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Evaluation and comparison of various sample preparation techniques for the analysis and quantitation of THC, synthetic cannabinoids, and metabolites by LC-MS/MS in human whole blood and urine

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**EVALUATION AND COMPARISON OF VARIOUS SAMPLE PREPARATION
TECHNIQUES FOR THE ANALYSIS AND QUANTITATION OF THC,
SYNTHETIC CANNABINOIDS, AND METABOLITES BY LC-MS/MS IN
HUMAN WHOLE BLOOD AND URINE**

by

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B.S., Colorado State University, 2015

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requirements for the degree of

Master of Science

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ABSTRACT

A cannabinoid refers to any natural or synthetic compound that interacts with the CB1 and CB2 receptors. There are currently three different groups of cannabinoids: endogenous cannabinoids, phytocannabinoids and synthetic cannabinoids. The most common phytocannabinoid is delta-9-tetrahydrocannabinol (THC), which is the active component in the *Cannabis sativa* or marijuana plant¹⁻³. Two examples of synthetic cannabinoids that are present in case reports from 2012 to 2018 are AB-FUBINACA and AB-PINACA⁴⁻⁷.

THC and synthetic cannabinoids are commonly encountered drugs in forensic toxicology cases, therefore, being able to extract these compounds and their metabolites is imperative for toxicological interpretation. There are a variety of commercially available sample preparation techniques for these analytes. Companies such as UCT, Biotage, Millipore-Sigma, Tecan, and Thermo Fisher Scientific manufacture these products. The focus of this research was to evaluate these techniques for their cleanliness, efficiency and cost effectiveness. Sample preparation techniques are designed to remove the different components of the matrix and other prescription or illicit substances present in the sample

that could interfere with the assay, increase the analyte recovery, extraction efficiency, decrease variability, and clean-up the sample to allow for less instrument downtime and longer column life⁸. This study focused on comparing a liquid-liquid extraction (LLE), solid phase extraction (SPE), and supported liquid extraction (SLE).

The primary purpose of this study was to develop and validate the three above mentioned sample preparation techniques for the analysis of THC, 11-hydroxy-THC, 11-nor-9-carboxy-THC (THCCOOH), AB-FUBINACA, AB-FUBINACA metabolite 3, and AB-PINACA in blood and urine.

Parameters assessed followed Academy Standards Board (ASB) Standard 036, Standard Practices for Method Validation in Forensic Toxicology, including recovery, suppression, and matrix effects.

For urine and blood analysis, the calibration range was determined to be 1 ng/mL to 50 ng/mL for all three techniques. Urine recovery was highest for the LLE method, with all compounds having a recovery greater than 50%. The SLE method had the lowest LOQ results for urine, with 0.5 ng/mL for 11-hydroxy-THC and THCCOOH, 0.75 ng/mL for THC, AB-FUBINACA and AB-FUBINACA metabolite 3, and 1 ng/mL for AB-PINACA. Ion suppression was reduced using the SLE method for urine along with having the shortest sample preparation time of 1 hr for up to 48 samples.

For blood analysis, the LLE method had the greatest recovery of all analytes. The LLE method also had reduced suppression and matrix effects compared to the SPE method. Sample preparation was shorter for the SPE method, consuming 2 hrs for an average sample batch, compared to 4 hrs for the LLE method, which included a 2 hr freezing step.

In conclusion, for urine analysis, all three sample preparation techniques were acceptable for the analysis of THC, synthetic cannabinoids, and their metabolites, with the SLE method being the preferred method. For blood analysis a LLE and SPE method were developed and are adequate for the analysis of THC, synthetic cannabinoids, and their metabolites, with the LLE method being the preferred method.

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LIST OF ABBREVIATIONS

| | |
|----------|--|
| ACS | American Chemical Society |
| ACN | Acetonitrile |
| ASB | Academy Standards Board |
| CA | California |
| Cal | Calibrators |
| CSA | Controlled Substances Act |
| DCM | Dichloromethane |
| DEA | Drug Enforcement Agency |
| DI | Deionized |
| ESI | Electrospray ionization |
| HPLC | High performance liquid chromatography |
| hr | hour |
| IRB | Institutional Review Board |
| LC-MS | Liquid chromatography mass spectrometry |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LLE | Liquid-liquid extraction |
| LOD | Limit of detection |
| LOQ | Limit of quantitation |
| MA | Massachusetts |
| mg | milligram |
| MI | Michigan |

| | |
|-----------------------|---|
| min | minutes |
| mL | milliliter |
| MO | Missouri |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MTBE | Methyl <i>tert</i> -butyl ether |
| <i>m/z</i> | mass to charge ratio |
| NC | North Carolina |
| NFLIS | National Forensic Laboratory Information System |
| ng | nanogram |
| NH ₄ OH | Ammonium Hydroxide |
| NJ | New Jersey |
| PTFE | Polytetrafluoroethylene |
| rpm | Rotations per minute |
| QC | Quality control |
| qual | qualitative |
| quant | quantitative |
| <i>r</i> ² | Coefficient of correlation |
| sec | seconds |
| SLE | Supported liquid extraction |
| SPE | Solid phase extraction |
| THC | delta-9-Tetrahydrocannabinol |

| | |
|---------|---|
| THCCOOH | 11-nor-9-carboxy-THC |
| TX | Texas |
| μg | microgram |
| μL | microliter |
| USA | United States of America |
| Δ | delta |
| % | percent |
| % CV | percent of the coefficient of variation |

1. INTRODUCTION

1.1 Cannabinoid Background

The name cannabinoid was originally applied to naturally occurring C₂₁ aromatic hydrocarbon compounds that can be found in the *Cannabis sativa* plant⁹. A cannabinoid now refers to any natural or synthetic compound that interacts with the same receptors in the human body as the *Cannabis sativa* compounds⁹. The human body consists of two receptors that belong in the G-protein class, CB1 and CB2, which are found in the human brain and give rise to effects on the central nervous system^{1,10-12}. CB2 receptors are also located in the periphery, predominantly in the immune system¹³. These two receptors are the binding sites for cannabinoids^{1,10-12}. Some cannabinoids, such as cannabinol have a greater affinity for binding to the CB2 receptor versus the CB1 receptor, influencing the different effects cannabinoids have on the human body¹³. The different cannabinoids that react with these endogenous cannabinoid receptors can be broken up into three different groups. The first group are the endogenous cannabinoids, such as anandamide and 2-archidonylglycerol^{12,14}. The next group of cannabinoids are phytocannabinoids². These are naturally occurring cannabinoids found in plants. The common source of phytocannabinoids is the *Cannabis sativa*, or marijuana plant, which contains hundreds of cannabinoids within the plant material². The final group of cannabinoids are synthetic cannabinoids. These compounds do not occur naturally, either in the body or in nature, and must be synthesized.

1.2 NFLIS Reports

The National Forensic Laboratory Information System (NFLIS) collects forensic analysis data from across the United States of America (USA) at the local, regional, and national level. This data is then compiled to give a comprehensive look at the drug use picture in the USA.

Early use of *Cannabis sativa* began with recreational or medicinal uses ^{1,2,15}. Cannabis was once the most commonly abused drug in the world but was surpassed by methamphetamine in 2017 ^{1,15,16}. The NFLIS-Drug 2017 Annual Report and the NFLIS-Drug 2018 Midyear Report show that cannabis and THC are the second most commonly reported drug in forensic cases in the United States ^{16,17}. In the 2018 Midyear Report, cannabis and THC account for 22.57% of the total drug cases reported, while the 2017 Annual Report shows cannabis and THC accounting for 21.76% of the total drug cases reported ^{16,17}. Since 2009, the number of cases reported to NFLIS have been decreasing with the highest number of cases being reported in the Northeast and Midwest regions ¹⁶. Cases in which cannabis and THC may be reported include driving under the influence, work place drug testing and postmortem cases ¹.

In NFLIS Special Report: Synthetic Cannabinoids and Synthetic Cathinones reported to NFLIS for 2013 – 2015, AB-FUBINACA and AB-PINACA were in the top four synthetic cannabinoids reported ⁷. AB-FUBINACA consisted of 11.69% of the total synthetic cannabinoid cases reported for 2013 – 2015 while AB-PINACA accounted for 8.84% ⁷. For both compounds, there was an increase in the number of cases reported from

2013 to 2014, while the number decreased from 2014 to 2015 ⁷. The case decrease falls along with the scheduling of AB-FUBINACA and a multitude of other synthetic cannabinoids in 2014 ⁴. In the 2017 Annual Report for NFLIS AB-FUBINACA was the number four most commonly reported drug in the synthetic cannabinoids drug group, bringing in 772 of the 24,501 cases reported ¹⁶. In the Midyear Report for 2018 AB-FUBINACA has seen a decrease in the number of cases reported with only 46 of 10,598 synthetic cannabinoid cases reported ¹⁷.

1.3 *Cannabis sativa*

The use of *Cannabis sativa* for its euphoric and psychotomimetic effects, dates back at least 5,000 years beginning in Central and Northeast Asia then spreading worldwide ^{1-3,15}. Throughout history, the uses of cannabis has ranged from recreational to medicinal to religious ^{1,2,15}.

Cannabis sativa contains over 400 natural chemicals ranging from cannabinoids, nitrogenous compounds, amino acids, hydrocarbons, flavonoids, alkaloids, and terpenes ^{1,2,14}. In 1898 and 1899 the first pure cannabinoid to be isolated from cannabis occurred. This compound was cannabitol ¹⁴. Of these 400 compounds, not all produce an effect on the body while others produce a range of effects. The cannabinoid with the most psychoactive effect found in cannabis is delta-9-tetrahydrocannabinol (THC) ¹⁻³.

1.3.1 Tetrahydrocannabinol

In 1964, the structure of THC was determined^{2,3,14}. THC is an acidic and lipophilic terpenoid with a pKa of 10.6.^{2,14,18,19}^{1,18}. Extraction of THC is made difficult due to it being a terpenoid, which are compounds composed of a hydrocarbon backbone, which can be modified by the addition of different functional groups and different orientations. These numerous modifications in structure lead to a tedious separation process¹⁴. THC consists of a tricyclic body and a five-carbon chain, totaling 21 carbons¹ (Figure 1). There are two numbering systems leading to the delta (Δ)-1 (Δ^1) and the Δ^9 naming, in which initial discovery and naming followed the Δ^1 numbering system, while more recent work uses the Δ^9 numbering system³. In 1971 THC was placed under the Controlled Substances Act (CSA) as a Schedule 1 drug, which are drugs that have no currently accepted medical applications and a high potential for abuse²⁰.

1.4 Synthetic Cannabinoids

Synthetic cannabinoids are compounds that have been synthesized in a laboratory and bind to the two cannabinoid receptors^{4,21}. These compounds, were initially developed by John W. Huffman to study the biological mechanisms of the cannabinoid receptor system as well as study novel compounds for therapeutic uses^{4,21}. Synthesis was conducted at universities and pharmaceutical companies. These compounds currently have no accepted medical uses in the United States⁴.

Synthetic cannabinoids are dissolved in solvents and then applied to plant material and dried, or used in e-cigarette appliances^{4,21}. The most common route of administration

of these substances is smoking ^{4,21}. Initial marketing and selling began in Europe as herbal incenses, and began appearing in the United States as early as 2008 ^{4,21}. Other names associated with synthetic cannabinoids, include but are not limited to Spice, K2, Blaze, Paradise, Spike, and Black Magic, while they may also be sold under pretext such as potpourri or herbal incense ⁴. Other marketing includes labeling as “legal alternative to marijuana” and “legal high” or “not for human consumption” ^{4,21}. The Drug Enforcement Agency (DEA) has been scheduling different synthetic cannabinoids as their popularity in case reports increases to protect the public’s health and safety ⁴. The vast majority of these compounds are scheduled as Schedule 1 compounds under the CSA. Two of these compounds to be scheduled under the CSA and appear on the National Forensic Laboratory Information System (NFLIS) annual reports are AB-FUBINACA and AB-PINACA.

1.4.1 AB-FUBINACA

AB-FUBINACA was first synthesized by Pfizer Inc. in 2009 and is classified as an indazole compound ^{6,22}. The chemical name for AB-FUBINACA is *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide ^{16,17,23} (Figure 1). The emergency temporary scheduling of AB-FUBINACA as a Schedule 1 compound under the CSA occurred in 2014 ²⁴. In 2016 the DEA permanently scheduled AB-FUBINACA as a Schedule 1 substance under the CSA ²⁵. Like THC, AB-FUBINACA binds to the CB1 and CB2 receptors in the human body and produce similar effects, however these effects may be more severe, such as hyperthermia, tachycardia and seizures ²².

1.4.2 AB-PINACA

AB-PINACA or *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-pentyl-1*H*-indazole-3-carboxamide, is classified as a pentyl indazole and was originally synthesized by Pfizer Inc. in 2009⁴⁻⁶ (Figure 1). It has similar psychoactive effects as THC as it acts on the same CB1 and CB2 receptors. Like AB-FUBINACA, the effects may be more severe than those of THC⁵. From 2010 to 2014, NFLIS reported 3,783 cases, with other agencies reporting at least three deaths and multiple overdoses that led to emergency hospital visits⁴. In 2015 the DEA emergency scheduled AB-PINACA as a Schedule 1 on the CSA⁵. Two years later AB-PINACA was permanently scheduled as a Schedule 1 under the CSA²⁶.

1.5 Pharmacokinetics and Pharmacodynamics

1.5.1 Use and Effects

THC is a psychoactive substance and its common route of administration is by smoking but can also be used orally^{1,27-29}. It also acts on the central nervous system as a depressant¹. The behavioral effects of THC include feeling euphoric, relaxed, an altered time perception, lack of focus and memory may be impaired. The person may also hallucinate, experience panic attacks, paranoia, and mood swings^{1,3}. Physical effects include impaired movement, slower reaction time and increased appetite¹.

A study by NMS Labs and Hartford Healthcare looked at different cases involving the ingestion of AB-FUBINACA. In all cases, patients believed they were ingesting “Molly”. The physical effects reported included sedation, tachycardia, hypothermia,

seizures, while the mental effects included unresponsiveness and an altered mental status³⁰. These reports expressed the different toxic effects AB-FUBINACA may cause after use.

In vitro and *in vivo* studies show that AB-PINACA is 2 to 14 times more potent than THC when acting on the CB1 and CB2 receptors⁵. Studies show that AB-PINACA is a full agonist for the cannabinoid receptors⁵. The effects include nausea, hallucinations, anxiety, paranoia, confusion, lethargy, tachycardia, and acute kidney failure to name a few symptoms⁵. Cases reporting the presence of AB-PINACA include motor vehicle accidents leading to death and impaired driving cases, delirium, loss of consciousness and seizures⁵.

1.5.2 Metabolism and Concentration

The main route of metabolism for THC in the human body consists of two phases. The first phase is hydroxylation in which the final product is 11-hydroxy-THC, an active metabolite^{1,28,31}. The second metabolite is not active and is formed by oxidation, creating 11-nor-9-carboxy-THC (THCCOOH)^{1,31}. When analyzing blood levels, THC appears rapidly in the blood after smoking, with peak levels between 4.8 to 10.2 minutes (min) at levels of 50 to 129 ng/mL for a low dose of 1.75% THC and 76 to 267 ng/mL for a high dose of 3.55% THC^{1,31}. The concentration decreases rapidly after the peak blood concentration but is still detectable up to 12 hours (hr) after smoking³¹. Peak 11-hydroxy-THC levels in blood occurred at 13.5 min with concentrations of 6.7 ng/mL for a low dose of 1.75% THC and 7.5 ng/mL for a high dose of 3.55% THC, with 11-hydroxy-THC no longer being detected before THC³¹. Unlike THC and 11-hydroxy-THC, the THCCOOH metabolite concentration levels plateaued once reaching peak blood levels³¹. Initial

detection time for THCCOOH is slower compared to THC and 11-hydroxy-THC, however once detected, the level is typically higher than 11-hydroxy-THC with the peak plateau levels ranging from 32 to 240 min after smoking ³¹. Peak levels averaged 24.5 ng/mL for a low dose of 1.75% THC and averaged 54 ng/mL for a high dose of 3.55% THC ³¹. Detection of THCCOOH ranged from 48 to 168 hr after smoking for a lower initial THC dose, while a higher THC dose ranged from 72 to 168 hr after smoking, with a slow decline in concentration as compared to THC and 11-hydroxy-THC ³¹. With the legalization of marijuana both medically and recreationally the THC potency has increased, with average levels as high as 20.59% in 2017 ³². Conversely, in 2014, the average percentage of THC was 11.84% ². It has been shown that THC, 11-hydroxy-THC, and THCCOOH display “low to moderate” levels of postmortem redistribution, with the redistribution increasing as the postmortem interval increases ¹⁸. The postmortem concentration of THC, 11-hydroxy-THC, and THCCOOH ranged from 0.5 to 50.5 ng/mL, 0.5 to 11.1 ng/mL, and 0.5 to 107.6 ng/mL respectively ¹⁸.

While the parent compound, AB-FUBINACA, may be detected in human blood or urine, the metabolites are predominately found in urine. AB-FUBINACA concentrations have been detected as low as 5.6 ng/mL in serum ³³. Due to the control status of AB-FUBINACA, metabolism studies must be carried out *in vitro* for humans or use an *in vivo* mouse model. The most common metabolite is AB-FUBINACA metabolite 3, which is an amide hydrolysis compound ²³.

A study completed in the state of Washington looked at reported concentration levels of AB-PINACA from impaired driving cases ³⁴. The levels detected were determined

using blood samples from 25 different cases, and ranged in concentration from 0.6 to 41.3 ng/mL³⁴. Of the 25 cases, five cases also reported the presence of other drugs along with AB-PINACA. One of the five cases contained THCCOOH with a concentration of 7.3 ng/mL, while the other four cases all contained such as AM-2201, JWH-210, THC, THCCOOH, MAM-2210, JWH-018, JWH-019, JWH-091 and JWH-250, ranging in concentrations of 0.2 ng/mL to 3.2 ng/mL³⁴.

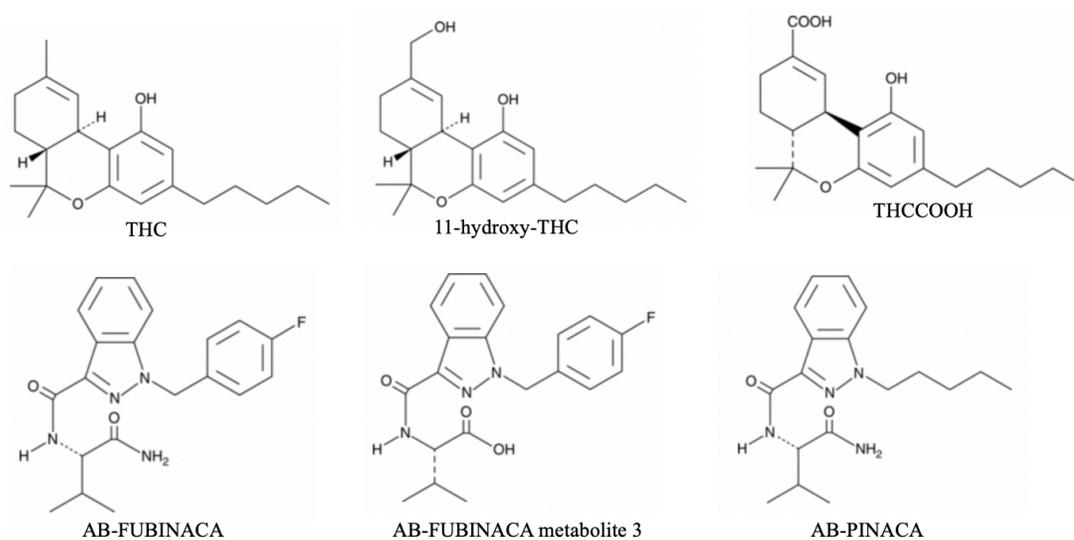


Figure 1. Chemical Structures of six analytes of interest

1.6 Sample Preparation Techniques

1.6.1 Purpose

Removing compounds of interest from a given matrix is important in forensic toxicology. Due to the complexity of matrices, such as blood and urine, the ability to clean up a sample and extract the analytes of interest while leaving interferences behind is critical. Blood is composed of plasma, red blood cells, white blood cells, and platelets³⁵⁻

³⁷. The plasma component makes up around 55% to 60% of blood and is composed of water, sugars, lipids, salts, and proteins such as albumin, enzymes, and clotting factors ^{8,36,37}. Urine's composition consists of urea, creatinine, uric acid, phosphate, sulfate, chloride, sodium and potassium ions, and a small amount of proteins and lipids ^{8,38}. Sample preparation techniques are designed to remove these different components of the matrix that may interfere, along with interferences due to other drugs present in the sample, , increase the analyte recovery, decrease variability, and protect the instrument allowing for less instrument downtime and longer column life ⁸.

There are a multitude of sample preparation techniques, beginning with the simplest technique of dilute and shoot, and moving into more complex extractions such as phospholipid depletion, protein precipitation, liquid-liquid extractions (LLE), solid phase extractions (SPE), solid phase microextractions, supported liquid extractions (SLE), and disposable pipette extractions. Each extraction has its advantages and disadvantages, but can all be used in forensic toxicology. This study focused on comparing LLE, SPE, and SLE.

1.6.2 Liquid-Liquid Extraction

The principle behind LLE are the solubility of the components present ³⁹. Two immiscible solvents are used, typically one aqueous and one organic. Once these solvents are added to the sample the analytes will travel into the solvent in which they are more soluble ^{1,39}. The solvents chosen depend on the pKa, polarity, are the compounds acidic or basic to determine which solvents will be used to increase the solubility of the analytes into

one of the immiscible layers ^{1,39}. Therefore, the different extractions can be acidic/neutral extractions or basic/neutral extractions. Depending on the method of analysis, the layer recovered can either be directly injected into the instrument, or dried down and reconstituted into a solvent that is more suitable for the instrument.

LLE are carried out in one container in which all solvents and compound of interest are added, mixed, centrifuged, and the organic layer is removed. There is the potential to repeat these steps to increase the analyte recovery ⁸. A few advantages for LLE are that compared to dilute and shoot, phospholipid depletion, and protein precipitation the extracts are cleaner and can be more selective. However, these extractions require intensive manual labor, they are more difficult to automate, and a possibility for variability due to the differences in layer removal while pipetting ⁸.

1.6.3 Solid Phase Extraction

When analytes of interest have similar polarities, or acid/base properties, LLE may not be suitable for their recovery. For these cases using SPE can be used ^{1,39}. Solid phase extractions use adsorption of components onto a solid phase to separate unwanted components and extract analytes of interest ^{1,39}. These extractions aid in removing interferences such as phospholipids found in blood, other interfering drugs, and other components found in the matrix, giving cleaner extracts. These packed columns consist of either silica or polymers ⁸. Sorbent beds composed of silica can only support a limited range of pH, the packing of the column is more variable, and the sorbent bed cannot dry while being conditioned and before the sample is loaded ^{1,8}. Comparatively, sorbent beds

made of polymers can support a larger pH range, the packing is more homogenous, and there is a higher sample capacity ^{1,8}. Solid phase extractions can be used for acidic, basic, or neutral compounds, in which the sorbent bed composition is adjusted to fit the type of analyte.

The traditional steps for SPE methods begin with conditioning the column. This adjusts the pH of the sorbent bed and activates the functional groups in the sorbent bed. The next step is loading the sample, followed by washing to remove any matrix interferences or other interferences present. The final step is eluting the analytes of interest ^{1,40}. Some advantages of using a SPE method are that the extracts are clean, due to matrix interferences such as phospholipids being removed during the washing step and increased reproducibility ^{1,8}. These methods can also be very selective, and there is the possibility for automation. Some disadvantages are that these processes can be time consuming with all the steps involved and there are costs involved due to buying the columns and ensuring all necessary equipment such as vacuums or positive pressure manifolds are purchased ⁸.

1.6.4 Supported Liquid Extraction

Supported liquid extractions are a modified LLE. They use the same principles of combining water immiscible solvents to create analyte extraction ^{41,42}. While a LLE method requires mixing the immiscible solvents by shaking, rocking, centrifuging, etc., SLE uses a stationary phase that captures the aqueous solution while the organic solvent flows through the stationary phase ^{41,42}. The stationary phase is composed of diatomaceous earth which has a high silica content ^{1,8,42,43}. Diatomaceous earth is composed of fossilized

diatoms that are high in silica content and found on the sea floor ^{42,44}. Diatoms are closely related to algae and have a hard shell or skeleton composed of silica, resulting in diatomaceous earth containing a rich silica content ^{42,44}. The diatomaceous earth is ground resulting in large, irregular shaped particles with high surface area leading to greater absorption increasing the extraction efficiency ^{1,8,42}.

The three steps involved in the SLE process are, loading the column, waiting while the matrix is absorbed into the column, and eluting the analytes of interest ^{8,41,42}. Some advantages of SLE methods are their reproducibility, increased analyte recovery, and decreased ion suppression. These methods give cleaner extracts compared to LLE methods due to phospholipids and other matrix interferences binding to the column and not eluting out. Supported liquid extractions can be more selective than LLE methods. Since there are no washing steps the eluent is not as clean as SPE samples and the selectivity is less than that of SPE methods, however the amount of time consumed is reduced compared to both LLE and SPE ⁸. Like SPE, automation is possible as well. Analysis is capable for acidic, basic, or neutral compounds and for all matrices.

1.7 Research Objectives

THC and synthetic cannabinoids are commonly encountered drugs in forensic toxicology cases, therefore, being able to extract these compounds and their metabolites is important. With a variety of sample preparation techniques available on the market determining the differences would be beneficial for choosing the most effective and efficient method.

The primary purpose of this study was to develop and validate three different sample preparation techniques for the analysis of THC, 11-hydroxy-THC, THCCOOH, AB-FUBINACA, AB-FUBINACA metabolite 3, and AB-PINACA. The three different sample preparation techniques consisted of LLE, SPE, and SLE methods. Analysis and quantitation were completed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Each method was validated to quantitate each of the six compounds of interest using the same linear dynamic range for each method. Additionally, the study looked at the ability to use the same sample preparation method on both human urine and whole blood. Validation parameters were assessed using American Standards Board (ASB) Standard 036, Standard Practices for Method Validation in Forensic Toxicology ⁴⁵. Following validation, the results of the three sample preparation techniques were then compared against each other, along with comparing other parameters such as the number of resources used and the amount of time consumed by each method.

2. MATERIALS AND METHODS

2.1 Instrumentation Theory

2.1.1 Liquid Chromatography

Liquid chromatography (LC) uses liquid to separate compounds and analytes of interest within a sample. Chemical principles of compounds are used for this separation. A system consists of two mobile phases that are controlled by pumps that cause the mobile phases to flow through the system where they are degassed to remove any bubbles and mixed together^{1,46}. The mobile phases may be used either as isocratic flow which consists of a consistent ratio of mobile phase A to mobile phase B, or gradient flow where the ratio of mobile phase A to mobile phase B changes over time¹. An autosampler houses the sample until it is injected into the mobile phase which then travels into the stationary phase, or column which is housed in an oven⁴⁶. A common system is a reverse phase system in which the mobile phases are more polar than the stationary phase¹. While in the column the compounds are separated based on their affinity, adsorption, ion exchange, and size exclusion for the stationary phase or the mobile phase. As the compounds elute from the column they then travel to the detector, in which a chromatogram is produced⁴⁶. The chromatogram plots the response as a function of the retention time, or the amount of time from injection to the compound reaching the detector⁴⁶. A variety of detectors are available for further analysis, such as ultraviolet-visible spectroscopy, infrared spectroscopy, and mass spectrometry^{1,46}.

2.1.2 Mass Spectrometry

Mass spectrometry (MS) is a technique used for the detection of ions present in a sample. Determining the molecule(s) present is based on the fragmentation and ionization patterns of molecules while they are in the gas phase^{39,47}. Molecules fragment in specific patterns that are distinctive to that molecule, allowing for elucidation of the different molecules present.

Mass spectrometers are composed of five components, the ion source, mass analyzer, ion detector, data system and a vacuum. There are multiple types of ion sources, including, electron ionization, chemical ionization atmospheric pressure chemical ionization, and electrospray ionization (ESI). The ion source is responsible for the ionization of molecules once introduced into the system⁴⁷. ESI, which is commonly coupled with LC-MS systems, uses atmospheric pressure and composed of a capillary tube and a counter electrode^{39,47}, which is heated to high temperatures. The capillary tube and counter electrode have a potential difference in which an electric current is created resulting in charge accumulation on the surface of the liquid as it emerges from the capillary tube^{39,47}. Droplets are multiplied further and further as solvent evaporates and the electric charge increases on the surface causing the droplets to split^{39,47}. The process repeats until the ions are ejected from the droplet. A nebulizing gas, typically nitrogen, can be used to assist in the formation of these droplets, along with maintaining a constant spray⁴⁷. The ions then pass through a sampling cone and are focused as they pass through vacuums and make their way to the analyzer³⁹.

Ions leaving the source are accelerated through the mass analyzer and focused using an electric field and finally separated by their mass to charge ratio (m/z)⁴⁷. Quadrupole mass analyzers are a commonly employed type of mass analyzer which consist of four parallel rods centered around a central axis⁴⁷. Radiofrequency and direct current are applied to the rods⁴⁷. As ions travel through the quadrupoles, only ions with a specific m/z for certain radiofrequency and direct current potentials will successfully reach the detector^{1,39,47}. Once the ions travel through the mass analyzer and reach the ion detector the relative intensity of each m/z is recorded creating a mass spectrum unique to that molecule⁴⁷. The data system then plots the relative intensity versus the m/z to give a visual representation of the mass spectrum. Both the mass analyzer and ion detector are under vacuum when using ESI.

2.1.2.1 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) is a type of mass analyzer that consists of multiple quadrupoles, most commonly used is a triple quadrupole, consisting of three quadrupole sets. The precursor ion is detected in the first quadrupole⁴⁷. As the precursor ions move into the second quadrupole the ions are fragmented into product ions due to radiofrequency only being applied to the rods, creating a collision cell^{39,47}. The third quadrupole is used as a mass analyzer like the first quadrupole and the m/z of the product ions are detected here⁴⁷. The system names these different quadrupoles as Q1, where the

precursor ion is detected, Q2 where collision energy is applied fragmenting the precursor ion, and Q3 where the product ions are detected ⁴⁷.

2.2 Reagents and Instrumentation

2.2.1 Standards/Reagents

Certified reference standards of Δ^9 -THC, (\pm)-11-hydroxy- Δ^9 -THC, (\pm)-11-nor-9-carboxy- Δ^9 -THC, AB-FUBINACA, AB-FUBINACA metabolite 3, and AB-PINACA, all with a concentration of 1 mg/mL, were purchased from Cayman Chemical (Ann Arbor, MI, USA). Δ^9 -THC- d_3 , (\pm)-11-hydroxy- Δ^9 -THC- d_3 , and (-)-11-nor-9-carboxy- Δ^9 -THC- d_3 , all at a concentration of 100 μ g/mL, AB-FUBINACA- d_4 , and AB-PINACA- d_9 , both with a concentration of 1 mg/mL, were used as certified reference internal standards, and were all purchased from Cayman Chemical.

Mobile phase and needle wash consisted of reagent grade Formic Acid, $\geq 95\%$, (Sigma-Aldrich, St. Louis, MO, USA), Optima® LC/MS Acetonitrile (ACN), Optima® LC/MS Methanol (Fisher Chemical, Fair Lawn, NJ, USA), and ultrapure deionized water (DI water) prepared using a Millipore Milli-Q Ultrapure (Type 1) water system (Millipore Sigma, Burlington, MA, USA).

For the extraction protocols HPLC Grade Hexanes, Optima® Ethyl Acetate, Certified ACS Plus Ammonium hydroxide (NH_4OH) (Fisher Chemical, Fair Lawn, NJ, USA), HPLC grade, 99+% Methyl *tert*-butyl ether (MTBE) (Alfa Aesar, Ward Hill, MA, USA), reagent ACS grade Glacial Acetic Acid (Acros, New Jersey, USA), DI water and ACN were used.

2.2.2 Specimen

Human urine was donated following approved institutional review board (IRB) guidelines (Boston University School of Medicine, Boston, MA, USA). Human whole blood was purchased from Equitech Enterprises (Kerrville, TX, USA), Biological Specialty Corporation (Colmar, PA, USA), and Boston Medical Center Blood Bank (Boston, MA, USA).

2.2.3 LC-MS/MS Instrumentation

Analysis was conducted on a Shimadzu Prominence Ultra-Fast Liquid Chromatograph consisting of a DGU-20A5R Degassing Unit, two LC-20AD pumps, one for each mobile phase, SIL-20AC HT Autosampler, CTO-20A column oven, and a CBM-20A communication bus module (Shimadzu, Kyoto, Japan). A 4000 Q Trap Tandem Mass Spectrometer (LC-MS/MS, SCIEX, Framingham, MA, USA), equipped with ESI as the detector. The chromatography was carried out on a XBridge® C18 3.5 μm , 2.1 x 50 mm column (Waters Corporation, Milford, MA, USA) or a Kinetex® 2.6 μm C18 100 Å, 50 x 2.1 mm column (Phenomenex, Torrance, CA, USA). Data collection was completed on Analyst® (version 1.6.2) software (SCIEX) and MultiQuant® (version 3.0.5373.0) software (SCIEX) was used for all data analysis. Statistical analysis was performed on Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

2.3 Methods

2.3.1 LC-MS/MS Parameters

The following parameters are for LC-MS/MS analysis on the SCIEX instrument. A XBridge® C18 3.5 µm, 2.1 x 50 mm column was used for LLE and SPE method analysis and a Kinetex® 2.6 µm C18 100 Å, 50 x 2.1 mm column was used for SLE method analysis. The flow rate was 0.8 mL/min with an injection volume of 10 µL. Mobile phase A was 0.1% formic acid in DI Water and mobile phase B was 0.1% formic acid in ACN. All mobile phases were prepared in the laboratory. A gradient flow was used beginning with 30% mobile phase B, ramping up to 90% mobile phase B and finishing back at 30% mobile phase B (Table 1, Figure 2). The total run time was 9 min with a needle rinse before and after each aspiration. Each needle rinsed was 1,000 µL of needle wash solution (40:40:20 ACN:methanol:DI water) which was prepared in the laboratory. The rinse time was 5 seconds (sec) with a rinsing speed of 35 µL/sec. The sampling speed was programmed to 3.0 µL/sec. The autosampler was set to 15°C and the column oven was set at 40°C.

Table 1. Mobile phase conditions.

| Gradient | | |
|------------|------------------|------------------|
| Time (min) | % Mobile Phase A | % Mobile Phase B |
| 0.01 | 70 | 30 |
| 0.5 | 70 | 30 |
| 5 | 10 | 90 |
| 8.5 | 10 | 90 |
| 8.51 | 70 | 30 |
| 9 | Stop | |

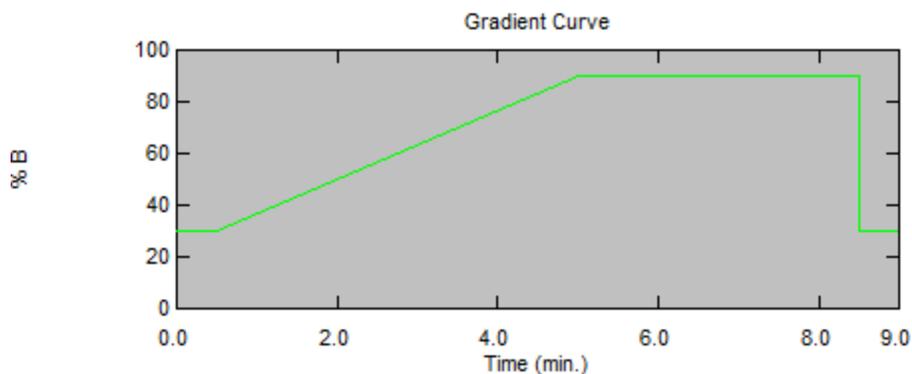


Figure 2. Gradient Curve for Mobile Phase B.

Each analyte of interest was monitored using two ion transitions, the quantitative (quant) and qualitative (qual) ions. The quant ion was used for all the quantitative calculations and studies. The qual ion was monitored to ensure the analyte's presence. Determining the ratio between the quant and qual ions for an analyte and monitoring the ion ratio during analysis ensures the compound's presence and not another compound with similar fragmentation. The internal standards were monitored using one transition. Table 2 shows the precursor ion (Q1 Mass) along with the quant and qual product ions (Q3 Mass) for each analyte and internal standard. The declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) are also included for each monitored transition.

Table 2. Parent compound and transition ions. The precursor mass, quant and qual ion transition masses, and DP.

| Compound | Precursor Ion (<i>m/z</i>) | Quant Ion (<i>m/z</i>) | Qual Ion (<i>m/z</i>) | DP (volts) |
|-----------------------------|---------------------------------------|-----------------------------------|----------------------------------|-------------------|
| Δ9-THC | 315.3 | 193.4 | 123.3 | 90.0 |
| 11-hydroxy-Δ9-THC | 331.3 | 193.1 | 313.4 | 64.0 |
| 11-nor-9-carboxy-Δ9-THC | 345.2 | 299.4 | 327.3 | 81.0 |
| AB-FUBINACA | 369.1 | 253.2 | 324.5 | 63.0 |
| AB-PINACA | 331.3 | 215.3 | 314.4 | 81.0 |
| AB-FUBINACA metabolite 3 | 370.2 | 109.2 | 253.1 | 72.0 |
| Δ9-THC-d3 | 318.3 | 196.2 | - | 84.0 |
| 11-hydroxy-Δ9-THC-d3 | 334.3 | 316.4 | - | 73.0 |
| 11-nor-9-carboxy-Δ9-THC-d3 | 348.2 | 330.3 | - | 86.0 |
| AB-FUBINACA-d4 | 373.1 | 328.3 | - | 66.0 |
| AB-PINACA-d9 | 340.3 | 224.2 | - | 66.0 |

2.3.2 Sample Preparation

2.3.2.1 Standard Solutions and Quality Controls

The certified reference standards, stored at approximately -20°C, were used to create working stock solutions containing all six compounds of interest. The certified reference standards were diluted with methanol to create the spiking mixes to prepare the calibrators, quality control (QC), LOD and LOQ samples (Table 3). Certified reference standards of the deuterated internal standards were diluted in methanol for a working solution of 250 ng/mL. For all experiments, 400 μL of blood or urine was used. For double blank samples no internal standard or calibrator/control was added, thus giving a base line for a clean sample. Blank samples were prepared by adding internal standard only.

Table 3. Preparation of calibration curve, QCs, LOD and LOQ in blood and urine.
The final concentration for the calibration curve and QCs starting with the working stocks and aliquoting into the urine and blood.

| Spike solution Conc. (ng/mL) | Aliquot Vol (μL) | Total Vol (μL) in blood/urine | Final Conc. (ng/mL) | Labeled As |
|-------------------------------------|------------------|-------------------------------|---------------------|------------|
| Calibrators in Blood/Urine | | | | |
| 10 | 40 | 400 | 1 | Cal1 |
| 25 | 40 | 400 | 2.5 | Cal2 |
| 50 | 40 | 400 | 5 | Cal3 |
| 100 | 40 | 400 | 10 | Cal4 |
| 200 | 40 | 400 | 20 | Cal5 |
| 300 | 40 | 400 | 30 | Cal6 |
| 500 | 40 | 400 | 50 | Cal7 |
| QCs in Blood/Urine | | | | |
| 30 | 40 | 400 | 3 | LQC |
| 150 | 40 | 400 | 15 | MQC |
| 400 | 40 | 400 | 40 | HQC |
| LODs and LOQs in Blood/Urine | | | | |
| 0.1 | 40 | 400 | 0.01 | LOD1 |
| 1 | 40 | 400 | 0.1 | LOD2 |
| 2.5 | 40 | 400 | 0.25 | LOD3 |
| 5 | 40 | 400 | 0.5 | LOD4 |
| 7.5 | 40 | 400 | 0.75 | LOD5 |
| 10 | 40 | 400 | 1 | LOQ1 |
| INTERNAL STANDARD | | | | |
| 250 | 40 | 400 | 25 | ISTD |

2.3.2.2 Liquid-Liquid Extraction Method

Extractions were carried out in 7 mL clear glass vials with PTFE lined screw tops (Supelco, Bellefonte, PA, USA). The urine or blood (400 μL), spiking solution (40 μL), and internal standard (40 μL) were added to the vials, along with 750 μL DI water, 200 μL 20% glacial acetic acid, and 4 mL of hexane:ethyl acetate:MTBE (80:10:10). The glacial

acetic acid and organic solution were prepared in the laboratory. The vials were rocked for 30 min followed by 10 min in the centrifuge at 3500 rotations per minute (rpm) then placed in a freezer at approximately -20°C for around 2 hours. The organic layer was transferred to 15 mL glass test tubes and evaporated to dryness on the nitrogen evaporator at 40°C for approximately 20 min (Organomation Berlin, MA, USA). The samples were reconstituted in 100 µL of 0.1% formic acid in DI water:0.1% formic acid in ACN (70:30) and transferred into LC vials with inserts and placed in the LC-MS/MS autosampler for analysis.

2.3.2.3 Solid Phase Extraction Method

For each extraction, 400 µL of urine or blood was transferred into a 6 mL glass test tube, with 40 µL of the internal standard and 40 µL of the appropriate spiking solution. The following sample pretreatment and the extraction parameters were modified from the UCT Application Note for the Styre Screen® THC 6 mL Tube ⁴⁸. To the test tube, 1 mL of ice cold ACN was added. The samples were vortexed, then centrifuged at 4,000 rpm for 10 min. The liquid was reduced down to 100 – 200 µL on an evaporator with nitrogen air flow and then 1 mL of DI water was added.

UCT Styre Screen® THC 3 mL 60 mg columns (United Chemical, Bristol, PA, USA) were used for the extraction. The columns were loaded onto a UCT positive pressure manifold. The matrix was then loaded onto the columns and absorbed into the column bed using positive pressure flow of 1 mL/min. The loaded column was washed with 1 mL of DI water:ACN:NH₄OH (84:15:1), made daily, with a positive pressure of 1 mL/min

followed by 11 min of pressure for drying. Clean test tubes were then placed under the columns for collection and 1.5 mL of hexane:ethyl acetate:glacial acetic acid (49:49:2) was added to the columns to elute the compounds of interest. Positive pressure with a flow rate of 1 mL/min was applied. The samples were then dried on an evaporator with nitrogen flow at 40°C for approximately 15 min and reconstituted in 100 µL of 0.1% formic acid in DI water:0.1% formic acid in ACN (70:30). The final solution was transferred to LC vials with inserts and placed in the LC-MS/MS autosampler tray.

2.3.2.4 Supported Liquid Extraction Method

Into 6 mL glass test tubes, 400 µL of urine or blood was added along with 40 µL of the internal standard and 40 µL of appropriate spiking solution. To this, 200 µL of 5% glacial acetic acid was added to each test tube and then vortexed. For the blood samples, after vortexing the sample were centrifuged for 5 mins at 4000 rpm. The supernatant was transferred onto Biotage Isolute® SLE+ 1 mL columns (Biotage, Charlotte, NC, USA) with 15 mL glass test tubes below to collect all liquid. After applying a quick pulse of pressure, the sample was absorbed onto the columns for five minutes using gravity flow. Following absorption, 3 mL of hexane:ethyl acetate:MTBE (80:10:10) was applied and allowed to flow for 5 minutes by gravity. This was repeated one more time. The extraction product was placed on a nitrogen evaporator and dried at 40°C for approximately 20 min. Following drying, 100 µL of 0.1% formic acid in DI water:0.1% formic acid in ACN reconstitution solution was added and the samples were then transferred to LC vials with inserts and placed in the LC-MS/MS autosampler tray.

2.3.3 Method Validation

Method validation was completed following the ASB Method Validation parameters ⁴⁵. These included calibration model, bias and precision, LOD, LOQ, analyte recovery, carryover, interferences, ion suppression and enhancement, dilution integrity, and processed stability ⁴⁵. Chromatography analysis was completed using Multiquant® software and statistical analysis was performed using Microsoft Excel.

Other parameters of interest for the comparison of the various sample preparation techniques were the amount of time each technique took, the number of reagents, the amount of reagent and the resources consumed.

2.3.3.1 Calibration Model

Calibration model was carried out over a series of five different prepared calibration curves for each of the six analytes of interest. Each curve was prepared separately, along with the three QCs prepared in triplicate by spiking negative blood or urine. Under ASB guidelines, the calibration curve must be validated with a minimum of six non-zero concentrations ⁴⁵. Seven points were used for this project, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 30 ng/mL, and 50 ng/mL. The three QCs fell between all 7 points, with a low QC of 3 ng/mL, a middle QC of 15 ng/mL, and a high QC of 40 ng/mL. The best fit curve was determined for each compound and the coefficient of correlation (r^2) value was recorded for each calibration curve. A residual plot was created for each analyte of interest over the calibration range to show whether the range was linear or quadratic.

The qual/quant ion ratio was also monitored and all values needed to fall within $\pm 20\%$ of this average value. This ratio was monitored for all validation parameters.

2.3.3.2 Bias

Bias studies were performed to determine the accuracy of the sample preparation technique for each analyte of interest. Bias studies were performed for each compound preparing the three QCs (3 ng/mL, 15 ng/mL, 40 ng/mL) in triplicate over five different runs. Bias was determined using the following equation and each value for each concentration needed to fall within $\pm 20\%$ ⁴⁵.

$$\text{Bias (\%)} = \frac{\text{Calculated Average} - \text{Actual Average}}{\text{Nominal Average}} * 100$$

These samples were prepared along with the calibration model samples and were also used for the precision studies.

2.3.3.3 Precision

Precision studies were performed to determine the reproducibility and repeatability for each sample preparation technique for each of the six analytes of interest. These values represent an analyst's ability to repeat these experiments from day to day and reproduce the same values. Precision studies were carried out using the same QC samples prepared in triplicate as the bias studies and carried out along with calibration model. Precision was recorded as the % coefficient of variation (% CV) and needed to fall within $\pm 20\%$ for the method to be validated.

Two forms of precision were calculated for each analyte, within-run precision and between-run precision. Within-run precision assesses the repeatability of the results during the same run. A within-run precision value was determined for each QC value for each individual run using the standard deviation and average calculated concentration.

$$\textit{Within-run Precision (\% CV)} = \frac{\textit{Standard Deviation}}{\textit{Average Calculated Concentration}} * 100$$

The overall within-run precision was calculated by averaging every within-run value.

Using the 15 samples from all five runs for each concentration, the between-run precision was determined. The between-run precision represents how close together the calculated concentration values were over all five runs. This value assesses the ability to repeat the experiment from day to day and be able to reproduce the results. The overall between-run precision was then determined by averaging the three QC between-run values.

$$\textit{Between-run Precision (\% CV)} = \frac{\textit{Standard Deviation of all samples}}{\textit{Grand Average Calculated Concentration}} * 100$$

Finally, the grand between-run precision was determined by taking the standard deviation of every QC sample over all five runs at all concentrations divided by the average accuracy for all 45 samples and multiplying this value by 100 to create a percentage.

2.3.3.4 Recovery

For analyte recovery, 10 different matrix lots were used in which two different concentrations were monitored, a low (10 ng/mL) and a high (30 ng/mL). Samples for all 10 matrix lots were prepared in duplicate and spiked with analyte and internal standard pre-extraction. Another set of all 10 matrix lots were prepared in duplicate and spiked with

analyte and internal standard post extraction. Recovery was then calculated using the following equation:

$$\% \text{ Recovery} = \frac{\text{Pre Extraction Area}}{\text{Post Extraction Area}} * 100$$

The area was monitored for this validation parameter; therefore, all low concentration samples were run on the same day and all high concentration samples were run on the same day.

2.3.3.5 Limit of Detection and Limit of Quantitation

For LOD, three different matrix lots were used, with each concentration of interest prepared in duplicate. Analysis was carried out over three runs using the established calibration model. The final concentrations consisted of 0.01 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 0.75 ng/mL, and 1 ng/mL. A double blank was also prepared in duplicate for each matrix lot. For each double blank, the signal area at the analyte of interest's retention time was manually integrated using MultiQuant®. This area was then averaged and a standard deviation was determined. The LOD was determined if the average signal area for a concentration was greater than the average double blank area plus 3.3 times the standard deviation, along with the qual/quant ion ratio within $\pm 20\%$ of the average qual/quant ion ratio and Gaussian peak shapes.

Limit of quantitation was determined using three different matrix lots prepared in duplicate, over three runs. A series of concentrations at or below the lowest calibrator were

run and the calculated concentration % CV needed to fall within $\pm 20\%$ for the value to be considered as a limit of quantitation.

2.3.3.6 Ion Suppression or Enhancement

Ion suppression and enhancement were determined by preparing 10 different lots of matrix in duplicate and spiking with analyte and internal standard post extraction. A neat solution was also prepared and injected six times. Suppression or enhancement was calculated with the following equation:

$$\% \text{ Ion Suppression or Enhancement} = \left(\frac{\text{Average Post Area}}{\text{Average Neat Area}} - 1 \right) * 100$$

Like analyte recovery, the peak area is of interest for this study instead of concentration. Analyte recovery and ion suppression or enhancement was carried out at the same time using the same post spiked samples.

2.3.3.7 Carryover, Interferences, Dilution Integrity, Stability

Carryover was determined by running double blanks directly after the highest calibrator and the peak area was determined. The average peak area needed to be less than 10% of the lowest calibrators peak area for there to be considered no carryover.

When looking at different interferences during analysis one source of potential interferences studied were different drugs and drug classes. These drugs may mask the analyte being analyzed. They may also enhance or suppress the signal therefore when using the method for quantitative purposes the calculated concentration may be greater than or less than the actual concentration. Common interferences were looked at by running

commonly encountered drugs (Table 11) through the extraction method, LC-MS/MS method, and quantitation method to ensure no interferences occurred. The drugs were prepared in three different drug mix groups, with a final concentration of 200 ng/mL once spiked in the matrix of interest (Table 11). Interference from the internal standard was studied by looking at negative matrix sources with only internal standard added and assessing peak area at the analyte retention time to. Blank sources of matrix were fortified with the highest calibrator only and the signal area was assessed at the internal standard retention to assess any interference with the internal standards. All area less than 10% of the lowest calibrator was considered as no interference.

Matrix interferences were analyzed by running 10 separate lots of matrix in double blank form and analyzing the signal area at the retention time of the analyte of interest. If the area was less than 10% that of the lowest calibrator there was considered no matrix interference.

Dilution integrity was studied to ensure that if an unknown sample had a concentration higher than the highest calibration point, the sample could be diluted and give an accurate result. This was completed by looking at a 1:10 and a 1:50 dilution. Each dilution was prepared in triplicate and run over five runs. Within-run precision, between-run precision, and bias were calculated to determine the integrity of diluting the samples.

To ensure a sample could be kept on the autosampler for more than one day due to atypical events processed sample stability was studied. Processed stability looked at the length of which a sample could sit after being processed before the results were no longer acceptable. The study was carried out over 72 hrs. The QCs were prepared in triplicate and

injected at 0 hr, 24 hr, 48 hr, and 72 hr. The ion ratio of quant ion:internal standard ion was compared back to 0 hr injection, and needed to fall within $\pm 20\%$.

3. RESULTS

3.1 Analyte Detection

For the MS parameters, the signal intensity was greater for the synthetic cannabinoids compared to the THC compounds, therefore, method development was more focused on the optimization of the THC compounds.

Initial analysis for determining the best column and LC parameters was completed using neat solutions, which consisted of the calibration or QC standards with internal standard prepared in mobile phase. A C18 and F5 (Phenomenex, Kinetex® 2.6 µm F5 100 Å, 50 x 3.0 mm) column were initially assessed for their use, with the C18 column being determined as the best column for analysis, due to the F5 column creating peaks with more tailing and less resolution of 11-hydroxy-THC and THCCOOH.

The retention times were determined using the earlier mentioned LC parameters (Table 4). Two different analytical columns with the same length and diameter, were used for validation analysis, a XBridge® C18 and a Kinetex® C18. Figure 3 shows the retention times of all six compounds in the chromatogram for the XBridge® C18 and Kinetex® C18 column.

Table 4. Retention time of analytes of interest. The retention times of the six analytes of interest in the order they elute beginning with the first compound to elute.

| Compound | XBridge® Retention Time (min) | Kinetex® Retention Time (min) |
|--------------------------|-------------------------------|-------------------------------|
| AB-FUBINACA | 2.09 | 2.33 |
| AB-PINACA | 2.46 | 2.64 |
| AB-FUBINACA metabolite 3 | 2.52 | 2.73 |
| 11-hydroxy-THC | 3.39 | 3.50 |
| THCCOOH | 3.49 | 3.56 |
| THC | 4.51 | 4.61 |

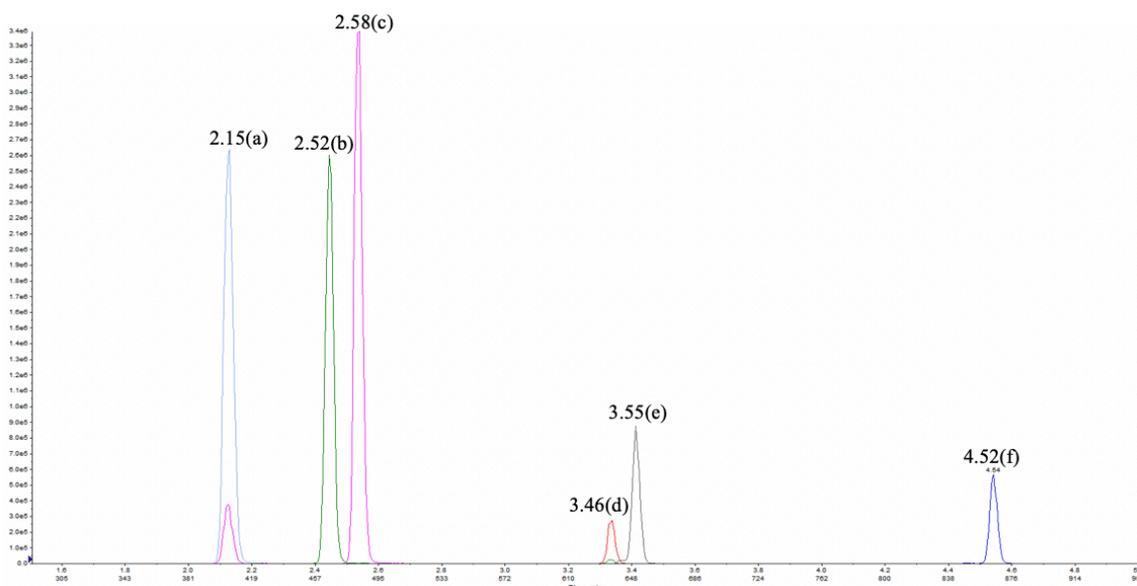


Figure 3. Liquid chromatogram for the XBridge® C18 column in a neat solution. All six compounds are resolved. (a) AB-FUBINACA, (b) AB-PINACA, (c) AB-FUBINACA metabolite 3, (d) 11-hydroxy-THC, (e) THCCOOH, (f) THC.

3.2 Urine Analysis

3.2.1 Calibration Range

The concentration range of interest for the calibration curves was determined from studies and commonly reported values of these compounds in the human body. With newer

synthetic cannabinoids and novel psychoactive substances having stronger effects with lower dosage the initial goal for the lower end of the calibration range was 0.01 ng/mL. Preliminary trial assessment revealed detection and quantitation of levels below 1 ng/mL would not be reproducible for the analytes of interest. Detection for the THC compounds was not possible at a concentration of 0.01 ng/mL either. To ensure repeatability and reproducibility, the lower end of the calibration curve was then set at 1 ng/mL, while the upper end was set to 50 ng/mL. The upper limit of the linear dynamic range was set above typically recorded levels of detection for the analytes of interest. To ensure the range of 1 ng/mL to 50 ng/mL was linear, five calibration curves were analyzed. The r^2 value was determined. This value needed to be 0.99 or greater for the curve to be accepted. A residuals plot for each of the five curves was also created to show the linearity of the range. One such plot can be seen below in Figure 4.

The r^2 values and residue plots were created for all six compounds being studied for each sample preparation technique. The r^2 values for each compound of interest are recorded in Table 5. For each analyte the r^2 value is compared to one another for each of the three sample preparation techniques. THC had the best r^2 value of 0.999 with the LLE and SPE method. 11-hydroxy-THC and THCCOOH had the best r^2 value of 0.999 for the SLE method. Looking at the synthetic cannabinoids, the LLE method had the best r^2 value, with 0.999 for AB-FUBINACA, 0.998 for AB-FUBINACA met 3 and AB-PINACA, while the SPE and SLE methods had values between 0.995 and 0.997 for the synthetic cannabinoids. All of these values are acceptable under the ASB guidelines and all three sample preparation techniques fall within the validation parameters for use.

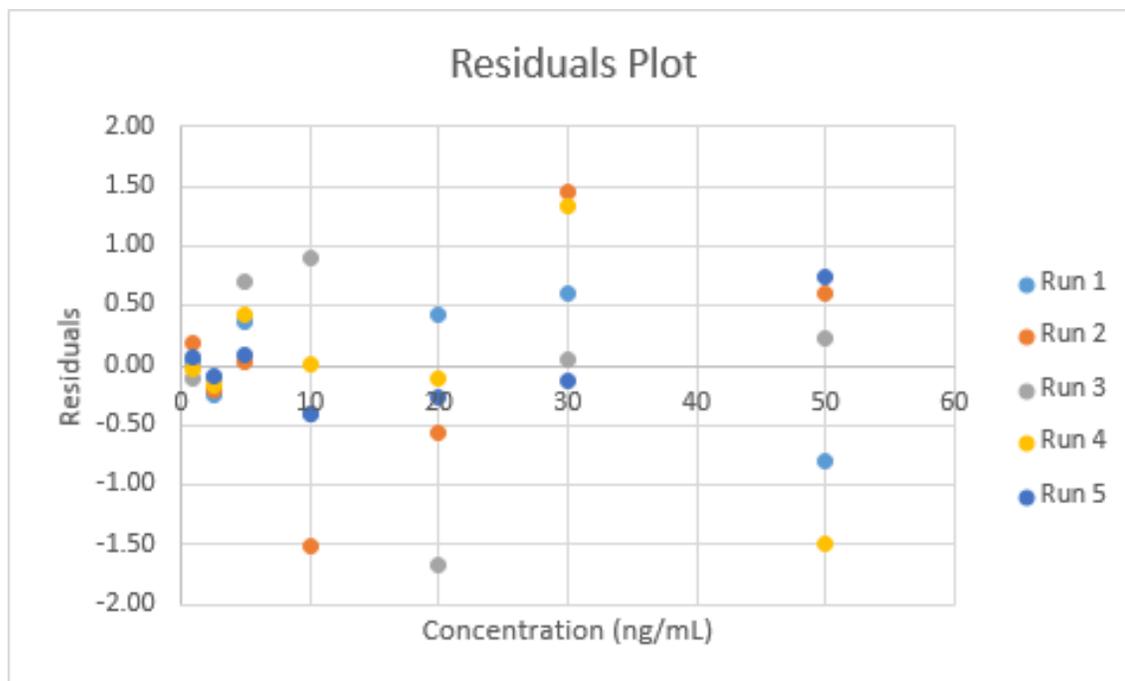


Figure 4. Residuals plot for THCCOOH using the LLE sample preparation technique for urine. This residuals plot shows random spacing around the zero value at the six calibration points expected of a linear range.

The residual plot looks at all seven calibration points. For each run the calculated concentration is subtracted from the actual concentration to give a residual value. These values are then summed together in which the sum must equal zero. If the curve is linear the residuals plot values will be spaced relatively evenly around zero and look scattered, while a quadratic curve will form a U-shaped plot⁴⁵. The residual plots give another visual representation of the shape of the curve being analyzed, and assist the analyst in determining the linear dynamic range for the analytes of interest. For each method, the linear dynamic range of 1 ng/mL to 50 ng/mL was appropriate according to the residues plot. For all three sample preparation methods, AB-FUBINACA, AB-FUBINACA

metabolite 3, and AB-PINACA residuals plots were beginning to resemble that of quadric curves with the linear dynamic range extending to 50 ng/mL (Figure 5). The r^2 values were still greater than 0.995, therefore the linear dynamic range was still acceptable, however, for future studies, using a range that ends of 40 ng/mL would be appropriate for synthetic cannabinoids. This value is also on the high end of levels detected in case samples.

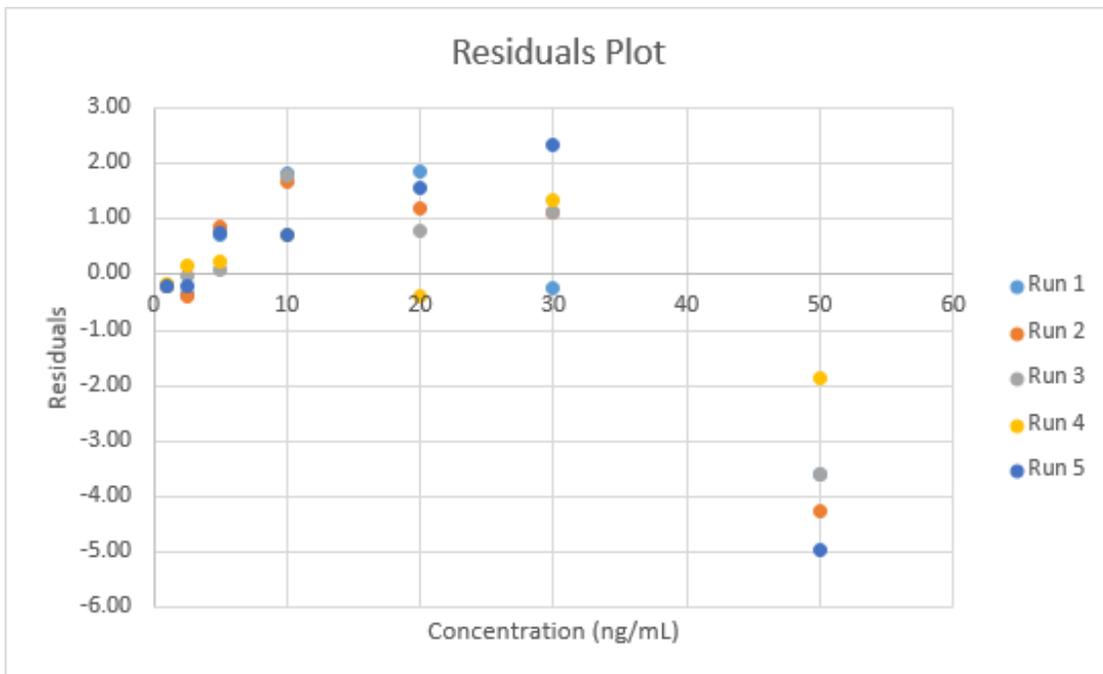


Figure 5. Residuals plot of AB-PINACA using the SLE method for urine. A U-shape is beginning to form over the concentration range of 1 ng/mL to 50 ng/mL, representing a range that is becoming quadric in nature.

Table 5. r^2 values for urine. A comparative look at the r^2 values for the LLE, SPE, and SLE sample preparation methods.

| Compound Name | r^2 values | | |
|-------------------|--------------|-------|-------|
| | LLE | SPE | SLE |
| THC | 0.999 | 0.999 | 0.998 |
| 11-hydroxy-THC | 0.999 | 0.999 | 0.999 |
| THCCOOH | 0.999 | 0.999 | 0.999 |
| AB-FUBINACA | 0.999 | 0.997 | 0.997 |
| AB-FUBINACA met 3 | 0.998 | 0.995 | 0.995 |
| AB-PINACA | 0.998 | 0.996 | 0.996 |

3.2.2 Bias and Precision

The three QCs, 3 ng/mL, 15 ng/mL, and 40 ng/mL were prepared in triplicate for five separate runs. These samples were used to calculate the bias and precision for each of the six analytes of interest for all three sample preparation techniques. The LLE method had the largest bias and poorest accuracy for all compounds except for AB-PINACA, which had as similar value as the SLE method (Table 6). For THC, its two metabolites and AB-FUBINACA metabolite 3, the SLE method had the best overall bias with THC, 11-hydroxy-THC, and AB-FUBINACA metabolite 3 having values under 2%, while THCCOOH had a value close to 3%. AB-FUBINACA and AB-PINACA had the best bias values for the SPE method with AB-PINACA having the best bias value compared to all compounds and all sample preparation techniques.

For the synthetic cannabinoids, the LLE had the greatest between-run precision, comparatively, THC and its two metabolites had the greatest between-run precision for the SPE method. The SLE method had the worst between-run precision for THC, 11-hydroxy-THC, AB-FUBINACA, and AB-PINACA, while the best between-run precision for AB-

FUBINACA metabolite 3 was using the SLE method. Both the LLE and SPE had similar between-run values for all compounds.

Looking at the within-run precision THC, 11-hydroxy-THC, and AB-FUBINACA had the best values for the SPE method. The LLE method had the best within-run precision for THCCOOH, while AB-FUBINACA metabolite 3 and AB-PINACA had the greatest within-run precision for the SLE method. All bias and precision values fell well within the accepted ASB guidelines of $\pm 20\%$, with all values falling within $\pm 10\%$. With all these values falling within $\pm 10\%$ the LLE, SPE, and SLE are sound methods to be used for forensic toxicology analysis.

Table 6. Comparison of bias and precision for urine matrix in LLE, SPE, and SLE.

| Compound Name | Average Overall Bias (%) | | | Average Between-Run Precision (%CV) | | | Average Within-Run Precision (%CV) | | |
|-------------------|--------------------------|-------|-------|-------------------------------------|------|------|------------------------------------|------|------|
| | LLE | SPE | SLE | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | -9.62 | -2.42 | -1.16 | 4.28 | 3.88 | 9.32 | 2.55 | 2.21 | 5.69 |
| 11-hydroxy-THC | -7.62 | -2.28 | -1.51 | 5.10 | 4.71 | 6.37 | 3.20 | 2.86 | 2.93 |
| THCCOOH | -8.52 | -3.07 | -3.02 | 4.89 | 3.72 | 4.66 | 1.81 | 2.50 | 3.01 |
| AB-FUBINACA | -6.06 | -1.05 | -3.11 | 3.66 | 3.87 | 4.81 | 2.04 | 1.68 | 2.21 |
| AB-FUBINACA met 3 | -6.39 | -2.25 | -1.64 | 5.90 | 6.47 | 5.55 | 3.06 | 4.10 | 2.36 |
| AB-PINACA | -2.60 | -0.61 | -2.68 | 6.53 | 6.75 | 7.08 | 4.10 | 5.04 | 4.01 |

3.2.3 Analyte Recovery

Recovery analysis was completed for each analyte over all three sample preparation techniques. A low concentration of 10 ng/mL and a high concentration of 30 ng/mL were

assessed for this study (Table 7) using 10 different lots of urine. The analyte with the lowest recovery was THC, with a recovery of around 25% for both the SPE and SLE methods, while the LLE method had a recovery around 45%. This low level of recovery is especially interesting for the SPE method because the SPE columns used were designed specifically for the extraction of THC. The transfer of the sample from different test tubes and vials during each method could result in a lower recovery for THC due to compound adhering to glass and not all sample being transferred to the next test tube. For the 11-hydroxy-THC and all the synthetic cannabinoids, the LLE method had a recovery of greater than 100%, with AB-FUBINACA having the greatest recovery average of 114.16%, while THCCOOH had a recovery averaging 99.72%. Compared to the SPE and SLE methods, the lower concentration studied had a greater recovery for all compounds, while the higher concentration had greater recovery for the SLE method. All analytes except THC had a higher recovery for the higher concentration studied for the SPE method. For all analytes, the SPE method had the lowest recovery, with each analyte other than THC having a recovery around 10 – 15% less than the SLE method. The synthetic cannabinoids had a recovery ranging from 76.68 – 82.06% when using the SLE method, while 11-hydroxy-THC and THCCOOH had a recovery ranging from 55.92 – 65.94% using the SLE method. The analyte with the greatest recovery for the LLE method was AB-FUBINACA, while the SPE and SLE analyte with the greatest recovery was AB-PINACA.

Table 7. Analyte recovery for urine. Two concentrations were assessed, 10 ng/mL and 30 ng/mL. Both are shown below along with a comparison of all three sample methods for each analyte.

| Compound Name | Recovery for 10 ng/mL (%) | | | Recovery for 30 ng/mL (%) | | |
|-------------------|---------------------------|-------|-------|---------------------------|-------|-------|
| | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | 52.21 | 25.5 | 25.98 | 43.47 | 21.24 | 28.64 |
| 11-hydroxy-THC | 109.86 | 47.2 | 60.82 | 98.74 | 49.16 | 65.94 |
| THCCOOH | 107.46 | 44.68 | 55.92 | 91.98 | 45.82 | 61.36 |
| AB-FUBINACA | 119.43 | 61.08 | 76.68 | 108.89 | 61.72 | 81.80 |
| AB-FUBINACA met 3 | 118.04 | 50.47 | 79.20 | 106.78 | 54.65 | 79.32 |
| AB-PINACA | 111.35 | 62.97 | 79.52 | 105.43 | 66.64 | 82.06 |

3.2.4 Limit of Detection and Limit of Quantitation

The limit of detection was less than 1 ng/mL for most of the compounds for all three sample preparation techniques (Table 8). AB-FUBINACA had consistent LOD (0.01 ng/mL) between the different sample preparation methods. Concentration values were the same between the LLE and SPE techniques for THCCOOH and AB-FUBINACA metabolite 3, 0.25 ng/mL and 0.1 ng/mL respectively, while AB-PINACA had the same value, 0.1 ng/mL, for the SPE and SLE techniques. THC had a lower LOD (0.25 ng/mL) using the LLE sample preparation technique while 11-hydroxy-THC had a lower LOD (0.25 ng/mL) for the SLE. The SPE method had the highest LOD for THC and 11-hydroxy-THC with a value of 1 ng/mL.

AB-FUBINACA was the only analyte that was able to reach the initial goal of 0.01 ng/mL for a level of detection for each of the three sample preparation techniques. AB-PINACA was able to reach 0.01 ng/mL for the LLE method, while AB-FUBINACA metabolite 3 was able to reach 0.01 ng/mL of the SLE method. THCCOOH had the lowest LOD, reaching 0.1 ng/mL for the SLE method, and 0.25 ng/mL for the LLE and SPE

method. The lowest concentration able to be detected for both THC and the first metabolite of THC, 11-hydroxy-THC, was 0.25 ng/mL. The SLE method had the lowest LOD for THCCOOH, 11-hydroxy-THC and AB-FUBINACA metabolite 3. The LLE method had the lowest LOD for THC and AB-PINACA.

Table 8. Limit of detection for urine. A comparative look at the LLE, SPE, and SLE sample preparation techniques for the limit of detection.

| Compound Name | LOD values (ng/mL) | | |
|-------------------|--------------------|------|------|
| | LLE | SPE | SLE |
| THC | 0.25 | 1 | 0.5 |
| 11-hydroxy-THC | 0.5 | 1 | 0.25 |
| THCCOOH | 0.25 | 0.25 | 0.1 |
| AB-FUBINACA | 0.01 | 0.01 | 0.01 |
| AB-FUBINACA met 3 | 0.1 | 0.1 | 0.01 |
| AB-PINACA | 0.01 | 0.1 | 0.1 |

When analyzing the LOQ for each compound a %CV of $\pm 20\%$ for the calculated concentration was needed to state a LOQ value for that compound. The lowest LOQ for each compound was determined to be 1 ng/mL for the LLE and SPE sample preparation methods. A lower LOQ was determined for each analyte using the SLE method (Table 9). AB-PINACA was the exception in that the lowest LOQ of 1 ng/mL was the same for all three sample preparation techniques. For the SLE method, 11-hydroxy-THC and THCCOOH had the lowest LOQ of all compounds at 0.5 ng/mL. THC, AB-FUBINACA and AB-FUBINACA met 3 had an LOQ of 0.75 ng/mL for the SLE method. For AB-FUBINACA and AB-PINACA, values such as 0.25 ng/mL and 0.5 ng/mL could sometimes be accurately calculated using the calibration curve for the run, however the % CV was always greater than the allowed 20%. Therefore, these values could not be determined as

the lower LOQ for the LLE, SPE, and SLE sample preparation techniques, even though the LOD was 0.01 ng/mL for AB-FUBINACA and 0.1 ng/mL or 0.01 ng/mL for AB-PINACA depending on the sample preparation technique. Combining the bias studies for the LLE in which bias was between 5% and 10% for most compounds, with the LOQ, having results in which 1 ng/mL were the lowest LOQ is reasonable due to the accuracy for the linear dynamic range being higher, therefore, concentrations that are on the lower end will have a greater % CV because the repeatability and reproducibility are not as anticipated. The SPE method having a LOQ of 1 ng/mL is not as expected due to the fact that the bias and precision for each compound was around 5%.

Table 9. Limit of quantitation for urine. A comparative look at all three sample preparation techniques for each analytes LOQ value.

| Compound Name | LOQ values (ng/mL) | | |
|-------------------|--------------------|-----|------|
| | LLE | SPE | SLE |
| THC | 1 | 1 | 0.75 |
| 11-hydroxy-THC | 1 | 1 | 0.5 |
| THCCOOH | 1 | 1 | 0.5 |
| AB-FUBINACA | 1 | 1 | 0.75 |
| AB-FUBINACA met 3 | 1 | 1 | 0.75 |
| AB-PINACA | 1 | 1 | 1 |

3.2.5 Other Validation Parameters

For all methods, each analyte signal was suppressed by the matrix (Table 10). The same two concentrations as recovery analysis were used for this study. The THC compounds and the synthetic cannabinoids were the least suppressed using the SLE

method. For all methods, suppression was the least for the synthetic cannabinoids. THC was the most suppressed using the LLE method, with the SPE and SLE having similar suppression results for THC. The SLE method was the most successful at removing components that affected suppression.

There was no carryover present for any of the analytes of interest during any of the validation studies for any of the methods.

Table 10. Ion suppression or enhancement study results for urine.

| Compound Name | 10 ng/mL (%) | | | 30 ng/mL (%) | | |
|-------------------|--------------|--------|--------|--------------|--------|--------|
| | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | -41.06 | -36.11 | -26.76 | -50.71 | -25.23 | -28.79 |
| 11-hydroxy-THC | -23.21 | -29.18 | -19.78 | -35.12 | -21.19 | -20.82 |
| THCCOOH | -34.52 | -34.91 | -18.04 | -43.09 | -26.94 | -20.80 |
| AB-FUBINACA | -19.07 | -25.37 | -3.54 | -29.03 | -15.56 | -10.73 |
| AB-FUBINACA met 3 | -16.36 | -27.13 | -4.77 | -23.41 | -17.25 | -5.37 |
| AB-PINACA | -14.83 | -18.12 | 1.81 | -24.20 | -10.77 | -5.15 |

For this study these drugs were broken down into three different mixes (Table 11). These compounds range from barbiturates, benzodiazepines, amphetamines, designer drugs, hallucinogens, opioids, analgesics, depressants, and novel psychoactive substances. For all 33 drugs studied as potential interferents, there was no interference present for any of the six analytes of interest or any of the internal standards.

Another form of interference is from the matrix itself. Like the potential drug interferences, the components found in urine, such as the urea, creatinine, potassium and sodium ions to name a few, may interfere with the analytes of interest causing either suppression or enhancement of the analyte, along with the potential to mask the analyte

signal as well. The urine study showed matrix interferences that were less than 10% the area of the lowest calibrator for urine. However, due to the suppression levels, interferences from the matrix were impeding the analysis of these compounds.

The last forms of interference assessed looked inward on the study to see if the internal standards interfere with the results in any way and if the analytes interfered with the internal standards. These results are important due to the analyte concentration being determined by the ratio of quant ion peak area to internal standard peak area. To assess if analyte effected the internal standard peak signal the highest calibrator was spiked into the urine and was extracted, however, no internal standard was added. For each analyte, there was no interference with the internal standards. To analyze if the analyte's internal standard effected the analyte peak signal, blanks were run, in which the matrix was spiked with internal standard solution only. All analytes except AB-PINACA did not have interference with their respective internal standards. For AB-PINACA, AB-PINACA-d9 (the AB-PINACA internal standard) did cause a peak in the blank samples. This signal was large enough that during the LOD studies, the peak area for the internal standard was the same as that of 0.01 ng/mL of AB-PINACA for the SPE and SLE methods, therefore the LOD was set at 0.1 ng/mL. For the LLE method, the internal standard interference was not as great allowing for the LOD to be 0.01 ng/mL for AB-PINACA. Due to this interference, for future studies, decreasing the concentration of AB-PINACA-d9 from 25 ng/mL would be advised. No other internal standard interferences were present.

Dilution integrity evaluated the capability of taking a sample which is too concentrated and diluting down, while still getting accurate calculated concentrations.

These studies looked at a dilution factor of 1:10 and of 1:50, determined their overall bias and precision, and established if samples may be diluted by a factor of 1:10 and 1:50 if needed. All analytes are able to be diluted by a factor of 1:10 or 1:50.

The final parameter assessed was the processed sample stability, assessing up to 72 hrs for each analyte. All methods were stable up to 72 hrs for each of the six analytes of interest.

Table 11. Potential drug interference mixes. The three mix groups and the different potential drugs that would cause an interference that are commonly encountered during case work. Each drug had a final concentration of 200 ng/mL in the matrix of interest.

| | |
|-----------|--|
| Mix 1 | Alpha-hydroxyalprazolam |
| | Clonazepam |
| | 7-aminoclonazepam |
| | Diazepam |
| | Etizolam |
| | Codeine |
| | Hydrocodone |
| | Methadone |
| | Morphine |
| | 6-monoacetylmorphine |
| | Fentanyl |
| | Oxycodone |
| | Benzoylcegonine |
| | Cocaine |
| | Norcocaine |
| Lidocaine | |
| Mix 2 | Amobarbital |
| | Butalbital |
| | Phenobarbital |
| Mix 4 | Amphetamine |
| | Methamphetamine |
| | 3,4-methylenedioxy-amphetamine (MDA) |
| | 3,4-methylenedioxy-N-ethylamphetamine (MDEA) |
| | 3,4-methylenedioxy-methamphetamine (MDMA) |
| | Ethylone |
| | Alpha-PVP |
| | 25I-NBOMe |
| | Amitriptyline |
| | Citalopram |
| | Fluoxetine |
| | Trazadone |
| | Phencyclidine (PCP) |
| | Lysergic acid diethylamide (LSD) |

3.3 Blood Results

3.3.1 Calibration Range, Bias and Precision

A r^2 value and a residuals plot were determined and created for each compound for each sample preparation technique. The r^2 values needed to be 0.99 or greater (Table 12). The LLE method had the greatest r^2 value for AB-FUBINACA metabolite 3 and THC. 11-hydroxy-THC had the greatest value using the LLE and the SLE methods. While THCCOOH, AB-FUBINACA, and AB-PINACA had greater r^2 values for the LLE and SPE methods. Similar to the urine method validation the residuals plots for THC, 11-hydroxy-THC, and THCCOOH represented a linear range from 1 ng/mL to 50 ng/mL for all three sample preparation methods. Also like the urine data the LLE residuals plots for AB-FUBINACA, AB-FUBINACA metabolite 3, and AB-PINACA, and the residuals plots for AB-FUBINACA and AB-PINACA using the SLE method were beginning to resemble that of a quadric curve with the concentration range of 1 ng/mL to 50 ng/mL. The main difference between the urine and blood results was in that the synthetic cannabinoids residuals plots for the SPE method, and AB-FUBINACA metabolite 3 for the SLE method did not resemble a quadric curve (Figure 6). The scattered look of the residuals values for each calibration point around the zero value represents that of a linear curve, while the LLE and SLE plots for the synthetic cannabinoids residual values plotted against the concentration form more of a U-shape, resembling the beginning of a quadratic curve. With the r^2 values being 0.995 or greater for all analytes over all three sample preparation techniques, using a linear curve fit is acceptable. Future studies of synthetic cannabinoids

may be best to analyze with an upper calibration point of 40 ng/mL or less. These values still fall above or near to what is typically reported for these compounds in the human body.

Table 12. r^2 values for blood.

| Compound Name | r^2 values | | |
|-------------------|--------------|-------|-------|
| | LLE | SPE | SLE |
| THC | 0.998 | 0.997 | 0.997 |
| 11-hydroxy-THC | 0.998 | 0.997 | 0.998 |
| THCCOOH | 0.999 | 0.999 | 0.998 |
| AB-FUBINACA | 0.999 | 0.999 | 0.997 |
| AB-FUBINACA met 3 | 0.999 | 0.998 | 0.996 |
| AB-PINACA | 0.998 | 0.998 | 0.995 |

For the bias studies completed on the blood matrix (Table 13), the SPE method had the best accuracy for all compounds except AB-FUBINACA metabolite 3. The SLE method was the most accurate for AB-FUBINACA metabolite 3, and more accurate compared to the LLE method for THC and THCCOOH. Except for analysis of AB-FUBINACA metabolite 3 and THCCOOH utilizing the LLE method and analysis of AB-PINACA utilizing the SLE method, all bias calculations were within $\pm 5\%$, with the above three situations having a bias within $\pm 7\%$. These values are all well within the $\pm 20\%$ validation criteria set by the ASB Guidelines. All further studies were not completed for blood analysis using the SLE method due to poor recovery of internal standards and analytes of the synthetic cannabinoids. Developing a new SLE method for the analysis of these compounds is needed to complete the study.

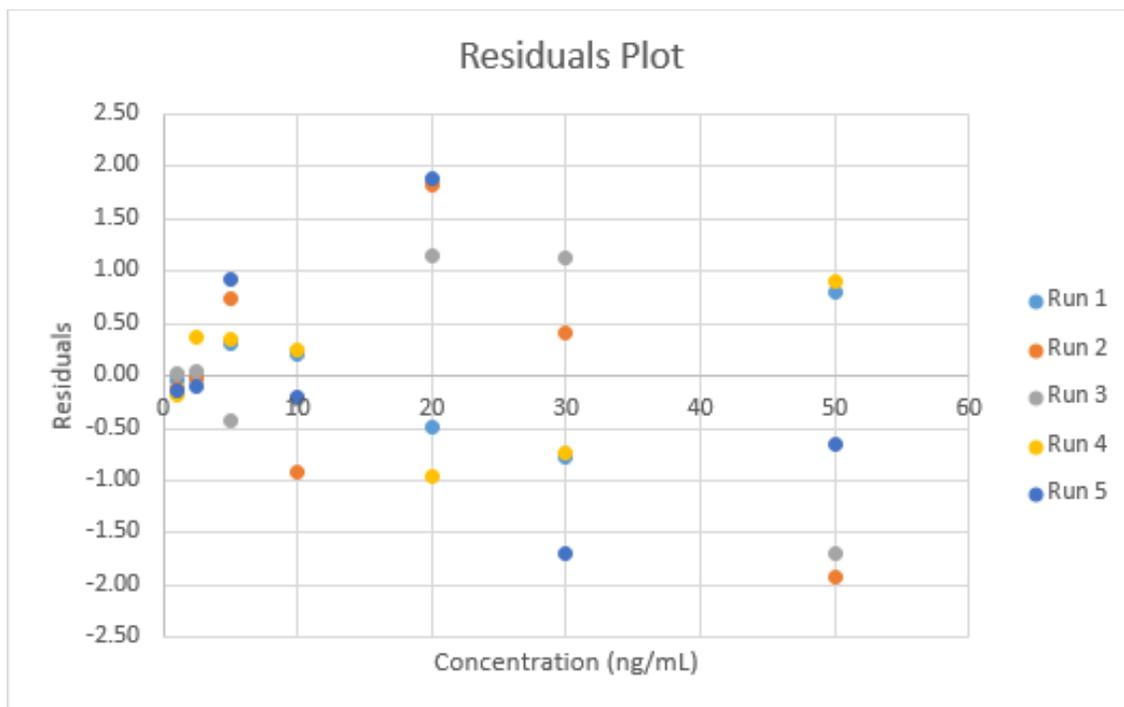


Figure 6. Residuals plot for AB-PINACA in blood using the SPE method. Unlike the LLE and SLE methods for blood, and the LLE, SPE, and SLE methods for urine, the AB-PINACA residuals plots were not beginning to resemble that of a quadric curve, but represented that of the expected plot of a linear curve with the residual values being scattered around the zero value for each calibration point.

Table 13. Comparison of bias and precision for blood matrix in LLE, SPE, and SLE.

| Compound Name | Average Overall Bias (%) | | | Average Between-Run Precision (%CV) | | | Average Within-Run Precision (%CV) | | |
|-------------------|--------------------------|-------|-------|-------------------------------------|------|-------|------------------------------------|------|------|
| | LLE | SPE | SLE | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | -3.28 | -1.33 | -2.90 | 8.46 | 7.27 | 11.90 | 5.53 | 5.08 | 6.87 |
| 11-hydroxy-THC | -2.69 | -0.04 | -2.82 | 5.39 | 7.11 | 6.63 | 4.18 | 5.58 | 5.17 |
| THCCOOH | -5.83 | -3.53 | -3.77 | 7.24 | 6.65 | 7.76 | 3.73 | 3.07 | 4.97 |
| AB-FUBINACA | -3.49 | -0.69 | -3.97 | 4.22 | 3.76 | 3.86 | 1.78 | 2.14 | 2.61 |
| AB-FUBINACA met 3 | -6.67 | -1.17 | -0.84 | 7.84 | 6.75 | 11.01 | 2.91 | 6.75 | 7.48 |
| AB-PINACA | -0.70 | -0.21 | -5.37 | 4.86 | 5.86 | 6.30 | 3.74 | 4.04 | 4.41 |

3.3.2 Analyte Recovery in Blood Matrix

Similar to urine, 10 different negative lots of blood were analyzed at two different concentrations, 10 ng/mL and 30 ng/mL, for the recovery study (Table 14). The recovery for the THC compounds using the SPE method was below 10% recovery. The parent compound THC had a recovery that averaged 1.18% between the two concentrations, with the low concentration of 10 ng/mL having a recovery of 0.65%. LLE method had a recovery for THC that averaged 8.8%, and like the SPE method had a recovery value below 10%. The two THC metabolites, 11-hydroxy-THC and THCCOOH, had greater recovery compared to the SPE method, with recovery values around 32% and 23% respectively. The synthetic cannabinoids had similar recovery values for both the LLE and SPE methods, with AB-PINACA having the highest recovery out of all analytes. The recovery was greatest for AB-PINACA using the LLE method, with a recovery averaging 64.5% while

the analyte recovery using the SPE method was 62%. Like AB-PINACA, AB-FUBINACA metabolite 3 had greater recovery utilizing the LLE method, with an average recovery of 56.7%, while AB-FUBINACA had a greater recovery utilizing the SPE method for an average recovery of 59.2%. Recovery was similar between both concentrations with the higher concentration having slightly higher recovery.

Table 14. Analyte recovery for blood. Two concentrations were analyzed for recovery analysis, a low concentration of 10 ng/mL and a high concentration of 30 ng/mL.

| Compound Name | Recovery for 10 ng/mL (%) | | | Recovery for 30 ng/mL (%) | | |
|-------------------|---------------------------|-------|-----|---------------------------|-------|-----|
| | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | 8.53 | 0.65 | - | 9.18 | 1.71 | - |
| 11-hydroxy-THC | 31.54 | 4.14 | - | 34.78 | 6.32 | - |
| THCCOOH | 23.34 | 3.93 | - | 24.83 | 8.02 | - |
| AB-FUBINACA | 52.57 | 61.25 | - | 58.71 | 57.17 | - |
| AB-FUBINACA met 3 | 53.08 | 43.68 | - | 60.43 | 60.58 | - |
| AB-PINACA | 59.66 | 56.57 | - | 69.39 | 67.43 | - |

3.3.3 LOD and LOQ for Blood Matrix

For the blood matrix, there was variation within the LOD results for the two studied methods (Table 15). For THC and 11-hydroxy-THC, the LLE method had a much lower LOD compared to the SPE method. Utilizing the SPE method for analysis of THC and 11-hydroxy-THC, the LOD was determined to be the same as the lowest calibrator, 1 ng/mL, for the linear dynamic range, while also being the same as the lower LOQ value. For the THC compounds using the LLE method of analysis, THC and THCCOOH had a lower LOD of 0.25 ng/mL compared to 11-hydroxy-THC, which had a LOD of 0.5 ng/mL. Compared to THC and 11-hydroxy-THC, AB-PINACA had a lower LOD with the SPE

method. The SPE method reached the initial goal of detection of 0.01 ng/mL. Assessing THCCOOH, AB-FUBINACA, and AB-FUBINACA metabolite 3, the LOD was the same for both the LLE and SPE methods. The LLE and SPE methods for blood results for the detection level mirror that of the urine results for all compounds except AB-PINACA. in which the LLE method has a lower detection level of 0.01 ng/mL while the SPE has a detection level of 0.1 ng/mL. Compared to the urine analysis for AB-PINACA, the blood results are opposite with the SPE method having a lower LOD of 0.01 ng/mL and the LLE detection limit being 0.1 ng/mL.

Moving to the LOQ analysis, the LOQ determined for all six analytes using both the LLE and SPE method as the mode of analysis, the LOQ value was determined to be 1 ng/mL which is the lowest calibrator in the linear dynamic range (Table 16). These results align with the SPE method for the analysis of THC, 11-hydroxy-THC, and THCCOOH considering the recovery of these compounds is below 10% for each analyte. With low recovery the amount of analyte reaching the detector creating a signal is decreased, therefore signal intensity is less and the likelihood of an accurate quantitative result for such low levels is reduced. For the LLE method as well, the recovery is below 35% for all THC compounds, creating a reduced chance of enough signal to accurately calculate the concentration at low levels. The LOQ of 1 ng/mL is unexpected for the synthetic cannabinoids for both sample preparation methods considering recovery was above 50% and a detection level of 0.01 ng/mL or 0.1 ng/mL was determined. However, at these lower concentrations such as 0.75 ng/mL or 0.5 ng/mL the %CV was always greater than the

allotted $\pm 20\%$, therefore quantitation is not possible. These quantitation results mirror that of the urine results for the LLE and SPE methods.

Due to recovery studies determining the method developed for the SLE technique was not adequate for the analysis of the synthetic cannabinoids no LOD or LOQ results were performed.

Table 15. Limit of detection for blood. Comparative look at the LLE, SPE, and SLE values for limit of detection.

| Compound Name | LOD values (ng/mL) | | |
|-------------------|--------------------|------|-----|
| | LLE | SPE | SLE |
| THC | 0.25 | 1 | - |
| 11-hydroxy-THC | 0.5 | 1 | - |
| THCCOOH | 0.25 | 0.25 | - |
| AB-FUBINACA | 0.01 | 0.01 | - |
| AB-FUBINACA met 3 | 0.1 | 0.1 | - |
| AB-PINACA | 0.1 | 0.01 | - |

Table 16. Limit of quantitation for blood.

| Compound Name | LOQ values (ng/mL) | | |
|-------------------|--------------------|-----|-----|
| | LLE | SPE | SLE |
| THC | 1 | 1 | - |
| 11-hydroxy-THC | 1 | 1 | - |
| THCCOOH | 1 | 1 | - |
| AB-FUBINACA | 1 | 1 | - |
| AB-FUBINACA met 3 | 1 | 1 | - |
| AB-PINACA | 1 | 1 | - |

3.3.4 Ion Suppression, Carryover, Dilution Integrity, Processed Stability

All analytes of interest were suppressed using the LLE and SPE methods for extraction purposes (Table 17). The amount of suppression was greater with the SPE

method allowing for interpretation that the SPE method did not adequately remove and clean up the blood samples. This data is counter intuitive given the principle behind SPE is to produce cleaner extracts compared to LLE. The final extracts supplied enough of a hampering effect on the analyte signal to create large suppression effects on each analyte. Suppression was greatest for THC and 11-hydroxy-THC when the LLE method was used, with THCCOOH resulting in similar suppression as the synthetic cannabinoids for the LLE method. Utilizing the SPE method, the suppression for all three THC compounds was the greatest. AB-FUBINACA metabolite 3 had the least ion suppression for the LLE method while looking at all six compounds, while, when utilizing the SPE method the ion suppression resembled that more of the THC compounds. AB-FUBINACA had the least ion suppression for the SPE method. The synthetic cannabinoids had the least amount of suppression of all analytes.

As per the urine studies, no carryover was present for any analyte using any of the three sample preparation techniques.

When a sample has too great a concentration, the sample may be diluted up to a factor of 1:50. These dilution integrity studies were performed at a dilution factor of 1:10 and 1:50, while the bias and precision were determined for each dilution factor. Both parameters were required to fall within $\pm 20\%$, which was accomplished at both 1:10 and 1:50 dilution factors.

Table 17. Ion suppression for blood.

| Compound Name | 10 ng/mL (%) | | | 30 ng/mL (%) | | |
|-------------------|--------------|--------|-----|--------------|--------|-----|
| | LLE | SPE | SLE | LLE | SPE | SLE |
| THC | -49.49 | -58.59 | - | -39.42 | -51.49 | - |
| 11-hydroxy-THC | -29.29 | -48.56 | - | -22.28 | -47.17 | - |
| THCCOOH | -41.72 | -56.01 | - | -31.55 | -53.12 | - |
| AB-FUBINACA | -27.08 | -31.52 | - | -12.59 | -22.32 | - |
| AB-FUBINACA met 3 | -19.55 | -41.53 | - | -6.75 | -34.11 | - |
| AB-PINACA | -24.00 | -32.50 | - | -18.72 | -29.22 | - |

For processed stability studies, THCCOOH, AB-FUBINACA, and AB-PINACA were all stable for 72 hrs for both the LLE and SPE method. THC and 11-hydroxy-THC were stable for 72 hrs using the SPE method. AB-FUBINACA metabolite 3 was stable for 72 hrs using the LLE method. The shorter stability of 48 hrs for THC and 11-hydroxy-THC using the LLE method, and AB-FUBINACA metabolite 3 using the SPE method all fell just outside the $\pm 20\%$ ratio range of the quant ion:internal standard ion.

3.3.5 Interferences

Using 33 different drugs placed in three different mixes, interference studies were completed (Table 11). As with urine, no interferences were caused by any of these analytes while utilizing any of the sample preparation techniques. The highest calibrator did not interfere with the internal standard and all internal standards but AB-PINACA did not interfere with the analytes. As with urine, the AB-PINACA internal standard caused a peak signal for AB-PINACA analyte. This had the most bearing on the LOD, in which the area for the peak at 0.01 ng/mL for the LLE method was the same as the area for the internal standard in the sample spiked only with internal standard. Therefore, the LOD was

determined to be 0.1 ng/mL. While the internal standard did create a peak in the blank sample spiked with internal standard for the SPE method, the peak area was smaller than that of the 0.01 ng/mL peak allowing for a detection level of 0.01 ng/mL to be set.

For matrix interference studies, all analytes had interferences less than 10% the average area of the lowest calibrator. One issue with completing the blood validation was acquiring negative blood samples for validation analysis. All blood lots were screened by gas chromatography – mass spectrometry (GC/MS) running a full screen, monitoring for any analytes present in the blood. A targeted analysis using the LC-MS/MS was then completed due to the lower LODs associated with targeted analysis. Due to the LLE method validation occurring first, all blood lots were screened using this method for a consistent screening process throughout the study. The most commonly encountered substances in the blood screen was THCCOOH and an endogenous peak with the same retention time. The endogenous interference was a large peak at 3.49 min that typically appeared with a twin peak at a retention time of 3.58 min (Figure 7). Blood lots containing an endogenous peak or THCCOOH were discovered in approximately half of the blood lots screened.

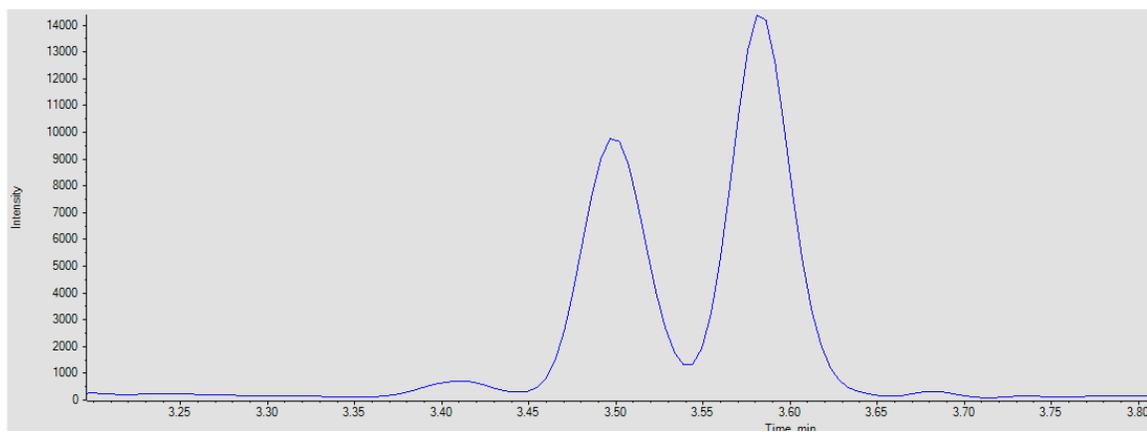


Figure 7. Negative blood sample with THCCOOH interference. The peak at 3.50 min is the same retention time as THCCOOH, therefore creating an unusable blood sample.

4. DISCUSSION

4.1 Method Development

To be able to compare each sample preparation technique to one another the amount of sample was held constant for all three sample preparation techniques. When determining if the same sample preparation method could be used for both blood and urine the matrix volume was kept the same along with keeping all steps the same between the two matrices. All method development began with blood as the matrix of interest due to the complexities of blood compared to urine. Once the method had been developed with blood the method was then transferred to urine.

This process was acceptable for developing methods for two of the three sample preparation methods. When developing the SLE method, this process yielded undesirable results. Analysis of validation parameters began with calibration model, bias, and precision, in which all six analytes had acceptable residual plots, r^2 values, bias percentages, and precision values. When moving to analyte recovery studies, the recovery results for AB-FUBINACA metabolite 3 were inconsistent between the 10 different blood lots. Matrix effects on AB-FUBINACA metabolite 3 were more significant for blood lots that had been stored in a refrigerator or freezer for longer periods of time or older blood samples. Due to AB-FUBINACA metabolite 3 not being determined to be a metabolite found in blood and only urine, the compound was removed from the analyzed compounds for blood. When further recovery studies were completed for the SLE method on blood the recovery of the ratio between the analyte ions and the internal standard ions for the synthetic cannabinoids was not the same as the neat solutions, causing the recovery studies

to fail for AB-FUBINACA and AB-PINACA. With this development it was determined that analysis on blood required a new SLE method. All parameters for urine were completed.

When developing each method, literature reviews supplied methods for the extraction of THC, 11-hydroxy-THC, and THCCOOH together and methods for the extraction of synthetic cannabinoids, while finding methods that extracted THC compounds and synthetic cannabinoids together in one method were not found or published. As a result, methods were typically developed around extraction of THC and its metabolites due to these compounds having lower signal intensity and the synthetic cannabinoids more readily extracting from the matrices.

4.1.1 Liquid-Liquid Extraction

The initial method development began with 200 μ L of blood. The first trials also began with 10% glacial acetic acid and a hexane:ethyl acetate (90:10) organic solvent ⁴⁹. These samples were reconstituted in 100 μ L of 0.1% formic acid in DI water after the organic layer was removed and dried down. Analysis on a Kinetex® F5, 2.6 μ m 100 Å, 50 x 3.0 mm column (Phenomenex, Torrance, CA, USA) showed the majority of the analytes of interest were not extracted from the matrix. AB-FUBINACA, AB-PINACA and AB-FUBINACA metabolite 3 were the only analytes that were extracted and able to be viewed on the chromatogram. All the working stock solutions were run as neat solutions to ensure all compounds were present and analysis showed a chromatogram with all analytes, confirming that the initial extraction method only extracted three analytes. Next steps

included increasing the acidity by using 20% glacial acetic acid, using 100% hexane, 100% dichloromethane (DCM), and 90:10 hexane:ethyl acetate. Once the organic layer was removed and dried down, all samples were reconstituted in 0.1% formic acid in DI water:ACN (70:30), due to this ratio being the starting mobile phase conditions. The 100% hexane trial did not extract THCCOOH. Using 20% glacial acetic acid and 90:10 hexane:ethyl acetate extracted all compounds; however, the signal intensity was weak. All analytes were extracted using 100% DCM and 20% glacial acetic acid, however there was significant peak tailing. Using the 100% DCM and 20% glacial acetic acid method, another trial was run on both blood and urine and the XBridge® C18 column (Waters Corporation, Milford, MA, USA) was used, which gave Gaussian peaks and better resolution of 11-hydroxy-THC and THCCOOH, while the resolution of AB-PINACA and AB-FUBINACA metabolite 3 was decreased as compared to the F5 column. However, the lower concentration levels had weak peak signal intensity. Another drawback using DCM was the organic layer was the bottom layer, therefore removal of the organic layer required the pipette tip moving through the blood layer, creating a final organic solution with residual amounts of blood in every sample. To remove the issue with DCM being the bottom layer and creating better extraction results of the compounds at lower levels, the acid was changed and the ratio of hexane:ethyl acetate was adjusted. For these trials 5% phosphoric acid was also evaluated⁵⁰ along with 90:10 hexane:ethyl acetate and 80:20 hexane:ethyl acetate. These trials had varying results with accuracy of the synthetic cannabinoids and which THC compounds were extracted. A literature search revealed a multitude of methods using 500 to 1,000 µL of matrix for extraction and reconstituting in 100 µL of

reconstitution solution to increase the signal intensity of THC and its metabolites. The volume of blood and urine was then increased to 400 μ L. The final method trials involved changing the organic solvent to include an ether, one trial involved diethyl ether while two other trials used MTBE⁵⁰⁻⁵² and evaluating 5% phosphoric acid and 5% glacial acetic acid. The diethyl ether did not extract THC or its metabolites, with the chromatograms resembling those of solvent blanks for the THC compounds. While both the phosphoric acid and glacial acetic acid extracted all compounds when combined with 80:10:10 hexane:ethyl acetate:MTBE the final method settled upon glacial acetic acid due to better signal for THC and its metabolites. If this study was focusing on synthetic cannabinoids only using phosphoric acid would be the better acid solvent due to greater signal intensity for the synthetic cannabinoids.

Due to the viscosity of the blood or urine being similar to the organic solvent, a freezing step was added before the organic layer was removed. This idea was adopted from The Center for Forensic Science Research and Education LLE method. By freezing the samples, the matrix layer would be either frozen solid or cold enough that it became more viscous allowing for removal of the organic layer to be more efficient. If any matrix was removed then pipetted back into the vial the emulsion that formed was decreased compared to no freezing step.

Comparing the blood and urine sample preparation, the urine samples took longer to freeze compared to the blood samples. While the freezing process was not included to increase the extraction efficiency but to increase the yield of organic layer removed while decreasing chances of matrix contamination while pipetting out the organic layer, the

freezing step may be removed or shortened to decrease the total time. Due to unexpected circumstances, blood samples were left in the freezer for 24 hrs before the organic layer was removed. These samples were then analyzed and the data interpreted. The calibration curve, three QCS, and other samples being analyzed passed for accuracy in calculating the expected concentration. Therefore, samples may be left in the freezer for 24 hrs before removing the organic layer and completing analysis.

4.1.2 Solid Phase Extraction

Styre Screen® THC columns were used for the SPE method. These columns were designed to be specific to THC, 11-hydroxy-THC, and THCCOOH. The columns were 3 mL columns with 60 mg sorbent bed composed of ultra clean, highly cross-linked styrene and divinylbenzene copolymer⁴⁸. The particle size ranged from 10 to 20 µm on average⁴⁸. The columns were designed to remove the conditioning step from a traditional SPE method, allowing the sample to be loaded directly onto the column. The developed method was modified from the application note, which was supplied along with the columns, for 6 mL columns with 100 mg sorbent bed⁴⁸. The matrix volume was reduced to 400 µL and the solvents were scaled appropriately according to the ratios used in the application note. The application note included a decanting step after the samples were centrifuged, which was removed for the final method due to the possibility of analyte loss during the transfer steps. Due to THC having an affinity to bind to glass, removing as many solvent transfer steps as possible increased the possibility of THC or any compound not to be left in a test tube and not extracted into the final solution that reached the LC autosampler.

4.1.3 Supported Liquid Extraction

For extraction, 1 mL Isolute® SLE+ columns from Biotage were used. The columns are composed of diatomaceous earth, silicon dioxide, and quartz ⁴³. Method development began with running trials of different application notes supplied by Biotage. The application notes focused on either extracting THC and its metabolites or extracting synthetic cannabinoids and their metabolites. Since SLE is built around the principles of LLE while adding a column to increase removal of matrix interferences and other interferences, the first step was to transfer the LLE method to the SLE columns. Using 1 mL columns, the initial volume of pretreated matrix could not exceed 1 mL. This was to not oversaturate the column which could lead to break through or decreased recovery due to matrix and interferences not binding to the column materials. Therefore, the 20% glacial acetic acid and DI water were combined into a 5% glacial acetic acid solution that the blood and urine were pretreated with. When the acid was added to the blood clots began to form which hindered the matrix from absorbing into the column beds and later hindered the absorption of the organic solvent into and through the column. Other trials were tried in which acid concentration was adjusted or the acid was changed. In the end, the samples were centrifuged causing the clots to sink to the bottom the of test tube and the supernatant could be removed.

The organic solvent was assessed to see if separating the three organic solvents, hexane, ethyl acetate and MTBE would increase recovery. A number of trials where 50:50 hexane:ethyl acetate followed by 100% MTBE were assessed. The final parameters for the method of 5% glacial acetic acid and 80:10:10 hexane:ethyl acetate: MTBE were chosen

due to these parameters having the best recovery of the THCCOOH and AB-FUBINACA metabolite 3 compounds, which were the analytes that were the most complicated analytes to extract.

4.1.4 Technique Comparison

Further comparison parameters include the amount of time each preparation technique required, the number of resources, the amount of solvents consumed, and the cost associated with supplies. Each technique required time spent moving through the method steps. The LLE method took the most amount of time, with the average number of samples taking 4 hrs from start of preparation to the samples being placed in the autosampler. The 4 hrs can be reduced by removing or decreasing the freezing time. The SPE method averaged 2 hrs for sample preparation while the SLE method consumed the least amount of time, taking 1 hr to complete.

When comparing the resources used, the preparation of mobile phases and reconstitution solution was excluded, due to their being no difference in volumes between the sample preparation techniques. The SPE method used the greatest number of different solvents with six. The LLE and SLE used less solvents, five for the LLE and four for the SLE. When totaling the solvent volumes consumed for a single sample the SLE method used the most solvent, with a total volume of 6.2 mL. The SPE used the least total volume of 4.5 mL of solvent during the extraction process. The LLE method was the least expensive technique, while the SLE and SPE methods were more expensive due to the cost of the columns. The SPE columns were the most expensive per column compared to the

SLE columns. Due to the solvents and glassware being similar between all three sample preparation techniques, the cost involved with purchasing these solvents, along with test tubes and vials did not influence the cost difference between all three methods analyzed.

5. CONCLUSIONS

LLE, SPE, and SLE methods were developed for the analysis and quantitation of THC, 11-hydroxy-THC, THCCOOH, AB-FUBINACA, AB-FUBINACA metabolite 3, and AB-PINACA. For the analysis of THC, synthetic cannabinoids, and their metabolites in urine all three sample preparation techniques were acceptable for forensic toxicology analysis. Looking at the validation parameters and time consumed, the preferred method for analysis is the SLE method. This is due to the reduced suppression effects, reduced matrix effects, the lower LOQ, and reduced sample preparation time. For blood, both the LLE and SPE method were acceptable for forensic toxicology analysis, however looking at validation parameters, the preferred method for blood analysis is the LLE method due to the increased analyte recovery, the lower number of different solvents, the reduced suppression and matrix effects. For the most cost-effective method with suitable results, the LLE method would be preferred for both urine and blood analysis due to.

6. FUTURE WORK

6.1 Additional Sample Preparation Techniques

One goal of this project was to use the same sample preparation method for both blood and urine. This was able to be accomplished for the LLE and SPE methods, however the SLE method was not adequate enough to validate the method for blood. A further study into creating a SLE method that can be used for both blood and urine in which both matrices are able to be fully validated. Work was begun to determine a SLE method in which blood could be fully validated and completing this work to ensure that this new method could be used for the analysis of THC, 11-hydroxy-THC, THCCOOH, AB-FUFINACA, AB-FUBINACA metabolite 3, and AB-PINACA in human whole blood and urine would fully complete this comparison of these various sample preparation techniques.

Looking into different SPE columns from UCT as well as from other companies, such as Waters, Biotage, and Tecan to see if better results are possible. Seeing if the addition of adding a phospholipid cartridge before using the SPE or SLE cartridges would be interesting to see if this could clean up the sample more and give a better limit of detection for the THC compounds. Also, looking into modifying the UCT Styre Screen® THC columns method to see if changing the sample pretreatment, the wash solution, or elution solution would aid in greater recovery of the THC compounds, especially since these columns are designed specifically for THC.

Another future project would be looking at a different LLE method to compare results. One method involves the DCM method mentioned above where the top layer is aspirated off matrix instead of pipetting through the matrix layer.

6.2 Additional Compounds

Adding in more metabolites of the synthetic cannabinoids such as AB-PINACA pentanoic acid and AB-FUBINACA metabolite 2a or metabolite 4. Including the analysis of newer synthetic cannabinoids found in case reports to determine if the three sample preparation methods also extract these compounds or if the sample preparation methods would need to be modified for analysis. Adding in additional phytocannabinoids, such as cannabidiol and cannabinol.

APPENDIX A: STANDARD REAGENTS

Table A. Certified Reference Material Lot Numbers. All reference material was purchased from Cayman Chemical.

| | | |
|-------------------------------------|-----------|------------|
| THC (1 mg/mL) | 0530060 | 0515871 |
| 11-hydroxy-THC (1 mg/mL) | 0504656 | 0504398 |
| THCCOOH (1 mg/mL) | 0497428 | - |
| AB-FUBINACA (1 mg/mL) | 0468160 | 0521258 |
| AB-FUBINACA metabolite 3 (1 mg/mL) | 0471112-9 | 0534117-2 |
| AB-PINACA (1 mg/mL) | 0468078 | 0534770 |
| THC-d3 (100 µg/mL) | 0481952 | 0481952 |
| 11-hydroxy-THC-d3 (100 µg/mL) | 0504666 | - |
| 11-nor-9-caborxy-THC-d3 (100 µg/mL) | 0428976-7 | 0428976-10 |
| AB-FUBINACA-d4 (1 mg/mL) | 0516865-1 | - |
| AB-PINACA-d9 (1 mg/mL) | 0514592-1 | - |

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CURRICULUM VITAE



