ANALYSIS AND DISTRIBUTION OF THC, 11-OH-THC AND THC-COOH IN POSTMORTEM FLUIDS AND TISSUES

By

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Abstract: Marijuana is the most commonly abused illicit drug worldwide. The psychoactive compound Δ^9-tetrahydrocannabinol (THC) can be found in *Cannabis sativa* and provides a euphoric feeling, distorted sense of time, and loss of inhibition. Numerous studies have also looked at THC impairment of memory, cognitive skills, and psychomotor skills. The Federal Aviation Administration's Civil Aerospace Medical Institute (CAMI) conducts toxicological analysis on aviation fatalities. Due to the severe trauma to the body associated with aviation accidents, the laboratory relies on tissues only for analysis in approximately 40% of cases. The purpose of this research is twofold: to develop a sensitive and robust method using liquid chromatography-tandem mass spectrometry to detect THC and its metabolites 11-OH-THC and THC-COOH in postmortem fluids and tissues; and to determine if there is a postmortem fluid or tissue that can be used as an interpretive aid when blood is not available. The analytical method was validated following SWGTOX guidelines and was used for the analysis of postmortem fluids and tissues from 11 aviation fatalities that had been previously found positive for cannabinoids by GC/MS. Specimens analyzed, when available, included: blood, urine, bile, vitreous humor, brain, lung, liver, kidney, spleen, muscle, and heart. The results of this study showed no consistent distribution of any of the analytes between blood and any other fluid or tissue. The specimens with the highest concentrations cannabinoids are blood, urine, lung, and kidney.

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CHAPTER I

INTRODUCTION

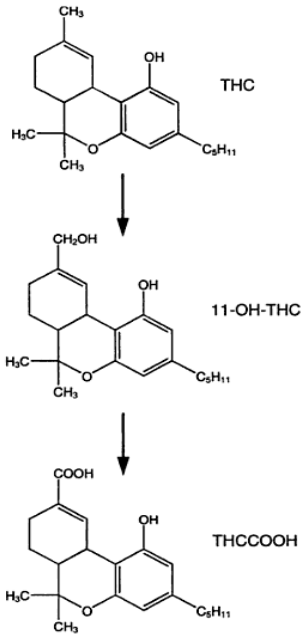
Approximately 147 million individuals worldwide currently use cannabis, making it the most used drug of abuse.[1] In fact, a survey conducted in 2010 showed that 6.9% of individuals 12 and older in the United States used marijuana in the preceding month.[2] The term marijuana can refer to the leaves, flowers, buds and/or stems that come from the plant *Cannabis sativa.* The main active compound in marijuana is Δ^9-tetrahydrocannibinol (THC). THC is rapidly metabolized to an equipotent psychoactive metabolite, 11-hydroxy-tetrahydrocannabinol (11-OH-THC), which is then metabolized to the inactive metabolite 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) (see Figure 1).[3] Because THC is a lipophilic compound, when it is smoked it quickly distributes throughout the blood and into the brain, giving the user a feeling of euphoria.[3]

An increasing number of states are now legalizing marijuana use, both recreationally and medicinally. However, THC has been shown to have detrimental effects on psychomotor skills, which is exemplified in numerous motor vehicle and aircraft simulator studies.[2,4,5,6] One study concluded that individuals who operate motor vehicles while under the influence have a 2-fold higher risk of being involved in an accident.[7] Another study compared ethanol ingestion with

marijuana smoking; they found a similar degree of both perceived impairment and actual impairment.[7]

With this in mind, there is an obvious need for a more comprehensive understanding THC and its metabolites within the body. Numerous studies have shown the THC and metabolite blood concentrations and impairment in antemortem samples; however, few studies have investigated the postmortem aspects of THC, such as distribution and postmortem redistribution.[3,4]

Figure 1: Structure of THC and its' metabolites.[3]



In conjunction with the Federal Aviation Administration's (FAA's) Civil Aerospace Medical Institute's (CAMI) Forensic Toxicology Research Laboratory, a method will be developed for the identification and quantitation of THC, 11-hydroxy-THC, and THC-COOH in postmortem blood, urine, and tissues. Because many of these specimens are putrefied, it is necessary to develop a robust method that will not be compromised by putrefactive byproducts. Furthermore, due to the severe nature of aviation accidents, often the only samples available for

toxicological analysis are tissue samples. Therefore, this project will also evaluate the fluid/tissue distribution of THC, 11-OH-THC, and THC-COOH in pilot fatalities.

The extraction method for this project is protein precipitation using acetonitrile, followed by a solid phase extraction method. The analysis portion of the method is being developed on a Waters® Xevo® TQ-S Acquity® Ultra Performance Liquid Chromatography (UPLC) to identify and quantitate THC, 11-OH-THC, and THC-COOH, in postmortem fluids and tissues at trace levels. The cases included in this study were previously found positive using gas-chromatography mass spectrometry (GC/MS). In this study, there are 11 cases with up to 11 different sample types per case to evaluate the distribution of THC, 11-hydroxy-THC, and THC-COOH.

Due to the lack of published studies related to the distribution of THC, 11-OH-THC, and THC-COOH in postmortem samples, the purpose of this study is not only to develop an extraction and analytical method for the detection of THC and its metabolites, but also to look at the distribution of THC and its metabolites to aid in the interpretation of non-blood specimen concentrations. It is anticipated that 1) this new extraction procedure will save significant time and solvent quantities over the current procedure, 2) it will provide a method which can identify and quantitate 11-OH-THC, which is not typically analyzed for in forensic laboratories, but will aid in the interpretation of time-of-use and impairment, 3) the LC/MS/MS will provide superior detection limits, and 4) the distribution data collected will aid in the interpretation of tissue concentrations.

CHAPTER II

LITERATURE REVIEW

2.1 Overview

Marijuana is classified as a Schedule I drug according to the Drug Enforcement Agency (DEA).[3] The National Survey on Drug Abuse and Health concluded that approximately 6,600 individuals tried marijuana for the first time every day in 2013.[8] This overwhelming number exemplifies the need for studies examining how marijuana affects an individual's short term and long term cognitive, motor, and psychomotor skills. In order to connect cognitive, motor, and psychomotor skill impairment with actual THC blood concentrations, analytical methods must be developed to determine THC and its metabolites in specimens.

The FAA's Toxicology Laboratory uses postmortem blood, when available, to evaluate whether persons involved in transportation accidents were impaired by drugs. Since the FAA's Toxicology Laboratory receives blood in approximately only 60% of the cases, it relies on tissues for interpretive aid in the remaining 40%. With this in mind, there is a need for a more thorough understanding of postmortem distribution of THC, 11-OH-THC, and THC-COOH. However,

limited studies exist pertaining to such distribution of THC, 11-OH-THC, and THC-COOH in postmortem tissues; thus, the need for this research.

2.2 Cannabis

Cannabis use can be dated back to 2727 B.C.[9] Cannabis has been used for centuries for medicinal purposes, but is more commonly known now for its recreational use. There are many different cannabis plant species, but the most common is *Cannabis sativa*. The plant is most commonly processed into marijuana, hash oil, or hashish for consumption. Marijuana refers to the dried buds, flowers, and stems of the cannabis plant, and is the most popular way of introducing THC to the body.[3] Hashish is the extracted resin of *Cannabis sativa,* which can also be smoked.[9] Hash oil contains a higher amount of THC and is the "refined oily extract of the cannabis plant."[9] Hash oil can be used to make edibles by making butter or it can lace marijuana cigarettes to increase the amount of THC introduced to the body.

The active compound in the cannabis plant is THC. In the body THC is oxidized into the metabolite 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC). This metabolite produces similar effects to THC, but 11-OH-THC has a longer half-life than THC, leaving it to exhibit its effects on the body for a longer period of time.[3] The inactive metabolite 11-nor-9-carboxyΔ^9-tetrahydrocannabinol (THC-COOH) is metabolized from 11-OH-THC and is found in abundance in the urine as a glucuronide, and is detectable for an extended period of time after the marijuana is consumed.[10]

2.2.1 Potency of Cannabis

Many different strains of cannabis plant have been created by cross breeding plants that vary in potency. In fact, certain cannabis plants have been found to have 40% Δ^9-tetrahydrocannabinol (THC).[3] It has been hypothesized that this drastic increase in potency has led to a greater number of individuals seeking out treatment for addiction.[11]

The concentration of THC in marijuana cigarettes has increased approximately 400% since 1995. From 1995-2014, the DEA analyzed the amount of THC in 37,606 cases.[12] Over the 10 year period of the study, the DEA found that the concentration of THC in the tested cannabis increased significantly.

The potency increase can largely be attributed to cannabis cultivation techniques and genetic modification of plants. Information on how to grow cannabis plants and ensure that it is the highest potency is easily obtainable from the internet. A search found that plants with the highest potency are grown indoors under strict conditions and are referred to as "skunk."[11] The most important factor in increasing potency is not allowing plants to be fertilized; unfertilized plants synthesize higher amounts of cannabinoids.[11]

2.3 Cannabinoid Pharmacokinetics & Pharmacodynamics

2.3.1 Pharmacokinetics

The most common way that individuals introduce THC to the body is through smoking. Over 400 chemicals are absorbed by the body when an individual smokes marijuana and within seconds THC is absorbed into the blood and distributed throughout the body.[13] The majority of THC in a marijuana cigarette is created during burning because the heat of the fire is able to decarboxylate tetrahydrocannabinolic acid (THCA).[3] This compound has a different structure than the metabolite THC-COOH included in this study.

The quick distribution associated with inhalation is why many abusers choose smoking as opposed to other ways of introduction, such as oral or rectal.[13] Bioavailability varies amongst individuals and depends on the amount that is inhaled, number of times an individual inhales, and the potency of the cigarette. All are factors which influence the amount of THC that is introduced into the body.[3]

The absorption and concentration profile of THC following smoking has been studied in blood and plasma in antemortem samples.[14–17] Smoking one marijuana cigarette that is 3.55%

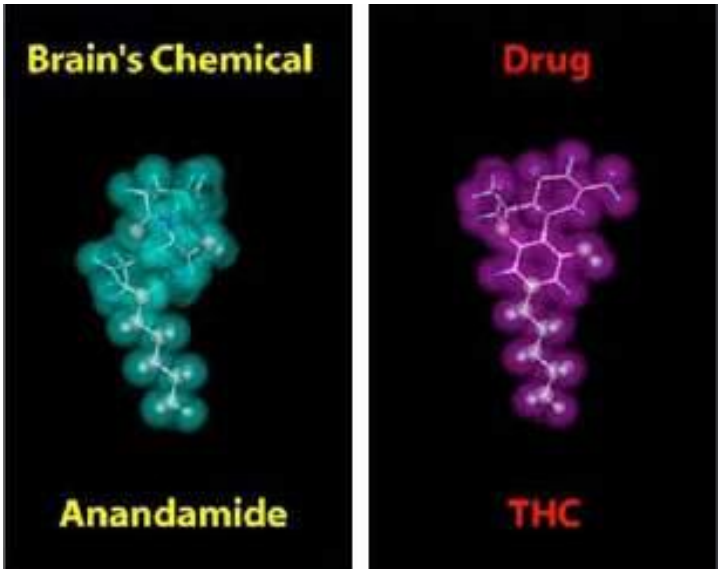
THC gives plasma concentrations of approximately 18 ng/mL within 5 minutes and reaches peak concentrations of approximately 162 ng/mL in 9 minutes.[14] THC quickly distributes into the organs of the body, resulting in blood concentrations returning to very low levels within 30 min, even though the person remains in a euphoric state for 2-4 hours.[13,17]

Concentrations of THC in the body also depend on how often the individual smokes. A study by Toennes et al. compared blood concentrations of THC, 11-OH-THC, and THC-COOH in occasional users with those of heavy users. Heavy users exhibited higher concentrations of THC and 11-OH-THC.

Oral administration of THC can come from a pill or food products. The oral pill dronabinol can be prescribed by a physician and contains a synthetic version of THC. This prescription is most commonly prescribed to AIDS patients who have a severe loss of appetite and cancer patients who experience nausea from chemotherapy. Oral administration of THC does not have as high of bioavailability as smoking and it does not produce immediate effects as a result of first-pass metabolism.[3]

THC binds to receptors, located in the brain, called cannabinoid receptor types 1 & 2 (CB1 and CB2 receptors). Naturally occurring proteins in the body called endocannabinoids, bind with these receptors. One specific endocannabinoid that THC mimics is Anandamide (see Figure 2). Anandamide assists in the regulation of cognitive functions, mood, judgment and emotions.[18] When THC binds to the receptors instead of Anandamide, interference in the regulation of these functions can occur.

Figure 2. Structure of Anandamide and THC.[18]



2.3.2 Pharmacodynamics

The body exhibits several side effects when cannabis is introduced to the body. THC is considered a Central Nervous System (CNS) depressant, which is able to slow the activity of the brain, reducing anxiety.[3] The most commonly known physiological effect is the feeling of euphoria that is felt quickly after THC is absorbed.

Other physiological effects, including increased heart rate, dry mouth, nausea, hallucinations, and psychotic breakdowns, are extremely common. [3,19] In 2006, there were 290,563 emergency room visits as a result of cannabis use.[3] Although there has been no report of death directly attributed to marijuana overdose, the effects of the drug on the body impacts an individual's ability to function normally, without impairment.

With the potency of marijuana rising, there have been an increased number of studies that have describe how this high-potency affects individuals. Several studies have shown that the use of high-potency cannabis increases an individual's risk of psychosis.[20–22] One study gave a questionnaire about cannabis use to 410 first-episode psychosis patients and found that 67%

considered themselves lifetime cannabis users and 53% of lifetime users preferred the "skunk-like" or high potency cannabis.[23]

2.4 Cannabis Effects on Human Performance

Numerous studies show how smoking marijuana affects an individual's cognitive, motor, and psychomotor skills. Both short-term and long-term cannabis users exhibit trouble with everyday cognitive activities such as memory, learning, and problem solving. Not only are cognitive functions inhibited, but psychomotor functions are also inhibited, making tasks such as driving a car or flying a plane dangerous.

The cognitive differences between long-term cannabis users, short-term cannabis users, and non-cannabis users have been well studied. Two of these studies looked at the cognitive skill of being able to remember a list of words in current long-term cannabis smoking individuals, individuals who were long-term cannabis smokers, but had only done so a few times over the past three months, and non-cannabis smokers over a period of 28 days.[24–26] Both studies concluded that the current long-term cannabis users had a much harder time recalling the specific list of words at days 0, 1, and 7, as opposed to the individuals who were non-cannabis smokers.[24–26]

A similar study looked at how cannabis smokers compared to non-cannabis smokers in concentrating/focusing on certain tasks such as reading. In a comparison between 20 non-cannabis smokers and 20 long-term cannabis smokers, it was found that cannabis smokers took longer to read the provided text, as well as how often they revisited text (a sign of cognitive impairment).[27]

2.4.1 Marijuana and psychomotor performance

To determine the effects that THC has on psychomotor performance, many studies utilize motor vehicle and aircraft simulators.[2,6,7,15,28,29] All of these studies have found that driving or operating an airplane while under the influence of marijuana affected the ability of individuals to perform psychomotor functions as well as when they were sober. In fact, one of these studies

showed some degree of performance decrement 1 day after smoking cannabis – long after the THC is believed to be eliminated from the body.[6,29] The reason for this "hangover" effect remains unclear, although the high lipophilicity of THC and potential for receptor downregulation may partially explain this phenomenon.

One area of public health concern is the psychomotor impairment associated with the use of cannabis while operating motor vehicles. The number of driving under the influence of drugs (DUID) fatal accidents has increased.[30] In fact, individuals arrested for DUID are most commonly seen to be under the influence of marijuana.[31] The term "DUID" specifically excludes alcohol, and driving under the influence of alcohol is simply termed "DUI." Studies have compared an individual's ability to operate a motor vehicle under the influence of marijuana versus operating a motor vehicle sober. Each of these studies found that cannabis impacted different areas of driving such as weaving in the lane, weaving outside of the lane, and the speed at which the individual was driving when the recklessness occurred.

An evaluation of marijuana only, alcohol only, and a combination of the two found that an individual who smoked a marijuana cigarette containing 3.55% THC exhibited the same amount of reckless driving as an individual that had a blood alcohol content (BAC) of 0.08, which is legally intoxicated in the United States.[2]

In addition to motor vehicle studies, there are several flight-simulator experiments that have showed similar results to DUID studies in automobiles. Impaired pilots made more errors when given a specific flying pattern than when they were sober.[28,29] In addition, being impaired led to deviations in altitude and pilots had difficulty following navigation directions provided to them over a radio.[28]

The impact that marijuana has on an individual's cognitive, motor, and psychomotor functions can be detrimental. The inability to operate motor vehicles or aircrafts under the influence has led to the need to understand how these cognitive and psychomotor impairments

may correlate with the concentrations and distribution of THC, 11-OH-THC, and THC-COOH in the body.

2.5 Postmortem Redistribution

Postmortem redistribution (PMR) occurs whenever a drug moves from higher concentrations to lower concentrations within the body after death. Drugs that often redistribute are lipophilic, have a large volume of distribution, and/or have a high pKa.[32]

Limited studies have been published that looked at the PMR of THC, 11-OH-THC, and THC-COOH.[32,33] One study showed the relationship between postmortem heart and iliac blood and found that blood from the heart had a slightly higher concentration of each compound.[32] A study using a pig to evaluate postmortem redistribution in fluids and tissues showed that PMR was observed for THC in the body. Overall, it was found that the possibility of PMR for cannabinoids can occur and how much it effects the concentrations depends on how quickly an autopsy occurs after death.

2.6 Conclusion

The manner in which THC, 11-OH-THC, and THC-COOH impact an individual's ability to perform everyday tasks such as operate transportation vehicles, and recall a list of words has been widely studied. However, the distribution of these three compounds in postmortem tissues has not been well studied. The goal of this research is to provide toxicologists with a better understanding of how THC, 11-OH-THC, and THC-COOH distribute in tissues and how the concentrations of each analyte relate to each other.

CHAPTER III

METHODOLOGY

3.1 Introduction

The current THC method used by the Federal Aviation Administration's (FAA's) Forensic Toxicology Research Laboratory in the Civil Aerospace Medical Institute (CAMI) is a solid-phase extraction coupled with chemical ionization (CI) gas chromatography- mass spectrometry (GC/MS). However, it has numerous limitations, including the lack of ability to identify THC-OH, the need for chemical derivatization, and a laborious extraction process. A new extraction and analytical method using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is desired to detect THC, 11-OH-THC, and THC-COOH at trace levels.

The purpose of this this new method is to provide the laboratory with the ability to identify trace levels of THC, 11-OH-THC, and THC-COOH in postmortem samples from aviation accident victims, and to evaluate the distribution of THC and its metabolites in different specimen types to aid in the interpretation of THC positive cases.

3.2 Chemicals & Reagents

THC, 11-OH-THC, THC-COOH were purchased from Cerilliant (Cerilliant Corp; Round Rock, TX) and Lipomed (Lipomed; Cambridge, MA) as 1.0 mg/mL methanolic standards. THC-d_3, 11-OH-THC-d_3, and THC-COOH-d_3 were purchased from Cerilliant as 100 ug/mL methanolic standards. Bovine blood was obtained from Country Home Meat Co. (Country Home Meat Co.; Edmond, OK). Selectrazyme (UCT; Bristol, PA) was used to hydrolyze urine specimens and a glucuronide control was made using a THC-glucuronide (Cerilliant Corp; Round Rock, TX) and THC-COOH glucuronide (Lipomed; Raleigh, NC).

A Millipore Direct Q-3 UV (Millipore Sigma; Billerica, MA) was used to generate deionized water (DI). Formic acid, LCMS grade water, and LCMS grade acetonitrile were purchased from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO). Mobile phase A (MPA) was made with LCMS grade water mixed with formic acid (999:1 v/v). Mobile phase B (MPB) was made with LCMS grade acetonitrile mixed with formic acid (999:1 v/v).

Sodium fluoride (NaF) (Sigma-Aldrich; St. Louis, MO) was used to make a 1% NaF solution used to dilute tissue specimens for homogenization. This was prepared by dissolving 10 g of sodium fluoride into 1000 g of water.

To make 1 L of 0.1 M Sodium Acetate Buffer pH 3.0, 8.2 g of Sodium Acetate was dissolved in 900 mL of DI water. The pH was adjusted to 3.0 using concentrated hydrochloric acid and diluted to 1 L with DI water. To make 1 L of 100 mM HCL:ACN, 0.830 mL of concentrated hydrochloric acid in 1 L of DI water. To make 95:5 100 mM HCL:ACN, 50 mL of ACN was added to 950 mL of 100 mM HCL. Both of these buffers have an expiration date six months after they are prepared.

3.3 Ultra-Performance Liquid Chromatography/Tandem-Mass Spectrometry Conditions

Analysis was completed using a Waters® Xevo® TQ-S Acquity® UPLC (Waters Corporation; Milford, MA). The UPLC column used was an ACQUITY® UPLC BEH C18 (2.1 x 100mm, 1.7 μm). This system consisted of a temperature-controlled autosampler (set at 10ºC) with a flow through needle that reduces the possibility of carryover, a binary solvent manager, and a tandem quadrupole featuring a StepWave ion guide. The StepWave ion guide has stacked ion rings that pull charged ions to the mass spectrometer and removing unwanted neutral ions, making the instrument more sensitive.

Waters IntelliStart™ (Waters Corporation; Milford, MA) was used to optimize the ionization and fragmentation of THC, 11-OH-THC, and THC-COOH. The source temperature was 150ºC, with a desolvation temperature of 500ºC. The cone gas flow was 150 L/hr, desolvation gas flow was 1,000 L/hr with a collision gas flow of 0.15 mL/min. IntelliStart™ optimized the analytes by evaluating their ability to be detected in positive and negative Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI) mode. All ions were detected and ionized the best using positive mode (+)ESI. A precursor ion and the product ions were selected for each compound according to their mass-to-charge (m/z) ratio and abundance. The precursor ion is captured in the first quadrupole, which selects the ion and sends the rest of the sample to waste. This captured precursor ion is hit with a collision energy producing three product ions in the collision cell, which are then selected for in the third quadrupole. All precursor and product ions can be seen in Table 1.

A Waters ACQUITY® UPLC BEH C18 (Waters Corporation; Milford, MA) column was used to achieve chromatographic separation. The column manager temperature was set at 30ºC. The UPLC flow rate was 0.60 mL/min and sample injection volume was 2 μL. The column was equilibrated for approximately 1 hour using the beginning mobile phase composition, 35:65 (MPA:MPB). During equilibration, the pressure was checked to make sure it was below 12,000

psi and the delta, the difference between the minimum and maximum pressure of the column, was less than 50. The mobile phase gradient was used to achieve the desired separation can be seen in see Table 2.

3.4 Method Development and Validation

Numerous methods have been created and validated on GC/MS for the analysis of THC in postmortem blood and urine.[32,34–37] The FAA Toxicology Laboratory currently has a validated GC/MS method for the detection of THC and THC-COOH in fluids and tissues. The literature does not indicate any validated methods using UPLC/MS/MS in the analysis of THC, 11-OH-THC, and THC-COOH in postmortem fluids and tissues.

The new UPLC/MS/MS method was validated using standards set by the Scientific Working Group for Forensic Toxicology (SWGTOX). The criteria evaluated in a quantitative analysis method are: linearity, limit of detection, limit of quantitation, intra-day variability, inter-day variability, stability, matrix effects, ion suppression, and recovery. The SWGTOX guidelines are of the highest standard for forensic toxicology and ensure that extractions and analytical methods are precise, accurate, and selective.[38]

3.4.1 Calibration and Carryover

Five extracted calibration curves were run to establish the linearity and consistency of the curves. Calibration curves began with the highest calibrator, followed by a blank, and then run from low to high. All values were within 20% of target concentration. Curves also needed to exhibit an r^2 value of 0.98 or greater to be accepted by the laboratory. Limit of detection and limit of quantitation were determined during the construction of the linear calibration curve.

Carryover was evaluated by running a blank sample after the highest calibrator. Area counts were used to determine how much, if any, carryover is present. This was completed five times and the actual carryover did not exceed 20% of the lowest calibrator.

3.4.2 Accuracy and Precision

A high, medium, and low control were run five times on five different runs. The three controls were representative of a high, medium, and low concentration within the curve. A large pool of each control was made and aliquoted into separate tubes. This large pool was sufficient to use for accuracy and precision, freeze-thaw, and dilution control studies. Samples for accuracy and precision remained in the refrigerator and five controls were extracted each day.

Accuracy was measured based on how close the value is to the target concentration and did not exceed 20% of this concentration. Precision was measured based on how reproducible results were and standard deviation was used to calculate the coefficient of variance.

3.4.3 Stability

Various stability factors were evaluated, including refrigerator stability, freeze/thaw stability, and process stability. Refrigerator stability was evaluated by looking at the accuracy and precision data collected over a 5 day period. This is detailed in the Accuracy and Precision section above.

Freeze-thaw cycles were evaluated as they may affect the analytes of interest. To do this, the low, medium, and high controls prepared for the accuracy and precision study were aliquoted on day one and then placed in the freezer. On day 2 all freeze/thaw controls were taken out and allowed to thaw five of each control level analyzed. The remaining controls are returned to the freezer. A total of three cycles were completed. The analytes were considered to be stable if they are within 20% of the target concentration.

Process stability was analyzed by extracting 5 controls of a low, medium, and high concentration on the first day and running them at one, two, three, and four days post-extraction. These samples sat on the instrument at a controlled temperature. Analyte stability was tested by extracting five controls of a low, medium, and high concentration and running them for five

consecutive days to show how stable they are sitting on the instrument. The analytes were considered to be stable if they were within 20% of the target concentration.

3.4.4 Ion Suppression/Enhancement, Drug Interference, and Recovery

Components of a matrix can interfere with the signal produced by the analytes. Therefore, ion suppression or enhancement of these analyte signals must be evaluated in different matrices that are commonly encountered in the laboratory. Ion suppression and enhancement were evaluated in the blood, urine, serum, liver, lung, brain, muscle, and kidney.

Since each tissue of a given tissue type will possess different putrefactive qualities and have different matrix effects, a homogenous mixture of each tissue type was prepared to determine ion suppression. The homogenous mixture consisted of five different samples. This was completed for each tissue type.

Five different sources of blood was used, including two different lots of bovine blood and then three different lots of human blood obtained from Oklahoma Blood Institute (OBI; Oklahoma City, OK). Five different sources of negative urine were used. For serum, three different bovine blood lots were used and two different lots of human serum.

Ion suppression was determined by spiking ten methanol blanks, and 5 post extracted blood, urine, serum, and tissue samples with the drugs of interest and their internal standards at final a concentration of 100 ng/mL of drug and 20 ng/mL of internal standard. These samples were analyzed and the area counts were compared between the methanol samples and the matrix samples to see if there was any analyte ion suppression or enhancement.

Another type if interference is ion suppression or enhancement due to the presence of other drugs. Five THC, 11-OH-THC, THC-COOH controls were spiked with very high concentrations of commonly encountered drugs, extracted, and analyzed to determine if they interfere with the detection of these compounds. The drugs evaluated for possible interference were acetaminophen and naproxen at 25,000 ng/mL and atenolol, atorvastatin, citalopram,

dextromethorphan, diphenhydramine, hydrocodone, methamphetamine, and sertraline at 1,000 ng/mL.

Recovery is the amount of drug that is retrieved after an extraction. To evaluate this, the area counts of spiked blanks, which contained no drug loss, were compared to the area of 5 extracted controls.

3.4.5 Dilution integrity

In cases where the determined concentration is above the highest calibrator, a dilution must be completed. To evaluate the effect of dilution on sample integrity, five 1:10 dilutions of the highest calibrator were completed. The value was acceptable if it did not exceed 20% of the target concentration.

3.5 Sample Selection and Storage

Fatal aviation accidents cases previously confirmed positive for cannabinoids were selected from the ToxFlo (ToxFlo™, DiscoverSoft Development, LLC; Oklahoma City, OK) toxicology database. All fatal cases were from accidents that occurred between 2014 and 2016. The cases selected had a majority of the desired biological specimens (blood, urine, vitreous humor, liver, lung, kidney, spleen, muscle, brain, and heart). Samples were stored at -20ºC in the laboratory.

3.6 Standard Preparation

Calibrators and controls were prepared from methanolic standards originating from different manufacturers. Class A volumetric flasks were used for the preparation of initial calibrator and control solutions. Calibration curves were prepared using a serial dilution and using bovine whole blood as the diluent.

A 10 ug/mL cannabinoid standard was prepared by adding 100 µL of 1.0 mg/mL THC, 11-OH-THC, and THC-COOH to a 10 mL volumetric flask and diluting to 10 mL with deionized (DI) water. Calibration curves were prepared by taking 100 µL of the 10 µg/mL working standard and diluting to 10 mL with bovine blood to create a 100 ng/mL calibrator. A serial dilution was carried out to make the 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.13 ng/mL, 1.56 ng/mL, and 0.78 ng/mL calibrators. The resultant calibration curve was from 0.78 – 100 ng/mL.

Control concentrations were prepared at 2, 20, and 80 ng/mL. First, 100 µL of 1.0 mg/mL THC, 11-OH-THC, and THC-COOH were diluted to 10 mL with DI water in a volumetric flask, resulting in a 10.0 µg/mL working solution. One mL of the 10.0 µg/mL solution was diluted to 10 mL resulting in a 1.0 µg/mL working solution in a volumetric flask. Eight hundred µL of the 1.0 µg/mL solution was diluted to 10 mL of bovine blood, resulting in an 80 ng/mL blood solution. A 20 ng/mL control was prepared by diluting 200 µL of the 1.0 µg/mL working solution to 10 mL with bovine blood. The 2 ng/mL control was prepared by diluting 20 µL of the 1.0 µg/mL working solution to 10 mL with bovine blood.

An internal standard solution of 100 ng/mL was prepared in the following manner. One hundred µL of 100 µg/mL THC-d3, 11-OH-THC-d3, and THC-COOH-d3 and diluting to 10 mL with DI water in a volumetric flack, producing a 1.0 µg/mL solution. One mL of the 1.0 µg/mL solution was diluted to 10 mL with DI water in a volumetric flask, producing a final working concentration of 100 ng/mL. Each calibrator, control, and sample was spiked with 100 µL of the 100 ng/mL internal standard solution, resulting is 10 ng of internal standard in each specimen.

3.7 Tissue Sample Preparation

Tissue samples must be homogenized before analysis. Samples were diluted 2:1 with 1% Sodium Fluoride (aqueous: tissue). The sample was then homogenized using an Omni Mixer Homogenizer (Omni International; Kennesaw, Georgia) until a smooth textured liquid is achieved.

3.8 Urine Sample Preparation

Since THC and THC-COOH form glucuronide bonds in the urine, samples must be hydrolyzed prior to analysis to break the drug-glucuronide bond. To accomplish this, 50 μL of Selectrazyme (UCT; Bristol, PA) and 2 mL of 1 M Sodium Acetate Buffer pH 5.0 was added to a 16x150 screw top tube containing 0.5 mL sample and 10 ng of internal standard. This was hydrolyzed for 3 hours at 65°C. Samples were then allowed to cool for 20 minutes at room temperature. Five milliliters of 0.1 M Sodium Acetate Buffer pH 3.0 was added and the pH was checked to ensure it was around 3.

3.9 Extraction Method

The best overall extraction method found to work for all three analytes was one that was recommended by UCT (UCT; Bristol, PA). Specimens were stored at -20ºC and allowed to thaw at room temperature prior to analysis. Test tubes used for the calibrators, controls, and specimens were silanized 16 x 100-mm screw top test tubes (Fisher Scientific; Waltham, MA). Sample size was 0.5 mL for each calibrator, control, and liquid sample. Each was placed into individually labeled, 16x150 mm screw top tubes. Since 0.5 mL of liquid samples was used, 1.5 grams of tissue homogenate was used, which equates to 0.5 g wet tissue sample. One hundred μL of internal standard was added to all specimens (10 ng). A protein crash was then carried out by adding 3 mL of ice cold acetonitrile to each sample. Samples were vortexed for ten seconds, placed on a rotary mixer for 5 minutes, followed by centrifugation at 2300 rpm for 12 minutes. After the sample centrifugation was finished, they were poured off into clean test tubes and 5 mL of 0.1 M Sodium Acetate Buffer pH 3.0 was added to each tube.

Extraction was carried out using the 10 mL Clean Screen THC (UCT; Bristol, PA) solid-phase extraction columns with 200 mg of sorbent using a positive-pressure manifold (UCT; Bristol, PA). Columns were conditioned with 2 mL methanol, 2 mL of DI water, followed by 1 mL of 0.1 M Sodium Acetate Buffer pH 3.0. Samples were then added and allowed to pass through the column at 1-2 mL per minute. Columns were then washed with 2 mL of DI water, 2

mL of 95:5 0.1 M HCL:ACN, and finally 200 µL of hexanes. SPE columns were then dried under full pressure for 5 minutes (approx. 25 PSI nitrogen).

THC was eluted from the SPE columns by adding 2 mL of hexanes and eluting into conical tubes at 1 mL per minute. 11-OH-THC and THC-COOH were then eluted into the same vial using 3 mL of a 50:50 mixture of hexanes:ethyl acetate at 1 mL per minute. Samples were then dried down in a water bath at 40 ºC under a gentle stream of nitrogen until only a small amount of water was left in the bottom of the conical tube. Drying down vigorously can result in the loss of THC in samples. Samples were reconstituted in 100 µL of LCMS grade methanol and transferred to a 350 µL silanized glass conical pulled-point insert (MicroLiter; Millville, NJ) inside of a 9mm screw thread glass vials (MicroLiter; Millville, NJ). Vials were capped with a 9mm red screw thread split cap (MicroLiter; Millville, NJ).

3.10 Data Analysis

All analyte data was collected using the Waters MassLynx software. Analyte concentrations was determined for all calibrators, controls, and samples using Waters TargetLynx software. The peaks had to be within +/- 5% retention time of the average calibrator, the product ion ratio had to be within +/- 20% of average calibrator, and the S/N had to be at least 1:10.

Concentrations of each tissue type were be compared to the concentration found in the blood. This was done for THC, 11-OH-THC, and THC-COOH separately. ANOVA was then used to compare the concentrations to each other to determine if a significant difference in concentrations exists between tissue types.

Statistical analysis were performed on the stability data to determine if the interday values are significantly different. ANOVA followed by Dunnett's Post Test were performed on the freeze-thaw stability and on-instrument stability.

3.11 Conclusion

This method was developed to find a way to extract THC, 11-OH-THC, and THC-COOH from postmortem blood, urine, and tissues. The matrix effects of tissues has lead to the desire of a cleaner extraction method that would allow for the UPLC/MS/MS to detect these compounds at trace levels.

CHAPTER IV

RESULTS

In this research project, up to 11 different fluid and tissue specimens in 11 aviation fatality cases were examined to evaluate the postmortem distribution of THC, 11-OH-THC, and THC-COOH. A new solid-phase extraction method and an analytical method using a Waters® Xevo® TQ-S Acquity® UPLC (Waters Corporation; Milford, MA) was developed and will save a significant amount of time and solvents compared to the previous method being used. THC, 11-OH-THC, and THC-COOH can be detected in postmortem fluids and tissues in one extraction with this method. The new analytical method on the UPLC/MS/MS is 4.0 minutes compared to the GC/MS method that took 5.67 minutes.

4.1 Analytical Method

The final validated analytical LC-MS/MS method was on the Waters® Xevo® TQ-S Acquity® UPLC. The UPLC column used was an ACQUITY® UPLC BEH C18 (2.1 x 100mm, 1.7 μm). The column manager temperature was set at 30ºC. The sample injection volume was 2 μL. The UPLC flow rate was 0.600 mL/min.

Waters IntelliStart was used to optimize the ionization and fragmentation of THC, 11-OH-THC, and THC-COOH. The ionization source was a positive electrospray

ionization. The source temperature was 150ºC, with a desolvation temperature of 500ºC. The cone gas flow was 150 L/Hr, desolvation gas flow was 1,000 L/Hr with a collision gas flow of 0.15 mL/min. A dwell time of 0.018 seconds for THC, and 0.009 seconds for 11-OH-THC and THC-COOH was used for recorded ions. Precursor ion and the product ions were selected for each compound according to their mass-to-charge (m/z) ratio and abundance and are listed in Table 1. The quantitation ions for THC, 11-OH-THC, and THC-COOH are 193.19, 193.19, and 327.31 respectively. The quantitation ions for THC-d3, 11-OH-THC-d_3, and THC-COOH-d_3 are 196.23, 196.24, 330.31 respectively.

Table 1. MS parameters

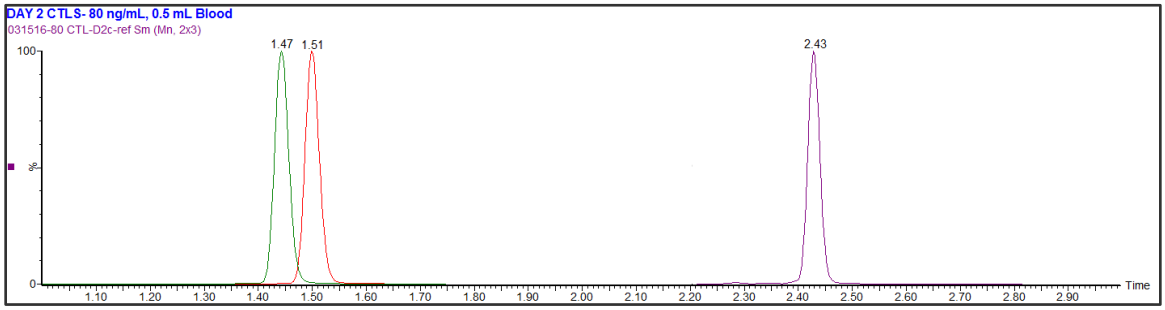
Compound	Retention Time	Cone Voltage (V)	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
THC	2.41	38.0	315.13	123.12 193.19	20.0 18.0
THC-d_3	2.41	19.0	318.15	123.11 196.24	33.0 21.0
11-OH-THC	1.43	20.0	331.19	193.19 313.32	25.0 13.0
11-OH-THC-d_3	1.43	20.0	334.14	196.24 316.30	23.0 13.0
THC-COOH	1.49	20.0	345.10	299.29 327.31	19.0 15.0
THC-COOH-d_3	1.49	20.0	348.12	302.29 330.31	19.0 15.0

The LC method utilized a gradient flow that allowed for separation of all compounds, as shown in Table 2. MPA consisted of LCMS grade Acetonitrile with 0.1% formic acid and MPB consisted of DI water with 0.1% formic acid. The gradient was 35:65 MPA:MPB to 10:90 MPA:MPB over 4minutes. It was found that 0.6 mL/min flow rate resulted in the sharpest peaks and the best separation. The sample injection volume was 2 μL using a flow-through needle to prevent carryover. The run time was a total of 4.0 minutes. A chromatogram of THC, 11-OH-THC, and THC-COOH can be seen in Figure 3.

Table 2. Gradient flow for analytical method.

Time (min)	Flow Rate (mL/min)	%MPA	%MPB
0.00	0.600	35.0	65.0
2.00	0.600	10.0	90.0
2.50	0.600	10.0	90.0
3.80	0.600	35.0	65.0
4.00	0.600	35.0	65.0

Figure 3. An 80 ng/mL control with (left to right) 11-OH-THC, THC-COOH, and THC.



4.2 Method Validation

The newly developed method uses SPE and LC/MS/MS to detect three common analytes found in specimens when an individual has been exposed to marijuana. The developed method has been successfully validated using SWGTOX guidelines and is shown to be robust.

4.2.1 Calibration Curves and Linearity

Five extracted calibration curves were run and each analyte was evaluated to determine the linearity. The linear dynamic range (LDR) was determined to be 0.78-100 ng/mL. The average correlation coefficients of THC, 11-OH-THC, and THC-COOH were 0.9992, 0.9984, and 0.9965 respectively with a weighting factor of 1/x (see Figures 4, 5, and 6). All values were within 20% of the target concentration and values can be seen in Tables 3, 4, and 5.

Table 3. THC Calibration Curve Concentrations

THC Calibration Curves									
Concentration	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**AVG**	**SD**	**%CV**	**%E**
100	101.5	99.7	99.65	98.82	97.05	99.34	1.6	1.6	0.7
50	49.28	49.28	49.94	55.43	53.23	51.43	2.8	5.4	-2.9
25	24.02	25.81	25.25	24.4	24.64	24.82	0.7	2.9	0.7
12.50	12.53	12.77	12.55	13.82	12.48	12.83	0.6	4.4	-2.6
6.25	6.35	6.11	6.38	6.75	6.48	6.41	0.2	3.6	-2.6
3.13	3.22	3.25	3.19	3.32	3.09	3.21	0.1	2.6	-2.8
1.56	1.58	1.57	1.47	1.49	1.44	1.51	0.1	4.1	3.2
0.78	0.76	0.74	0.79	0.65	0.81	0.75	0.1	8.3	3.8

Figure 4. Calibration Curves THC

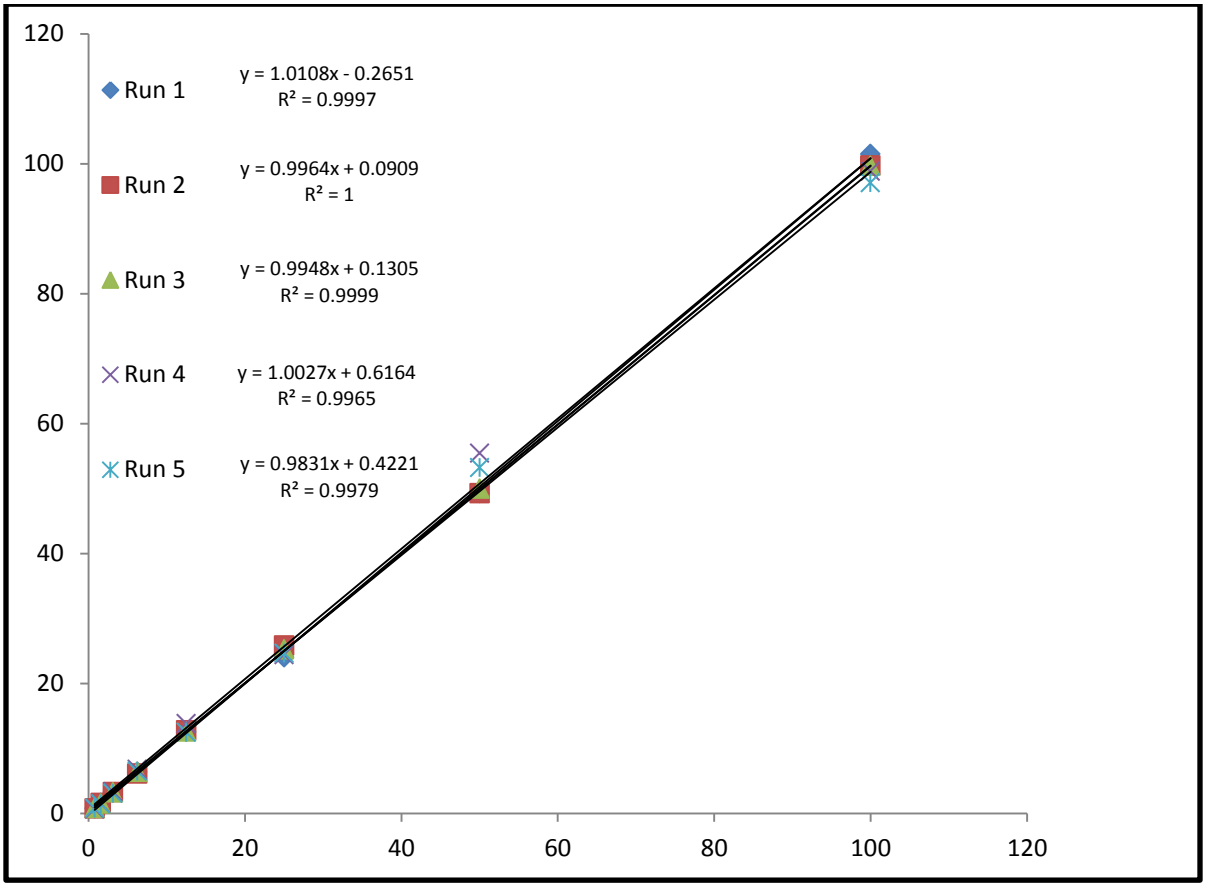


Table 4. 11-OH-THC Calibration Curve Concentrations

11-OH-THC Calibration Curves									
Concentration	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**AVG**	**SD**	**%CV**	**%E**
100	103.40	101.79	102.21	101.88	101.83	102.2	0.7	0.7	-2.2
50	47.12	49.59	48.02	57.20	50.75	50.54	4.0	7.9	-1.1
25	24.87	25.81	25.45	23.05	23.57	24.55	1.2	4.9	1.8
12.50	12.31	12.77	11.76	12.20	11.54	12.12	0.5	4.0	3.1
6.25	6.21	6.11	6.39	6.31	6.12	6.23	0.1	1.9	0.4
3.13	2.82	3.25	2.80	3.35	3.08	3.06	0.2	8.1	2.1
1.56	1.3	1.57	1.83	1.73	1.30	1.61	0.2	12.4	-3.3
0.78	0.86	0.74	0.76	0.70	1.02	0.816	0.1	15.70	-4.6

Figure 5. Calibration Curves 11-OH-THC

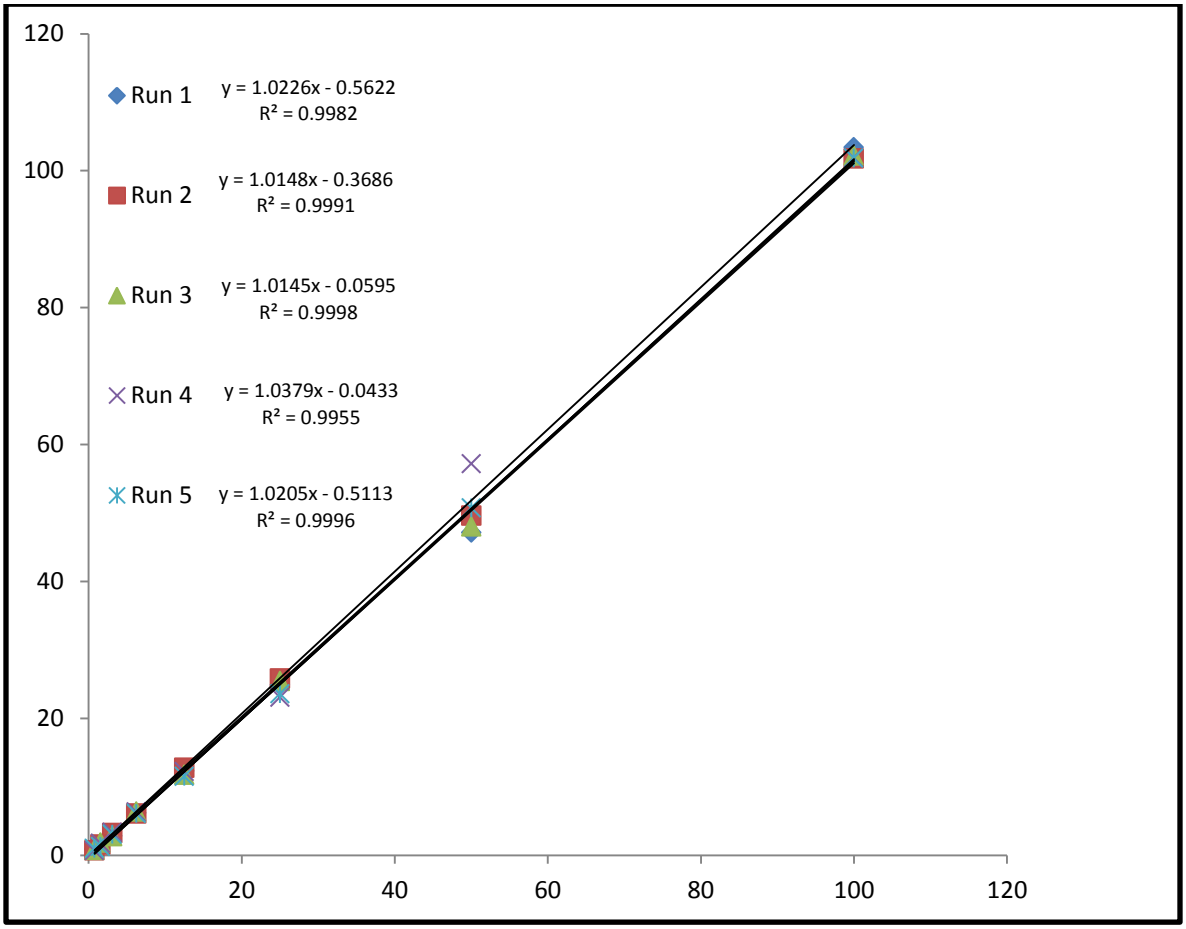
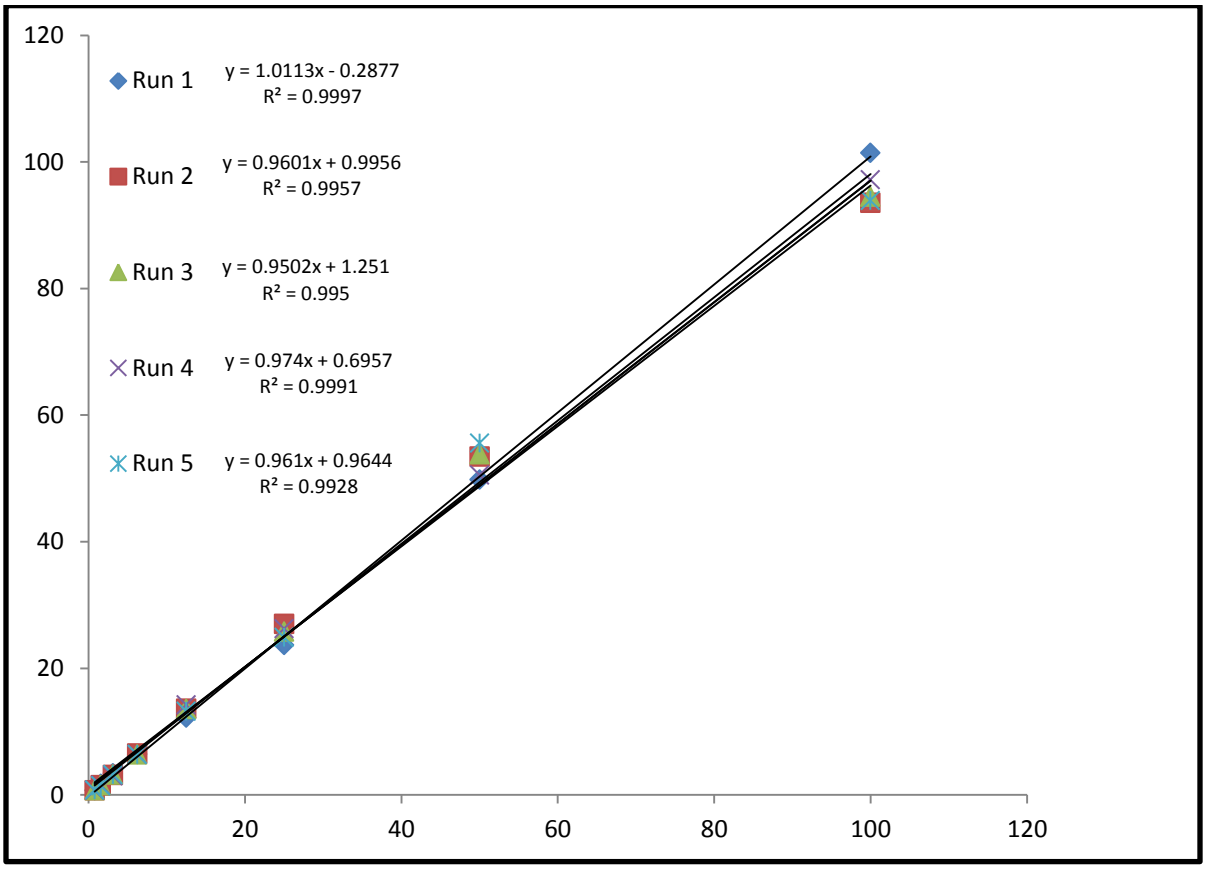


Table 5. THC-COOH Calibration Curve Concentrations

THC-COOH Calibration Curves									
Concentration	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**AVG**	**SD**	**%CV**	**%E**
100	101.4	93.5	76.45	97.17	93.89	96.08	3.3	3.4	3.9
50	49.79	53.4	53.78	50.47	55.57	52.60	2.4	4.6	-5.2
25	23.66	27.00	25.75	26.20	24.84	25.49	1.3	5.0	-2.0
12.50	12.17	13.6	13.52	14.20	13.20	13.34	0.7	5.6	-6.7
6.25	6.40	6.50	6.37	6.36	6.35	6.40	0.1	1.0	-2.3
3.13	3.37	3.10	3.19	2.99	3.18	3.16	0.1	4.4	-1.3
1.56	1.69	1.50	1.49	1.51	1.38	1.51	0.1	7.4	2.9
0.78	0.69	0.70	0.68	0.70	0.76	0.71	0.0	4.4	9.5

Figure 6. Calibration Curves THC-COOH



4.2.2 Limit of Detection and Lower Limit of Quantification

The LOD and LLOQ for each analyte was 0.78 ng/mL, which is the lowest calibrator. While detection of analytes below this limit was possible, the FAA Toxicology Laboratory decided that this level was sufficiently sensitive for the laboratory's purposes. All specimens below the lowest calibrator were considered to be negative, regardless if they met all identification criteria.

4.2.3 Carryover

To evaluate carryover, the highest calibrator was run followed by a methanol blank. The areas of the methanol blank were evaluated to see if analytes were present and if they were the area counts were compared to the area of the lowest calibrator. It is required that the carryover in the methanol blank not be more than 20% of the lowest calibrator. There was no observed carryover for any analytes. This is most likely because the instrument has a flow-through needle, which helps prevent carryover.

4.2.4 Accuracy and Precision

Five low, medium, and high controls were extracted five times on five different runs to determine accuracy and precision. Concentrations of the controls were 80 ng/mL, 20 ng/mL, and 2 ng/mL. A large pool was made of each concentration and was aliquoted out into tubes that were then used for accuracy and precision and freeze-thaw stability. Accuracy and precision samples were left in the refrigerator at 4°C and five samples were extracted on the first day and then the next four days. Freeze-thaw stability samples were stored in the freezer at -20°C and are discussed in detail below.

All controls were within 20% of the target concentration. The accuracy expressed as a % error was calculated by subtracting the target concentration from the measured value, dividing it

by the target concentration, and multiplying it by 100. The largest % error occurred on the fifth day.

Precision is expressed as the coefficient of variation, which is calculated by dividing the standard deviation by the mean and multiplying by 100. The largest coefficient of variation was 8% and was for the 11-OH-THC 2 ng/mL control on Day 1. Accuracy and precision can be seen in Tables 6, 7, and 8.

Table 6. Intra and Inter-Day Accuracy and Precision for THC

	DAY 1			DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.83 ± 0.02	1	-9	1.69 ± 0.02	1	-15	1.73 ± 0.04	2	-13	1.73 ± 0.05	3	-14	1.69 ± 0.01	1	-15	1.73 ± 0.06
20	21.39 ± 0.15	1	7	20.38 ± 0.19	1	2	19.47 ± 0.26	1	-3	22.67 ± 0.22	1	13	23.84 ± 0.46	2	19	21.55 ± 1.59
80	80.69 ± 2.28	3	1	79.54 ± 1.32	2	-1	76.50 ± 0.56	1	-4	88.63 ± 0.85	1	1	95 ± 0.97	1	19	84.07 ± 6.91

Table 7. Intra and Inter-Day Accuracy and Precision for 11-OH-THC

DAY 1				DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.84 ± 0.15	8	-8	1.72 ± 0.02	1	-14	1.68 ± 0.09	6	-16	1.94 ± 0.11	6	-3	1.79 ± 0.06	3	-11	1.84 ± 0.15
20	20.53 ± 0.43	2	3	19.36 ± 0.59	3	-3	17.83 ± 0.52	3	-11	20.28 ± 0.88	4	1	19.17 ± 0.58	3	-4	20.53 ± 0.43
80	80.20 ± 2.09	3	0	82.25 ± 1.78	2	3	73.47 ± 1.89	3	-8	81.51 ± 1	1	2	83.98 ± 0.90	1	5	80.20 ± 2.09

Table 8. Intra and Inter-Day Accuracy and Precision for THC-COOH

DAY 1				DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.72 ± 0.04	2	-14	1.79 ± 0.08	4	-11	1.83 ± 0.07	4	-8	1.72 ± 0.04	2	-14	1.86 ± 0.08	4	-7	1.78 ± 0.08
20	19.82 ± 0.29	1	-1	21.45 ± 0.64	3	7	20.83 ± 0.29	1	4	20.99 ± 0.32	2	5	22.12 ± 1.14	5	11	21.04 ± 0.99
80	79.90 ± 0.68	1	0	85.38 ± 1	1	7	85.17 ± 1.40	2	6	88.38 ± 1.59	2	10	87.28 ± 1.65	2	9	85.22 ± 3.22

4.2.5 Stability

Analyte stability was evaluated for process stability, refrigerator stability, and freeze-thaw stability. Process stability was evaluated by reanalyzing all of day one controls left on the instrument on days two, three, and four and five post-extraction. The extracted specimens remained on the instrument in a temperature controlled autosampler that was set at 10°C. It was found that THC, 11-OH-THC, and THC-COOH were all stable for five days. Results of the processed stability can be seen in Tables 9, 10, and 11.

The refrigerator stability was evaluated while running the accuracy and precision study, where controls were prepared on day one and stores in the refrigerator at 4°C until extraction. As seen in Tables 9, 10, and 11, all specimens were within 20% of their target concentrations over the 5 days stored in the refrigerator.

Freeze-thaw stability was also evaluated. Controls of low, medium, and high concentration were frozen on day one and underwent three freeze-thaw cycles. On day two, all of the controls were taken out of the freezer and allowed to thaw for one hour. Five controls of low, medium, and high concentration were analyzed and the remaining controls were placed back in the freezer. The same process occurred on days 3 and 4, resulting in three freeze-thaw cycles. All values were within 20% of the target concentration and can be seen in Tables 12, 13, and 14.

Table 9. Process Stability for THC.

DAY 1				DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.8 ± 0.02	1	-9	1.83 ± 0.07	4	-9	1.85 ± 0.05	3	-8	1.88 ± 0.02	1	-6	1.94 ± 0.04	2	-3	1.87 ± 0.06
20	21.39 ± 0.15	1	7	20.59 ± 0.42	2	3	20.70 ± 0.13	1	4	21.03 ± 0.42	2	5	21.68 ± 0.48	2	8	21.08 ± 0.54
80	80.69 ± 2.28	3	1	76.16 ± 2.76	3	-1	78.85 ± 2.35	3	-1	80.65 ± 3.36	4	1	82.25 ± 4.42	5	3	79.72 ± 3.76

Table 10. Process Stability for 11-OH-THC

DAY 1				DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.86 ± 0.16	9	-7	1.84 ± 0.17	9	-8	1.94 ± 0.21	11	-3	1.80 ± 0.09	5	-10	1.86 ± 0.10	5	-7	1.86 ± 0.16
20	20.54 ± 0.45	2	3	21.46 ± 0.58	3	7	21.08 ± 0.72	3	5	21.20 ± 0.61	3	6	21.22 ± 0.56	3	6	21.10 ± 0.66
80	80.20 ± 2.10	3	0	85.96 ± 1.78	2	7	85.60 ± 1.89	2	7	84.60 ± 1.00	1	6	83.98 ± 6.65	8	5	84.07 ± 3.94

Table 11. Process Stability for THC-COOH

DAY 1				DAY 2			DAY 3			DAY 4			DAY 5			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.72 ± 0.04	2	-14	1.76 ± 0.12	7	-12	1.66 ± 0.08	5	-17	1.70 ± 0.06	4	-15	1.70 ± 0.06	4	-15	1.71 ± 0.08
20	19.82 ± 0.29	1	-1	20.02 ± 0.21	1	0	19.90 ± 0.31	2	-1	19.96 ± 0.30	2	0	19.48 ± 0.23	1	-3	19.84 ± 0.33
80	79.90 ± 0.68	1	0	79.66 ± 0.90	1	0	80.36 ± 1.04	1	0	80.56 ± 1.56	2	1	83.16 ± 2.79	3	4	80.73 ± 2.02

Table 12. Freeze/Thaw Stability for THC

FRESH				CYCLE 1			CYCLE 2			CYCLE 3			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.83 ± 0.02	1	-9	1.92 ± 0.05	3	-4	1.79 ± 0.10	5	-10	1.67 ± 0.01	1	-17	1.80 ± 0.11
20	21.39 ± 0.15	1	7	22.23 ± 1.15	5	11	19.68 ± 1.03	5	-2	22.68 ± 1.87	8	13	21.50 ± 1.67
80	80.69 ± 2.28	3	1	81.84 ± 1.08	1	2	74.10 ± 2.06	3	-7	84.15 ± 1.43	2	5	80.80 ± 4.14

Table 13 Freeze/Thaw Stability for 11-OH-THC

FRESH				CYCLE 1			CYCLE 2			CYCLE 3			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.86 ± 0.15	8	-8	1.86 ± 0.10	5	-7	1.83 ± 0.10	5	-9	1.91 ± 0.09	5	-5	1.86 ± 0.12
20	20.53 ± 0.43	2	3	22.03 ± 0.96	4	10	20.48 ± 1.21	6	2	21.54 ± 0.93	4	8	21.15 ± 1.14
80	80.20 ± 2.09	3	0	87.96 ± 2.94	3	10	77.89 ± 3.19	4	-3	81.54 ± 1.53	2	2	81.90 ± 4.51

Table 14. Freeze/Thaw Stability for THC-COOH

FRESH				CYCLE 1			CYCLE 2			CYCLE 3			
Target (ng/mL)	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Mean (ng/mL)	CV%	E%	Grand Mean (ng/mL)
2	1.72 ± 0.04	2	-14	1.96 ± 0.07	4	-2	2.14 ± 0.08	4	7	2.06 ± 0.10	5	3	1.97 ± 0.18
20	19.82 ± 0.29	1	-1	21.75 ± 1	5	9	22.63 ± 3	13	13	23.81 ± 1.28	5	19	22.00 ± 2.25
80	79.90 ± 0.68	1	0	84.22 ± 1.18	1	5	86.12 ± 3.77	4	8	87.70 ± 2.03	2	10	84.48 ± 3.68

4.2.6 Ion Suppression/Enhancement, Drug Interference, and Recovery

Ion suppression was observed for all tissue specimens for all analytes. However, the deuterated internal standard used also suffered similar suppression. Therefore, the suppression observed affected the drug and internal standard in the same way, and suppression did not affect the quantitation of any compounds.

Cross-contribution and ion suppression/enhancement from THC, 11-OH-THC, and THC-COOH to one another was evaluated. After a comparison of the area counts of each analyte, it was found that there was no observed interference or cross contribution observed.

Drug interference was evaluated by spiking an 80 ng/mL of THC, 11-OH-THC, and THC-COOH control with 10 commonly encountered drugs to determine if they effected the amount of drug detected in a specimen. It was found that none of the tested compounds interfered with the detection of THC, 11-OH-THC, or THC-COOH, as all of the controls hit within 20% of the target concentration.

Recovery was evaluated by comparing the area count of spiked blanks to extracted 2, 20, and 80 ng/mL controls. The response ratios were evaluated to determine the percentage of recovery. Recovery for all analytes at each target concentration can be seen in Table 17.

Table 15. Ion suppression for 2 ng/mL

Ion Suppression 2 ng/mL							
Analyte	**Blood**	**Urine**	**Serum**	**Liver**	**Lung**	**Brain**	**Muscle**
THC	-18.1	-3.2	-20.3	-81.8	-68.4	-52.3	-46.6
THC-d_3	-10.3	-0.4	-18.1	-79.0	-69.6	-50.6	-43.5
11-OH-THC	-13.7	-15.8	-22.5	-51.3	-41.8	-39.3	-33.0
11-OH-THC-d_3	-8.8	-4.6	-9.7	-46.3	-40.9	-33.0	-25.6
THC-COOH	-23.5	-22.5	-40.8	-68.3	-74.9	-52.0	-56.7
THC-COOH-d_3	-17.3	-12.8	-46.0	-69.9	-69.3	-50.2	-56.2

Table 16. Ion suppression for 80 ng/mL

Ion Suppression 80 ng/mL							
Analyte	**Blood**	**Urine**	**Serum**	**Liver**	**Lung**	**Brain**	**Muscle**
THC	4.2	2.5	-12.3	-72.4	-69.7	-48.2	-80.3
THC-d_3	2.0	2.0	-13.4	-72.3	-62.5	-47.4	-80.1
11-OH-THC	-1.3	2.4	-8.1	-20.4	-44.8	-29.8	-49.5
11-OH-THC-d_3	-2.5	2.1	-7.0	-24.4	-48.4	-32.0	-51.1
THC-COOH	-13.4	-10.2	-32.4	-49.7	-65.5	-53.2	-63.8
THC-COOH-d_3	-16.1	-10.9	-41.3	-54.7	-66.8	-45.5	-66.0

Table 17. Recovery of analytes in percentage.

Concentration ng/mL	THC %	11-OH-THC %	THC-COOH %
2	35	11	26
20	32	12	31
80	34	10	31

4.2.7 Dilution integrity

Some cases have specimens have concentrations above the highest calibrator and required dilution to get the value within the curve. Therefore, we evaluated whether dilution of a specimen affects it determined concentration. Five 1:10 dilutions of an 80 ng/mL and 1:100 of a 1 ug/mL control were extracted and evaluated. All dilutions were within 20% of the expected concentration.

4.3 Data Analysis

ANOVA and Dunnett's Post Test evaluated statistical difference for the freeze-thaw stability and on-instrument stability for the 80 ng/mL controls. For THC, the second and third freeze thaw cycles were significantly different than the first day with values of $p < 0.001$ and $p < 0.05$ respectively. For THC-COOH, the values were significantly different with $p < 0.05$, $p < 0.01$, and $p < 0.001$ for freeze-thaw cycles 1, 2, and 3, respectively. There was no significant

difference amongst any of the cycles for 11-OH-THC. Although there was significant difference, all values were still within 20% of the 80 ng/mL.

For the on-instrument stability, there was no significant difference for THC and 11-OH-THC between the fresh control and the same controls that were ran for five consecutive days after. THC-COOH had a significant difference of $p < 0.05$ on the fifth day of post-extraction analysis, but all values were still within 20% of the 80 ng/mL.

4.4 Postmortem Fluid and Tissue Concentrations

A search of the ToxFlo database resulted in 11 cases between 2014- 2016 that had most of the desired specimens. The tested fluids and tissues in these cases, if available, were blood, urine, bile, liver, lung, kidney, spleen, muscle, brain, heart, and vitreous humor.

4.4.1 Urine Glucuronide Control

A THC and THC-COOH glucuronide control was run with all urine specimens. The target concentration was 25 ng/mL. The control hit within 20% with the THC value being 25.2 ng/mL and the THC-COOH being 20.4 ng/mL.

4.4.2 Case Studies

The concentrations of THC, 11-OH-THC, and THC-COOH are displayed in Tables 18, 19, and 20 respectively. Cases 4 and 7 had the highest concentrations of THC and were able to provide a lot of data on 11-OH-THC. In these tables, "neg" represented that the specimen did not have a detected concentration and "—" represented that a specimen was not available for analysis.

4.4.3 Postmortem Fluid Concentrations

Blood

Concentration of THC in the blood ranged from 0.76 ng/mL to 8.39 ng/mL. Only four cases showed data for 11-OH-THC and ranged between 1.03 to 4.59 ng/mL. THC-COOH was detected in all specimens and ranged from 2.37 to 70.2 ng/mL. In the specimens where 11-OH-THC was detected, the concentration for THC-COOH was the highest.

Urine

Concentration of THC in the urines ranged from 1.25 to 32.2 ng/mL. The specimen with the highest THC concentration also had the highest 11-OH-THC and THC-COOH concentrations with 620 ng/mL and 970 ng/mL respectively. The concentration of 11-OH-THC ranged from 11.7 to 620 ng/mL and was detected in all of the cases where urine was available for analysis. The concentration of THC-COOH ranged from 24.2 to 970 ng/mL.

Bile

THC was detected in bile in 5 of 9 cases and ranged from 0.73 to 50.39 ng/mL. Both 11-OH-THC and THC-COOH were detected in all bile specimens that were available. Concentrations for 11-OH-THC were 1.93 to 227 ng/mL. Bile provided the highest concentrations for THC-COOH of all the specimens with ranges from 93.0 to 3,307 ng/mL. The same specimen had the highest concentration for all three analytes.

Vitreous Humor

There were only two cases that had vitreous humor available for analysis. Neither case detected THC or 11-OH-THC. One case detected THC-COOH at 2.7 ng/mL.

4.4.4 Postmortem Tissue Concentrations

Liver

THC in liver was only detected in two cases. These concentrations were 22.3 and 52.2 ng/mL. Six livers were positive for 11-OH-THC and ranged from 1.27 to 66.1 ng/mL. The two cases with the highest concentrations of 11-OH-THC were 38.6 and 66.1 ng/mL, which were the same two cases where THC was detected. THC-COOH was detected in all liver samples and the concentration ranged from 28.5 to 2,238 ng/mL.

Lung

The observed concentrations of THC in the lungs was 1.82 to 151ng/mL. Two of the lungs were unsuitable for analysis because neither the drug nor the internal standard could be detected after extraction. If the concentration of THC in the lung was higher, as observed in cases 4, 5, 7, and 9, 11-OH-THC could also be detected. The range of 11-OH-THC was 2.18 to 42.6 ng/mL. THC-COOH was detected in all lung specimens between 1.31 to 205ng/mL. The lung with the highest concentration of THC also had the highest concentration of THC-COOH.

Kidney

THC was detectable in 8 of 11 kidneys studied, two kidneys were negative and one was unsuitable for analysis. These concentrations ranged from 0.99 ng/mL to 450 ng/mL. Four cases detected 11-OH-THC, ranging from 1.34 to 17.9 ng/mL. Concentrations of THC-COOH were high ranging from 10.1 to 1,774 ng/mL.

Spleen

THC was detected in spleen in concentrations ranging from 0.77 to 20.0 ng/mL. THC-COOH was detected in 9 of the 11 cases, ranging from 2.06 to 284 ng/mL. The two cases with the highest THC concentrations, 16.9 and 20.0 ng/mL, were the only cases where 11-OH-THC was

detected, with concentrations of 19.8 and 13.8 ng/mL respectively. Additionally, these two cases had the highest THC-COOH concentrations with 284 and 62.9 ng/mL.

Muscle

Ten of eleven cases showed concentrations of THC in the muscle. These concentrations ranged from 1.19 to 377 ng/mL. The second highest concentration of THC in the muscle was 81.4 ng/mL. Only two muscles detected 11-OH-THC and this was at 10.9 and 12.0 ng/mL.

Brain

THC was detected in 6 of 11 cases ranging from 1.34 to 43.6 ng/mL. Each brain that had detectable THC also had detectable 11-OH-THC, that ranged in concentration from 0.99 to 37.4 ng/mL. Each brain that was tested showed a concentration of THC-COOH. These concentrations ranged from 0.83 to 73.4 ng/mL. The same specimen had the highest concentration for all three analytes. Specimens where THC was not detected had the lowest values of THC-COOH.

Heart

Concentrations in the heart varied ranging from 1.70 to 158 ng/mL for THC. Only two hearts had a concentration of 11-OH-THC, which were 8.60 and 29.9 ng/mL. These two hearts were the ones that had the highest concentration of THC. THC-COOH was detected in 4 hearts ranging from 3.16 to 346 ng/mL. The two hearts that contained concentrations of 11-OH-THC had the highest concentrations of THC-COOH.

Postmortem distribution coefficients can be seen in Tables 21, 22, and 23. The postmortem distribution coefficient was calculated with the equation below. The purpose of the distribution coefficient is to determine if one specimen has a concentration that is consistently

higher or lower than the blood concentration. If the blood did not have a concentration of an analyte, a coefficient could not be calculated.

$$Distribution\ Coefficient = \frac{[fluid\ or\ tissue]}{[blood]}$$

The ratio of 11-OH-THC to THC is displayed in Table 24 and the ratio of THC-COOH to THC is displayed in Table 25. This ratio would show how the concentration within a specimen related to each other to possibly find a consistency. These ratios were calculated in each specimen using the equations shown below. If a fluid or tissue did not have a concentration of one of the analytes, a ratio could not be calculated.

$$Ratio = \frac{[11-OH-THC]}{[THC]}$$

$$Ratio = \frac{[THC-COOH]}{[THC]}$$

Table 18. THC Fluid and Tissue Concentrations (ng/mL)

THC Fluid and Tissue Concentrations (ng/mL)											
Case	**Blood**	**Urine**	**VH**	**Liver**	**Lung**	**Kidney**	**Spleen**	**Muscle**	**Brain**	**Heart**	**Bile**
1	Neg	1.25	--	Neg	Neg	Neg	0.77	5.21	Neg	Neg	0.73
2	4.03	—	—	Neg	14.1	22.56	1.31	7.37	3.71	Neg	14.17
3	0.76	1.26	—	Neg	1.82	1.91	Neg	2.43	Neg	Neg	Neg
4	1.78	1.43	Neg	52.17	143.3	450	16.91	81.35	43.59	150.47	50.39
5	3.45	Neg	—	Neg	42.57	32.32	5.14	11.88	—	1.7	6.35
6	Neg	1.35	Neg	Neg	Neg	Neg	Neg	5.31	Neg	6.58	Neg
7	4.67	32.19	—	22.33	150.5	16.38	19.99	376.9	30.57	158	—
8	1.5	—	—	Neg	48.1	9.08	0.96	1.19	1.34	5.35	Neg
9	5	—	—	Neg	62.61	5.21	5.58	3.24	16.4	54.7	—
10	Neg	—	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
11	8.39	3.62	—	Neg	Unsuit	0.99	6.39	3.1	8.8	Neg	5.6

Table 19. 11-OH-THC Fluid and Tissue Concentrations (ng/mL)

11-OH-THC Fluid and Tissue Concentrations (ng/mL)											
Case	**Blood**	**Urine**	**VH**	**Liver**	**Lung**	**Kidney**	**Spleen**	**Muscle**	**Brain**	**Heart**	**Bile**
1	Neg	13.47	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	5.13
2	Neg	—	—	Neg	Neg	Neg	Neg	Neg	3.31	Neg	21.17
3	Neg	13.39	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	16.81
4	2.02	60.56	Neg	66.11	12.69	17.87	19.76	10.89	37.4	29.9	227.4
5	Neg	43.28	—	4.04	1.15	1.34	Neg	Neg	—	Neg	20.64
6	Neg	11.66	Neg	1.27	Neg	Neg	Neg	Neg	Neg	Neg	0.98
7	4.59	620	—	38.61	6.95	10.47	13.79	11.96	30.82	8.6	—
8	Neg	—	—	Neg	Neg	Neg	Neg	Neg	0.99	Neg	1.93
9	1.03	—	—	Neg	2.18	1.77	Neg	Neg	8.97	Neg	—
10	Neg	—	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	1.81
11	1.74	25.92	—	8.17	Neg	Neg	Neg	Neg	3.01	Neg	61.66

Table 20. THC-COOH Fluid and Tissue Concentrations (ng/mL)

THC-COOH Fluid and Tissue Concentrations (ng/mL)											
Case	**Blood**	**Urine**	**VH**	**Liver**	**Lung**	**Kidney**	**Spleen**	**Muscle**	**Brain**	**Heart**	**Bile**
1	2.37	24.23	—	28.47	1.81	12.14	2.06	0.83	0.83	Neg	290.6
2	52.29	—	—	59.86	7.93	47.43	7.54	Neg	4.66	Neg	1000
3	3.13	109	—	29.08	3.11	10.1	Neg	0.8	0.98	Neg	564.7
4	68.02	102	2.7	333.1	204.8	1774	284.4	66.83	73.41	345.8	3307
5	31.97	219	—	351.9	30.7	225.8	25.8	7.23	—	Neg	231.28
6	2.62	87.55	Neg	57.25	1.31	33.57	2.83	1.17	2.52	3.32	201.1
7	70.16	970	—	2238	31.29	1482	62.86	27.02	28.79	59.64	—
8	4.6	—	—	76.97	2.66	45.62	4.63	1.76	2.54	3.16	309.8
9	9.32	—	—	46.3	5.94	107.2	10.54	0.87	7.51	Neg	—
10	4.46	—	—	36.62	3.55	65.13	Neg	2.34	1.85	Neg	93.07
11	65.72	189	—	528.5	50	222.2	15.71	10.15	12.25	Neg	301.0

Table 21. THC Distribution Coefficients

THC Distribution Coefficients										
Case	**Urine/ Blood**	**VH/ Blood**	**Liver/ Blood**	**Lung/ Blood**	**Kidney/ Blood**	**Spleen/ Blood**	**Muscle/ Blood**	**Brain/ Blood**	**Heart/ Blood**	**Bile/ Blood**
1	—	—	—	—	—	—	—	—	—	—
2	—	—	—	3.50	5.60	0.325	1.83	0.921	—	3.52
3	1.66	—	—	2.40	2.51	—	3.20	—	—	—
4	0.80	—	29.3	80.5	253	9.50	45.7	24.5	84.5	28.3
5	—	—	—	12.3	9.37	1.49	3.44	—	0.493	1.84
6	—	—	—	—	—	—	—	—	—	—
7	6.89	—	4.78	32.2	3.50	4.28	80.7	6.55	33.8	—
8	—	—	—	32.1	6.05	0.640	0.793	0.893	3.57	—
9	—	—	—	12.5	1.04	1.12	0.648	3.28	10.9	—
10	—	—	—	—	—	—	—	—	—	—
11	0.43	—	—	—	0.118	0.762	0.369	1.05	—	0.667
n	4	0	2	7	8	7	8	6	5	4
Mean	2.4	—	17	25	35	2.5	17	6.2	27	9
SD	2.6	—	12	25	82	3.0	28	8.4	31	11
CV %	108	—	72	101	234	119	164	136	117	133

Table 22. 11-OH-THC Distribution Coefficients

11-OH-THC Distribution Coefficients										
Case	**Urine/ Blood**	**VH/ Blood**	**Liver/ Blood**	**Lung/ Blood**	**Kidney/ Blood**	**Spleen/ Blood**	**Muscle/ Blood**	**Brain/ Blood**	**Heart/ Blood**	**Bile/ Blood**
1	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—
4	30.0	—	32.7	6.28	8.85	9.78	5.39	18.5	14.8	113
5	—	—	—	—	—	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—	—
7	135	—	8.41	1.51	2.28	3.00	2.61	6.72	1.87	—
8	—	—	—	—	—	—	—	—	—	—
9	—	—	—	2.12	1.72	—	—	8.71	—	—
10	—	—	—	—	—	—	—	—	—	—
11	14.9	—	4.70	—	—	—	—	1.73	—	35.4
n	3	0	3	3	3	2	2	4	2	2
Mean	60	—	15	3	4	6	4	9	8	74
SD	53	—	12	2	3	3	1	6	6	39
CV %	89	—	81	64	76	53	35	68	78	52

Table 23. THC-COOH Distribution Coefficients

THC-COOH Distribution Coefficients										
Case	**Urine/ Blood**	**VH/ Blood**	**Liver/ Blood**	**Lung/ Blood**	**Kidney/ Blood**	**Spleen/ Blood**	**Muscle/ Blood**	**Brain/ Blood**	**Heart/ Blood**	**Bile/ Blood**
1	10.2	—	12.0	0.764	5.12	0.869	0.350	0.350	—	123
2	—	—	1.15	0.152	0.907	0.144	—	0.089	—	19.1
3	34.8	—	9.29	0.994	3.22	—	0.256	0.313	—	180
4	—	0.040	4.90	3.01	26.1	4.18	0.983	1.08	5.08	48.6
5	6.85	—	11.0	0.960	7.06	0.807	0.226	—	—	7.23
6	33.4	—	21.9	0.500	12.8	1.08	0.447	0.962	1.27	76.8
7	13.8	—	31.9	0.446	21.1	0.896	0.385	0.410	0.850	—
8	—	—	16.7	0.578	9.92	1.01	0.383	0.552	0.687	67.3
9	—	—	4.97	0.637	11.5	1.13	0.093	0.806	—	—
10	—	—	8.21	—	14.6	—	0.525	0.415	—	20.9
11	2.88	—	8.04	0.761	3.38	0.239	0.154	0.186	—	4.70
n	6	1	11	10	11	9	10	10	4	9
Mean	17	0.04	8	0.9	11	1	0.4	0.5	2	61
SD	13	—	8	0.7	7	1	0.2	0.3	2	56
CV %	74	—	71	85	71	97	62.4	60.8	92	91

Table 24. Ratio of 11-OH-THC to THC

Ratio of 11-OH-THC to THC											
Case	**Blood**	**Urine**	**VH**	**Liver**	**Lung**	**Kidney**	**Spleen**	**Muscle**	**Brain**	**Heart**	**Bile**
1	—	10.8	—	—	—	—	—	—	—	—	7.03
2	—	—	—	—	—	—	—	—	0.892	—	1.49
3	—	10.6	—	—	—	—	—	—	—	—	—
4	1.14	42.4	—	1.27	0.089	0.040	1.17	0.134	0.858	0.199	4.51
5	—	—	—	—	0.041	0.041	—	—	—	—	3.25
6	—	8.64	—	—	—	—	—	—	—	—	—
7	0.983	19.3	—	1.73	0.046	0.639	0.690	0.032	1.01	0.054	—
8	—	—	—	—	—	—	—	—	0.739	—	—
9	0.206	—	—	—	0.035	0.340	—	—	0.547	—	—
10	—	—	—	—	—	—	—	—	—	—	—
11	0.207	7.16	—	—	—	—	—	—	0.342	—	11.0
n	4	6	0	2	4	4	2	2	6	2	5
Mean	0.6	16	—	1.5	0.05	0.3	0.9	0.08	0.7	0.1	5
SD	0.4	12	—	0.2	0.02	0.2	0.2	0.05	0.2	0.1	3
CV %	68.0	74	—	15.3	40.36	93.6	25.8	61.45	30.8	57.3	61

Table 25. Ratio of THC-COOH to THC

Ratio of THC-COOH to THC											
Case	**Blood**	**Urine**	**VH**	**Liver**	**Lung**	**Kidney**	**Spleen**	**Muscle**	**Brain**	**Heart**	**Bile**
1	—	19.4	—	—	—	—	2.68	0.159	—	—	398
2	13.0	—	—	—	0.562	2.10	5.76	—	1.26	—	70.6
3	4.12	86.5	—	—	1.71	5.29	—	0.329	—	—	—
4	38.2	71.3	—	6.39	1.43	3.94	16.8	0.822	1.68	2.298	65.6
5	9.27	—	—	—	0.721	6.99	5.02	0.609	—	—	36.4
6	—	64.9	—	—	—	—	—	0.220	—	0.505	—
7	15.0	30.1	—	100	0.208	90.5	3.15	0.072	0.942	0.377	—
8	3.07	—	—	—	0.055	5.02	4.82	1.48	1.90	0.591	—
9	1.86	—	—	—	0.095	20.6	1.89	0.269	0.458	—	—
10	—	—	—	—	—	—	—	—	—	—	—
11	7.83	52.2	—	—	—	224	2.46	3.27	1.39	—	55.2
n	8	6	0	2	7	8	8	9	6	4	5
Mean	12	54	—	53	0.7	45	5	0.8	1.3	0.9	125
SD	11	23	—	47	0.6	73	5	1.0	0.5	0.8	137
CV %	95	43	—	88	89.1	163	85	120.1	37.3	83.4	109

CHAPTER V

DISCUSSION

With the number of adult marijuana users increasing, there is a need for a robust and sensitive method to detect THC, 11-OH-THC, and THC-COOH.[39] A previous study has also shown that the average blood concentration of THC in marijuana-positive pilots has increased from 2.7 ng/mL to 7.2 ng/mL from 1997-2006.[40] This finding is consistent with studies that have shown an increase in the potency of cannabis.[11,41] This increase in prevalence and the fact that the FAA laboratory relies on tissues in 40% of its cases has emphasized the need to understand the distribution of cannabinoids throughout the body.

Following the successful development and validation of an extraction method and LC/MS/MS separation and quantitation method, postmortem fluids and tissues from 11 cases were analyzed to evaluate the postmortem concentrations and distribution of cannabinoids. Since approximately 40% of cases received by the FAA do not have blood available, an understanding of the postmortem distribution of cannabinoids could aid in the interpretation of THC concentrations in postmortem tissue specimens.

5.1 Method Development

The primary goal of this research was to develop a rapid, sensitive, and robust method for the identification and quantitation of THC, 11-OH-THC, and THC-COOH in postmortem fluids and tissues. The method that was developed was not only able to detect all three analytes at levels as low as 0.78 ng/mL, but it has also provided the laboratory with an extraction procedure that saves time and solvent quantities.

A Waters® Xevo® TQ-S Acquity® UPLC was chosen for this particular project because the Waters Xevo excels at small molecule detection and the UPLC, through the nature of the very small diameter column packing material, is able to provide superior separation compared to conventional HPLC. Chromatographic separation was achieved using a gradient method with a flow rate of 0.60 mL/min through an Acquity UPLC® BEH C18 1.7 μm, 2.1 x 100mm column. The analytical method is 4 minutes and the retention times of THC, 11-OH-THC, and THC-COOH are 2.41, 1.43, and 1.48, respectfully. Although 11-OH-THC and THC-COOH do not have baseline separation, the two analytes have different precursor ions, so there was no interference or cross-talk.

5.1.2 Attempted Extractions

Numerous extraction methods were evaluated for the extract of THC, 11-OH-THC, and THC-COOH from postmortem fluids and tissues. Different liquid-liquid extractions and solid-phase columns were evaluated to determine the best extraction for all specimen types. Although a simple "crash and shoot" method or a liquid-liquid extraction was desired because of the simplicity and reduction in extraction time, such extractions did not provide enough clean-up of tissue specimens and was unable to extract all of the desired analytes to a suitable level needed for our research samples.

A review of the literature showed that a large number of extractions utilized some composition of hexane:ethyl acetate in liquid-liquid extractions.[36,42–44] We initially evaluated

different hexane:ethyl acetate compositions for the liquid-liquid extraction, including 50:50, 90:10, and 75:25. Using 0.5 mL of liquid sample or 1.5 mL of tissue homogenate and buffering the specimen with 0.1 M Sodium Acetate buffer pH 4.0, we found that the 75:25 hexane:ethyl acetate worked extremely well for blood and urine. However, the liquid-liquid extraction was unsuitable for tissue specimens.

Numerous solid-phase extractions (SPE) were attempted. These extraction methods were found from literature and application procedures obtained from various vendor websites.[45,46,46–48] Many of these worked for blood and urine, but were unable to extract the desired analytes from tissue samples.

The current laboratory procedure that analyzes THC and THC-COOH by GC/MS was used to see if it is suitable for all three analytes. This extraction begins with a protein crash of blood and tissue samples using ice cold acetonitrile. Specimens are centrifuged, the supernatant transferred to a new tube, dried down to approximately 2 mL, and 4 mL of 0.1 M Sodium Acetate pH 7.0 was added to each tube. The solid-phase extraction columns used are Bond Elute-LRC Certify II, 200 mg sorbent bed. Columns are conditioned with 2 mL of methanol and 2 mL of 0.1 M Sodium Acetate buffer pH 7.0. Samples are then run through the column at approximately 1 mL/min. Columns are rinsed with 1 mL of 0.1 M Sodium Acetate buffer pH 7.0 and then dried for 5 minutes. THC is eluted by passing 3 mL of 95:5 hexane:ethyl acetate through the column into conical tubes. The columns are then rinsed again with 4 mL of 1:1 methanol:DI water. THC-COOH is eluted with 3 mL of 75:25 hexane:ethyl acetate with 1% acetic acid into the same conical tube as the THC elution. The tubes were dried down and reconstituted with 50 μL of methanol. When used for GC/MS analysis, specimens are derivatized so they can be analyzed using GC/MS; however, since specimens will be analyzed using LC/MS/MS, derivatization is not necessary. This extraction worked to extract all analytes; however, the THC was extremely noisy so other options were explored.

5.1.3 Working Extraction Method

The method found to work used a SPE column from UCT, CSTHC column. After an acetonitrile crash to remove proteins and cellular debris, samples were centrifuged and poured into a new tube and immediately buffered with 5 mL of 0.1 Sodium Acetate buffer pH 3.

During the elution of 11-OH-THC and THC-COOH, it was noticed that a small amount of water came off of the columns. At first, evaporation until dryness was attempted followed by reconstitution with 100 µL methanol, but the area counts of multiple 100 ng/mL standards were inconsistent. A test of ten 100 ng/mL controls where tubes were evaporated to dryness and ten 100 ng/mL controls where a small amount of water was left in the bottom was conducted. The amount of water in each of the "water" tubes was measured and was all the amounts were between 55-65 µL. Each of the samples were then reconstituted in 100 µL methanol. It was found that the area counts of the analytes where water was left were reproducible, whereas the standards that were taken to dryness varied greatly. It was decided that for the best results, samples should be dried down until all of the hexane and ethyl acetate was removed and the water should be kept.

This extraction was successful in all desired tissue types for all desired analytes. The extraction is simple and robust, allowing for accurate quantitation of THC, 11-OH-THC, and THC-COOH in postmortem fluids and tissues.

5.2 Postmortem Distribution of Fluids and Tissues

Whenever an aviation accident occurs, specimens are sent to the FAA's Forensic Toxicology Research Laboratory for toxicological analysis. There were 11 postmortem cases that were chosen for analysis between the years of 2014-2016. These cases had been previously confirmed positive by GC/MS. If available, the fluids and tissues tested in this study were: blood, urine, bile, liver, lung, kidney, spleen, muscle, brain, heart, and vitreous humor.

Although there are many studies that discuss the pharmacokinetics and pharmacodynamics of THC, 11-OH-THC, and THC-COOH, this information is beyond the scope of this paper. This study focused on developing a suitable analytical method and the postmortem distribution of THC, 11-OH-THC, and THC-COOH .

5.2.1 Postmortem Fluid Concentrations

It was expected that concentrations of THC in the blood would be low due to the high volume of distribution of THC.[3,13] However, detection of higher concentrations of THC-COOH than THC in the blood agreed with another study, that concluded that THC-COOH was able to be detected in the blood for a longer period of time than THC or 11-OH-THC.[14]

In the urine, THC, 11-OH-THC, and THC-COOH form a glucuronide that must be cleaved before analysis.[13] A 25 ng/mL THC and THC-COOH glucuronide control was hydrolyzed, extracted, and run with specimens to ensure that they hydrolysis method chosen would produce accurate results. The control hit the target concentration within 20% with a THC concentration of 25.2 and a THC-COOH concentration of 20.4 ng/mL. High levels of 11-OH-THC and THC-COOH were seen in all urine specimens. Studies have suggested that it is possible that higher levels of 11-OH-THC could mean that an individual is a chronic smoker and/or has smoked marijuana recently.[13,44]

High concentration of THC-COOH in the bile were similar to findings in another distribution study conducted that looked at the concentrations of THC-COOH in urine, heart blood, vitreous humor, and bile.[49] It was expected to see high concentrations of THC-COOH, but not to the extent to which they were seen in these samples. Although only two cases had vitreous humor available for analysis, the results were consistent with a study that found very small concentrations of cannabinoids, if any, in vitreous humor specimens.[45]

5.2.2 Postmortem Tissue Concentrations

It was expected that the tissue specimen with the highest concentration of THC would be detected in the lung, and the study confirmed this result. Analysis of the lung is especially important in this distribution study since inhalation is the most common route of administration of THC. After smoking marijuana, THC is quickly absorbed by the lungs due to its state of being highly perfused.[13] Higher concentartions of THC in the lung also showed detectable levels of 11-OH-THC.

Another distribution study also found high concentrations of THC-COOH in several postmortem tissues and found that liver had one of the highest retentions of cannabinoids.[34] The liver contains the enzyme cytochrome P450, which is responsible for the metabolism of THC to 11-OH-THC and further to THC-COOH.[13] This could be a reason why 11-OH-THC was detected in the liver in half of the cases.[50]

Cannabinoid concentrations in the kidney were surprising. Case 4 had a THC concentration of 450 ng/mL, which was the highest of all cases. It also had the highest amount of THC for the lung, liver, brain, heart, and bile. The most likely reason the THC-COOH concentrations were high is because the function of the kidneys is to filter and help remove waste. High concentrations of THC-COOH in the kidney was also found by Kemp et al. and Brunet et al.[34,51] The data shows that the kidneys would be a good specimen to analyze to analyze because it will most likely have THC and THC-COOH present.

The brian is also a highly perfused organ and THC is quickly able to cross the blood brain barrier. The detection of cannabinoids in the brain, even if they were absent in the blood agreed with a study conducted by Mura et al.[52] This study also tested different portions of the brain to compare the concentrations and found that different portions of the brain result in different concentrations. The portion of the brain that was analyzed for the 11 cases in this thesis were unknown. Concentrations of THC and 11-OH-THC in the brain were found in 6 of 9 cases,

while THC-COOH was detected in all 9. Although the portion of the brain tested is unknown, the brain would be a good tissue to analyze for the presence of cannabinoids.

Interpretation of the muscle and the heart can be difficult because it is unknown what muscle and what portion of the heart is being tested. This could be a reason why there is such a wide range of concentrations detected. However, it was consistent that only high levels of THC showed detectable concentrations of 11-OH-THC.

5.3 Distribution and Ratio Coefficients of Cannabinoids

There was no consistent distribution coefficient of any of the evaluated cannabinoids within any of the specimens tested. The coefficient of variations for distribution coefficients was high for all of the analytes throughout the different specimen types. As a result of no consistency in distribution coefficients between fluid or tissue specimens and blood, concentrations from non-blood fluids and tissues relatively meaningless and have little value as an interpretive aid in estimating the concentrations of THC, 11-OH-THC, or THC-COOH in blood. he ratios of 11-OH-THC to THC and THC-COOH to THC also did not show a consistency among any fluid or tissue specimens.

There are numerous reasons why there could be a lack of correlation of the distribution between tissues. Individualizing characteristics such as gender and body composition could influence how the analytes distribute through the body. Since THC is a lipophilic, if an individual has a higher BMI, it is possible that more of the THC could be stored in adipose tissue rather than organs.

The Forensic Toxicology Laboratory has no control of the specimens that arrive after an aviation accident. It is often unknown where blood samples are collected from and the entire organ is not always submitted for analysis. It is also possible that degradation of the analytes may have occurred.

Concentrations detected in tissue specimens could also vary based on the portion of the tissue that is provided for analysis. For example, the concentration of the brain in one portion of the brain may differ than another region. Although homogenization helps keep the concentration consistent within a given sample, it can still vary based on the location of the organ that is tested.

When the body dies, postmortem changes begin instantly. Cells begin degrading and postmortem redistribution can occur. The concentration of analytes that are subject to postmortem redistribution vary based on sampling site and the time sample occurs after death.[53] A study that examined PMR of cannabinoids between central and peripheral blood found that the longer amount of time between death and sampling, the greater amount of postmortem redistribution would occur.[32]

There are also many factors concerning the cannabis itself that could affect the distribution. Since these are postmortem cases, much information regarding the individual's cannabis intake is unknown, such as time of last use, how often they used cannabis, the potency, and if they smoked marijuana or consumed the THC orally by pill or in an edible.

Concentrations within the body also would vary based on how often the individual used cannabis. Studies show that chronic users could have a concentration of THC and THC-COOH in their blood after 7 days of not using cannabis.[54] This could mean that the pilot smoked marijuana several days before the aviation accident and it was just still able to be detected in the fluid and tissue specimens.

The increase in cannabis potency could also lead to a difference in the distribution and how long it remains in the body. A previous study concluded that there has been an increase in the concentration of THC detected in aviation accidents between 1997 and 2006.[55] Since this study was conducted ten years prior to this study, it was important to find other studies that looked at the increase of cannabis since 2006. These findings have supported that the potency has continued to rise.[11,12]

Route of administration and the concentration of THC introduced to the body could affect the concentration in the body and where analytes are detected at higher levels. For example, if an individual was to eat "pot brownies," the concentration of THC would not be as high in the lungs as it would if the individual would have smoked marijuana. This is because of the first-pass metabolism, where THC goes through the liver and is metabolized into 11-OH-THC.[13] Since ingesting marijuana takes longer for effects to be felt, an individual could consume more, trying to obtain the desired euphoric effect.[56]

5.4 Conclusion

The method developed for the extract and analysis of THC, 11-OH-THC, and THC-COOH from postmortem fluids and tissues was sensitive and robust. There are no existing studies in the literature that look at the distribution in almost every tissue type with all three of these analytes.

This study was able to look at the distribution of tehse three analytes in 11 cases. These cases had most of the desired specimens available for analysis. It was found that THC was most commonly detected in the lung, kidney, spleen, and muscle. In the brain, 11-OH-THC was detected in four cases and in three cases for lung, liver, kidney, and urine. THC-COOH was detected in almost every fluid and tissue sample, but was found to be the highest in the liver, kidney, and bile.

Limitations of this study are not knowing where blood was obtained from in the body, what muscle was being analyzed, as well as the demographics of the decedent. It is possible that the distribution is much different based on the composition of the decedent. A larger number of cases could in fact provide better distribution results and aid in interpretation of the distribution of THC, 11-OH-THC, and THC-COOH. Although a consistent distribution pattern was not observed, it was found that the best specimens to analyze were the blood, urine, lung, and kidney.

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