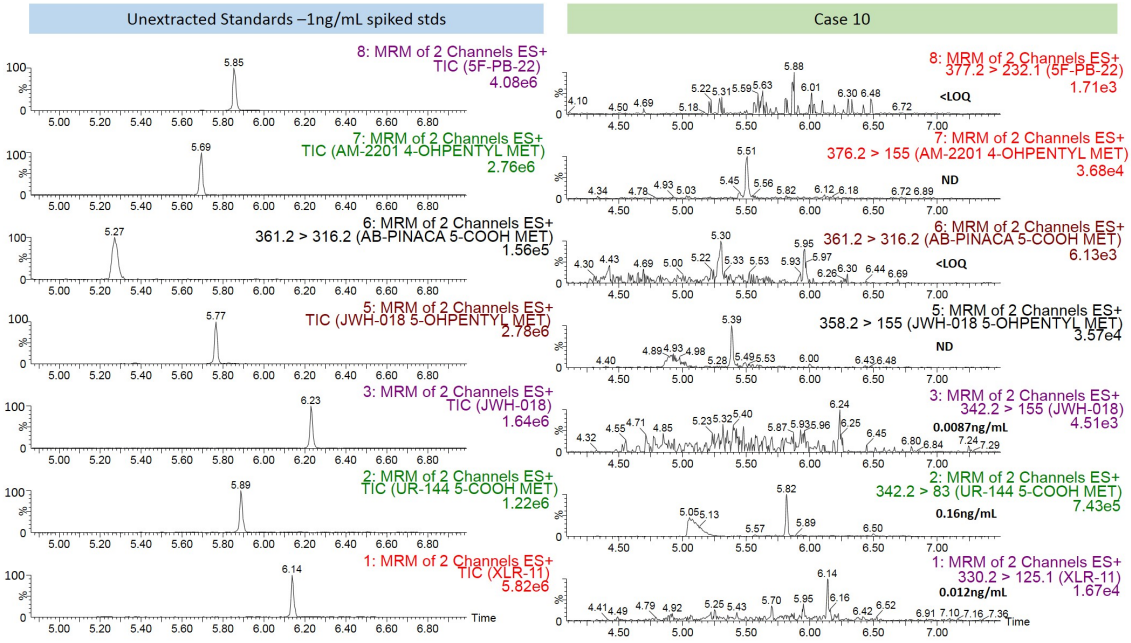

Figure H: Case 10 Results

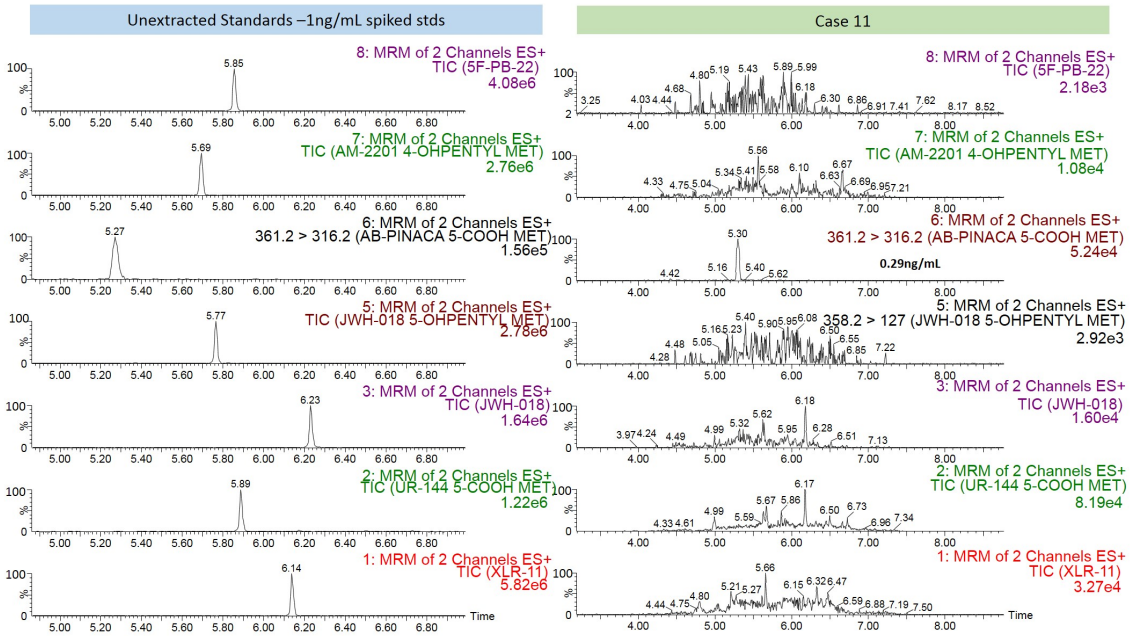

Figure I: Case 11 Results

LIST OF JOURNAL ABBREVIATIONS

Accid Anal Prev	Accident Analysis & Prevention
Anal Bioanal Chem	Analytical and Bioanalytical Chemistry
Anal Chem	Analytical Chemistry
Anal Chim Acta.	Analytica Chimica Acta
Clin Chem Lab Med	Clinical Chemistry and Laboratory Medicine
Clin Pharmacol Ther	Clinical Pharmacology & Therapeutics
Curr Addict Rep	Current Addiction Reports
Drug Alcohol Depen	Drug and Alcohol Dependence
Drug Test Anal	Drug Testing and Analysis
Forensic Sci Int	Forensic Science International
Forensic Toxicol	Forensic Toxicology
Front Behav Neurosci	Frontiers in Behavioral Neuroscience
Int J Legal Med	International Journal of Legal Medicine
J Anal Toxicol	Journal of Analytical Toxicology
J Chromatogr A	Journal of Chromatography A
J Chromatogr B	Journal of Chromatography B
J Gen Intern Med	Journal of General Internal Medicine
Life Sci	Life Sciences
Microchim Acta	Microchimica Acta
Prog Neuro-Pyschoph	Progress in neuro-psychopharmacology & biological psychiatry

Psychopharmacology (Berl).	Psychopharmacology Berlin
Trends Anal Chem	Trends in Analytical Chemistry

Bibliography

1. Spaderna M, Addy PH, D'Souza DC. Spicing things up: synthetic cannabinoids. Psychopharmacology (Berl). 2013 Aug;228(4):525-40.

2. Khullar V, Jain A, Sattari M. Emergence of new classes of recreational drugs-synthetic cannabinoids and cathinones. J Gen Intern Med. 2014 Aug;29(8):1200-4.

3. Seely KA, Lapoint J, Moran JH, Fattore L. Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids. Prog Neuro-Pyschoph. 2012 Dec 03;39(2):234-43.

4. Spinelli E, Barnes AJ, Young S, Castaneto MS, Martin TM, Klette KL, et al. Performance characteristics of an ELISA screening assay for urinary synthetic cannabinoids. Drug Test Anal. 2015 Jun;7(6):467-74.

5. Elsohly MA, Gul W, Wanas AS, Radwan MM. Synthetic cannabinoids: analysis and metabolites. Life Sci. 2014 Feb 27;97(1):78-90.

6. Znaleziona J, Ginterova P, Petr J, Ondra P, Valka I, Sevcik J, et al. Determination and identification of synthetic cannabinoids and their metabolites in different matrices by modern analytical techniques - a review. Anal Chim Acta. 2015 May 18;874:11-25.

7. Scheidweiler KB, Huestis MA. Simultaneous quantification of 20 synthetic cannabinoids and 21 metabolites, and semi-quantification of 12 alkyl hydroxy metabolites in human urine by liquid chromatography-tandem mass spectrometry. J Chromatogr A. 2014 Jan 31;1327:105-17.

8. EMCDDA | Perspectives on drugs: synthetic cannabinoids in Europe. 2016 [updated 2016; cited]; Available from: http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids.

9. Schedules of controlled substances: temporary placement of three synthetic cannabinoids into schedule I. Final order. Federal register. 2015/03/04 ed; 2015;5042-7.

10. Fattore L, Fratta W. Beyond THC: The New Generation of Cannabinoid Designer Drugs. Front Behav Neurosci. 2011;5.

11. Vandrey R, Dunn KE, Fry JA, Girling ER. A survey study to characterize use of Spice products (synthetic cannabinoids). Drug and Alcohol Depen. 2012 Jan 01;120(1-3):238-41.

12. Wohlfarth A, Scheidweiler KB, Castaneto M, Gandhi AS, Desrosiers NA, Klette KL, et al. Urinary prevalence, metabolite detection rates, temporal patterns and evaluation of

suitable LC-MS/MS targets to document synthetic cannabinoid intake in US military urine specimens. Clin Chem Lab Med. 2015 Feb;53(3):423-34.

13. Tai S, Fantegrossi WE. Synthetic Cannabinoids: Pharmacology, Behavioral Effects, and Abuse Potential. Curr Addict Rep. 2014 Jun 01;1(2):129-36.

14. Barnes AJ, Young S, Spinelli E, Martin TM, Klette KL, Huestis MA. Evaluation of a homogenous enzyme immunoassay for the detection of synthetic cannabinoids in urine. Forensic Sci Int. 2014 August 2014;241:27–34.

15. Tuv SS, Krabseth H, Karinen R, Olsen KM, Oiestad EL, Vindenes V. Prevalence of synthetic cannabinoids in blood samples from Norwegian drivers suspected of impaired driving during a seven weeks period. Accid Anal Prev. 2014 Jan;62:26-31.

16. Diao X, Huestis MA. Approaches, Challenges, and Advances in Metabolism of New Synthetic Cannabinoids and Identification of Optimal Urinary Marker Metabolites. Clin Pharmacol Ther. 2016.

17. Namera A, Kawamura M, Nakamoto A, Saito T, Nagao M. Comprehensive review of the detection methods for synthetic cannabinoids and cathinones. Forensic Toxicol. 2015;33(2):175-94.

18. Aldlgan AA, Torrance HJ. Bioanalytical methods for the determination of synthetic cannabinoids and metabolites in biological specimens. Trends Anal Chem. 2016;80:444-57.

19. Wohlfarth A, Scheidweiler KB, Chen X, Hua-fen L, Huestis MA. Qualitative Confirmation of 9 Synthetic Cannabinoids and 20 Metabolites in Human Urine Using LC–MS/MS and Library Search. Anal Chem. 2013 March 18, 2013;85(7):3730-8.

20. Davies BB, Bayard C, Larson SJ, Zarwell LW, Mitchell RA. Retrospective Analysis of Synthetic Cannabinoid Metabolites in Urine of Individuals Suspected of Driving Impaired. J Anal Toxicol. 2016 Mar;40(2):89-96.

21. Jang M, Shin I, Kim J, Yang W. Simultaneous quantification of 37 synthetic cannabinoid metabolites in human urine by liquid chromatography-tandem mass spectrometry. Forensic Toxicol. 2015;33(2):221-34.

22. Zaitsu K, Nakayama H, Yamanaka M, Hisatsune K, Taki K, Asano T, et al. High-resolution mass spectrometric determination of the synthetic cannabinoids MAM-2201, AM-2201, AM-2232, and their metabolites in postmortem plasma and urine by LC/Q-TOFMS | SpringerLink. Int J Legal Med. 2015;129(6):1233-45.

23. Capitan-Vallvey LF, Valencia MC, Nicolas EA. Flow Injection Analysis with On-Line Solid Phase Extraction for Spectrophotometric Determination of Ponceau 4R and its

Subsidiary Unsulfonated Dye in Sweets and Cosmetic Products | SpringerLink. Microchim Acta. 2002;138(1):69-76.

24. Wang X, Mackowsky D, Searfoss J, Telepchak MJ. Determination of Cannabinoid Content and Pesticide Residues in Cannabis Edibles and Beverages. 2016 [updated 2016; cited 34 10]; 20-7]. Available from: http://www.chromatographyonline.com/determination-cannabinoid-content-and-pesticide-residues-cannabis-edibles-and-beverages.

25. Jang M, Yang W, Choi H, Chang H, Lee S, Kim E, et al. Monitoring of urinary metabolites of JWH-018 and JWH-073 in legal cases. Forensic Sci Int. 2013 Sep 10;231(1-3):13-9.

26. Knittel JL, Holler JM, Chmiel JD, Vorce SP, Magluilo J, Jr., Levine B, et al. Analysis of Parent Synthetic Cannabinoids in Blood and Urinary Metabolites by Liquid Chromatography Tandem Mass Spectrometry. J Anal Toxicol. 2016 Apr;40(3):173-86.

27. Jang M, Yang W, Shin I, Choi H, Chang H, Kim E. Determination of AM-2201 metabolites in urine and comparison with JWH-018 abuse. Int J Legal Med. 2014 Mar;128(2):285-94.

28. Special Report: Synthetic Cannabinoids and Synthetic Cathinones Reported in NFLIS, 2013-2015. Drug Enforcement Administration's National Forensic Laboratory Information System; 2016.

29. Engvall E. Enzyme immunoassay ELISA and EMIT. Methods in Enzymology: Academic Press; 1980;419–39.

30. HPLC Separation Modes. Waters Corporation; 2016 [updated 2016; cited]; Available from: http://www.waters.com/waters/en_US/HPLC-Separation-Modes/nav.htm?cid=10049076&locale=en_US.

31. Maurer HH. Current role of liquid chromatography–mass spectrometry in clinical and forensic toxicology | SpringerLink. Anal Bioanal Chem. [Review]. 2007;388(7):1315-25.

32. Lewis SW, Lenehan CE. Liquid and Thin-Layer Chromatography. In: Houck M, editor. Forensic Chemistry - 1st Edition. 1 ed: Elsevier; 2015;79-81.

33. Jiang G, Stenzel JR, Chen R, Elmashni D. UHPLC/MS Analysis of Illicit Drugs. In: Xu QA, editor. Ultra-high performance liquid chromatography and its applications. First ed: Wiley; 2016;253-70.

34. What is MS and How does it Work? : Waters. Waters Corporation; [cited]; Available from: http://www.waters.com/waters/en_US/What-is-MS-and-How-does-it-Work%3F/nav.htm?locale=101&cid=10073253.

35. Overview of Mass Spectrometry. ThermoFisher Scientific; [cited]; Available from: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-mass-spectrometry.html.

36. Smith R. Mass Spectrometry. In: Houck M, editor. Forensic Chemistry - 1st Edition: Elsevier; 2015;85-8.

37. Stoll DR, Li X, Wang X, Carr PW, Porter SEG, Rutan SC. Fast, comprehensive two-dimensional liquid chromatography. J Chromatogr A. [Review]. 2007 19 October 2007;1168(Issues 1–2):3–43.

38. Mallet CR, Murphy BP. Multi-Dimensional Chromatography: Trap & Elute vs. At-Column Dilution. Waters Corporation; 2015 [updated 2015; cited]; Available from: http://www.waters.com/waters/library.htm?cid=511436&lid=134844733.

39. Mallet C, Botch-Jones S. Illicit Drug Analysis Using Two-Dimensional Liquid Chromatography/Tandem Mass Spectrometry. J Anal Toxicol. 2016;40(8):617-27.

40. Karinen R, Tuv SS, Oiestad EL, Vindenes V. Concentrations of APINACA, 5F-APINACA, UR-144 and its degradant product in blood samples from six impaired drivers compared to previous reported concentrations of other synthetic cannabinoids. Forensic Sci Int. 2015 January 2015;246:98–103.

41. Behonick G, Shanks KG, Firchau DJ, Mathur G, Lynch CF, Nashelsky M, et al. Four Postmortem Case Reports with Quantitative Detection of the Synthetic Cannabinoid, 5F-PB-22. J Anal Toxicol. 2014 2014-10-01;38(8):559-62.

42. Hasegawa K, Wurita A, Minakata K, Gonmori K, Nozawa H, Yanagishi I, et al. Postmortem distribution of MAB-CHMINACA in body fluids and solid tissues of a human cadaver | SpringerLink. Forensic Toxicol. 2015;33(2):380-7.

43. Home | Cayman Chemical. [cited]; Available from: https://www.caymanchem.com/Home.

44. Urine HEIA. Immunalysis Corporation; 2013 [updated 2013 2013-03-21; cited]; Available from: http://immunalysis.com/products/urine-heia/.

45. Lacroix C, Saussereau E. Fast liquid chromatography/tandem mass spectrometry determination of cannabinoids in micro volume blood samples after dabsyl derivatization. J Chromatogr B. 2012 15 September 2012;905:85–95.

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