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Detection of delta 9-Tetrahydrocannabinol and metabolites in the meibomian lipids of tear samples through LC-MS/MS

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**DETECTION OF DELTA 9-TETRAHYDROCANNABINOL AND
METABOLITES IN THE MEIBOMIAN LIPIDS OF
TEAR SAMPLES THROUGH LC-MS/MS**

by

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B.A., Providence College, 2019

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

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ABSTRACT

Δ 9-Tetrahydrocannabinol (THC) is the most psychoactive substance out of the over 80 active cannabinoids. Due to its psychoactive and impairing properties, detection and quantitation is important to determine impairment levels of individuals. With an increased use of recreational marijuana, the risk of Driving Under the Influence of Drugs (DUID) is steadily increasing. Current legislation outlaws driving under the influence of Marijuana however there exists limitations with current methods of detection of drug analyte. Δ 9-Tetrahydrocannabinol, 11-Hydroxy- THC, and 11-nor-carboxy-THC, were used in detection because these analytes are produced in the metabolism of THC. Since THC is very lipid soluble, it is present in lipid rich environments in the body. Due to the lipid rich nature of meibomian fluid, a component of tears, and the presence of Fatty Acid Binding Protein (5) FAPB5, a protein known to bind to cannabinoids, tear fluid could be used as a less-invasive biological matrix to test for the presence of THC and its metabolites.

This project optimized a collection of tear fluid, along with a simple buffer extraction, to create a method suitable for direct injection using LC-MS/MS. Collection was completed by BVI Weck-Cel® Sterile Cellulose strips, measuring approximately 2 x 20 mm, and placed in Thompson eXtreme PVDF 0.2 μ m, pre-slit, red cap, filter vials containing Quantisal buffer solution for extraction. All analysis and calibrations were

completed with fortified matrix standards with concentrations ranging from 0.25 - 250 ng/mL. Validation was consistent with American Academy of Forensic Sciences Academy Standards Board (ASB) Standards of Forensic Toxicology Standard 036, First Edition 2018.

Tear samples were collected from volunteer patients according to Institutional Review Board (IRB) standards before and after administration of Marijuana. Samples were collected approximately 30 minutes post administration in order to capture tears when the analyte is most potent in the body. Samples and calibration standards were analyzed using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) with the QSight® 220 CR LC-MS/MS and the Halo® C18 3.0x50 mm (2.7 µm) column. Limit of Detection (LOD) and Limit of Quantitation (LOQ) for THC was calculated at 0.25 ng/mL. Limit of Detection of THCOOH was detected at 0.25 ng/mL and Limit of Quantitation was calculated at 1 ng/mL.

Upon analysis of Patient Samples, it was determined that THC and metabolites could be detected and quantitated in tear fluid. However, it is noted that insufficient sample volume in collection of this type of sample is an issue that leads to poor quantitation and should be optimized in future research.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BAC	Blood Alcohol Concentration
CNS	Central Nervous System
DED	Dry Eye Disease
DI	Deionized
DUID	Driving Under the Influence of Drugs
ESI	Electrospray Ionization
FABP	Fatty Acid Binding Protein
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
IRB	Institutional Review Board
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MRM	Multiple Reaction Monitoring
m/z	Mass to Charge ratio
PVA	Polyvinyl Alcohol
THC	Δ 9-Tetrahydrocannabinol
THCA-A	Δ 9-Tetrahydrocannabinolic acid A

THCA-B	Δ 9-Tetrahydrocannabinolic acid B
THCOOH	11-nor-carboxy-THC
11-OH-THC	11-Hydroxy-THC

1. INTRODUCTION

1.1 Background

Δ^9 -Tetrahydrocannabinol (THC) is the primary psychoactive compound in Marijuana that creates adverse, impairing effects for the user. Concentration of analyte is determined through toxicological analysis of biological fluids, also referred to as matrices. In current practice, blood and urine are matrices primarily used for the detection of THC. However, there are inherent limitations in using these matrices even though they are used with the primary methods for determination of Driving Under the Influence of Drugs (DUID). The legalization and decriminalization of Marijuana on a state and federal level has raised questions for impaired driving statutes, specifically DUID. In current practice, there is variation in legislation as to what is a legal limit of operating a motor vehicle while under the influence of Marijuana¹.

A good matrix used in detection of drug analytes involving DUID should be able to determine concentration at the time of a roadside stop. The current inherent issue with blood is the nature of time sensitivity as an invasive method. In order to collect a sample, the sample cannot be collected roadside and must be done by a certified phlebotomist. The meibomian lipids within tear samples are hypothesized to be an efficient matrix for the detection of THC due to the lipophilic nature of THC and could reduce the limitations of traditional collection methods.

1.2 Δ 9-Tetrahydrocannabinol

It has been reported that cannabis use has progressively increased over the past decade². Consumption varies among individuals, both in terms of method of consumption and frequency of consumption¹. Cannabis, contains over 421 chemical compounds and over 60 cannabinoids, with more being discovered³. Within the classification of Cannabinoids exists the sub-category of THC. Δ 9-Tetrahydrocannabinol is contained in the fruits, leaves and resin of the plant matter of Cannabis Sativa⁴. Approximately 95% of THC found within Cannabis are a combination of monocarboxylic acids that are subject to decarboxylation when heated⁴. When decarboxylated, due to heating or drying, THC is absorbed in the body and the user is able to feel the psychoactive effects⁴.

THC has 9 known cannabinoids with C1-C5 side chains^{3,5}. THC precursors can be designated into two categories: Δ 9-Tetrahydrocannabinolic acid A (THCA-A) and Δ 9-Tetrahydrocannabinolic acid B (THCA-B). These two categories represent the biogenic precursors, THCA-A being the major precursor and THCA-B being the minor precursor, being available at a much lesser percent⁵.

1.2.1 Pharmacodynamics of Δ 9-Tetrahydrocannabinol

Δ 9-Tetrahydrocannabinol is a cannabinoid with psychoactive properties, meaning that the compound has the capacity to alter the mental state⁶. THC has been shown to alter vital processes such as increased heart rate, as well as alter subjective processes. Effects include: creating dysphoria and euphoria, increased somatic effects, a mimic of stimulant effects, as well as alteration of intellectual efficiency and energy⁷. Mentioned in *Effects of*

THC on Behavioral Measures of Impulsivity in Humans (McDonald et al.), THC is more sedative in nature and shares this parallel with ethanol⁷. Marijuana has been known to have therapeutic effects as THC and Cannabidiol (CBD) have been utilized for anti-convulsion, analgesic, hypnotic, anti-emetic, and anxiolytic properties^{8,9}.

Cannabinoids effect natural processes by interacting with endogenous cannabinoid receptors^{10,11}. Distribution of specific receptors have been linked to the cerebral cortex, limbic areas, basal ganglia, cerebellum, thalamus, and brainstem¹⁰. In turn, Cannabis effects approximately every system in the body and effects both mood and cognition. As stated previously, THC is sedative in nature and produces what is described as a “high” or extreme euphoria. The high can occur seemingly instantly and can reach a plateau in which the effects could last 2 hours or more¹⁰. Spatial perception is altered when under the influence of THC and psychomotor performance is significantly impaired¹⁰. This is aligned with a dampened reaction time, impaired perception, motor incoordination, short term memory alteration, and issues in concentration¹⁰.

1.2.2 Pharmacokinetics of Δ 9-Tetrahydrocannabinol

Δ 9-Tetrahydrocannabinol can be administered in different ways. The most common practice of administration is smoking. Smoking creates a simplistic and efficient way to administer THC because of the direct heating of the Marijuana. Smoking produces a rapid method of drug delivery to the Central Nervous System^{4,12}. Within seconds of inhalation, THC can be measured in plasma⁴. THC can be administered orally and is absorbed due to its high octanol/water partition coefficient¹². THC has been administered

rectally, sublingually, and dermally although these are not common routes of administration¹².

Smoking in common practice allows approximately 20-25% of the inhaled THC to be biologically available. It has been determined by Marilyn A. Huestis that an actual dose of 0.2 to 4.4 mg of 100% THC is enough to cause psychoactive effects¹². This was calculated by taking distribution data, discovered by Adams and Martin in 1996¹³. Their study outlined a dose of 2 to 22 mg, however this did not account for the approximate 75-80% loss^{13,14}. THC distribution is a rapid process because of its affinity to lipid rich areas and metabolism by the liver. The heart, lungs, brain, and the liver are lipid rich organs that receive the THC well because of its lipophilicity¹⁴. THC has a high volume of distribution at approximately 10 l/kg and it is 95-99% protein bound to lipoproteins in plasma^{14,15}. When exposed for a long period of time, concentrations of THC are significantly increased in the body's fat molecules and can be retained for extended periods of time^{14,16}.

THC is metabolized through hepatic metabolism in two phases¹⁴. Phase I is an oxidation of the THC in which the molecule undergoes allylic and aliphatic hydroxylation, oxidation of ketones and alcohols, beta-oxidation, and degradation of the pentyl side chain¹⁴. Phase II is a glucuronidation reaction¹⁴. All pathways are visualized in Figure 1. The metabolism of THC yields an equally potent metabolite, 11-hydroxy-THC (11-OH-THC) as well as an inactive metabolite, 11-nor-9-carboxy-THC (THCCOOH), both of these metabolites being the primary metabolites of THC^{14,17,18}.

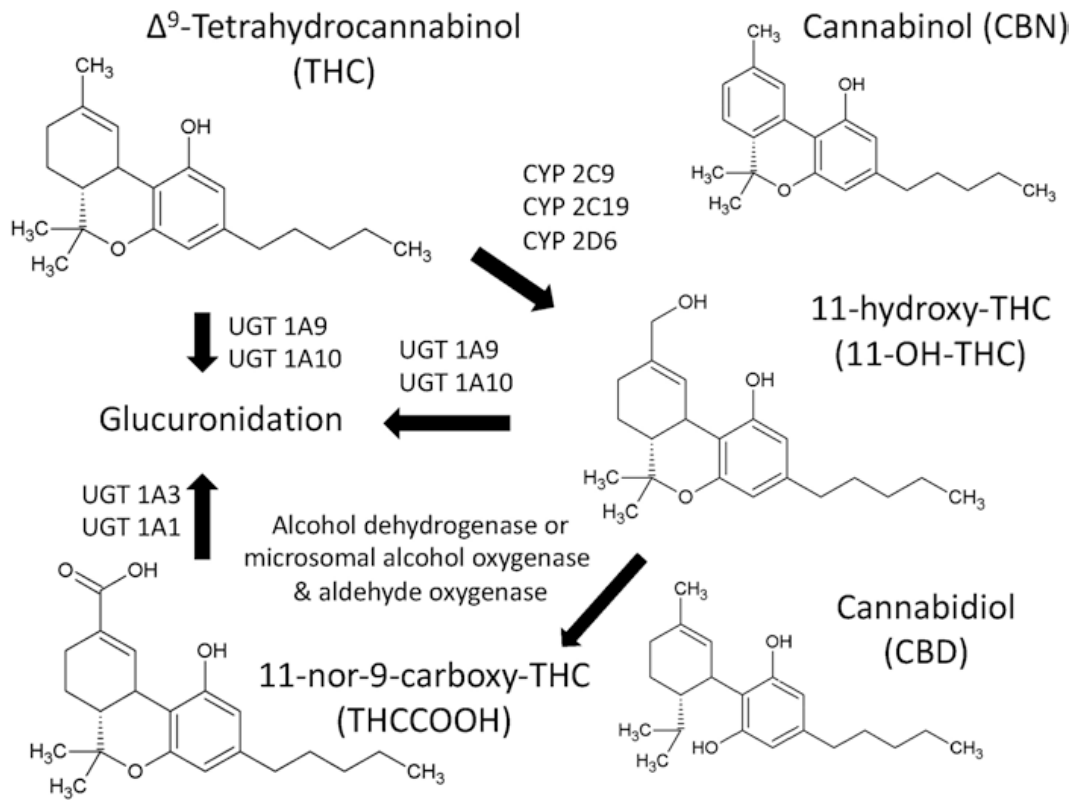


Figure 1. Metabolic process of THC¹⁹

1.2.3 Δ^9 -Tetrahydrocannabinol Affinity to Lipids

As seen in section 1.2.2, the nature of distribution of THC is extremely rapid and lipid focused. As stated before, THC has an affinity to lipid rich tissues and fluids such as brain, lung, liver, plasma, and other adipose tissues¹⁴. Normally, the lipophilic nature of THC and other cannabinoids pose problems for detection because it imposes a limitation on what materials, procedures, and matrices can be utilized. It would be of extreme benefit

to optimize a detection procedure that utilizes a lipid rich matrix that is non-invasive of nature for sample collection .

1.3 Ophthalmology: Meibomian layer of tear fluid as a probative matrix

1.3.1 Tear Composition

It is our hypothesis that THC and its metabolites can be detected from tear fluid due to the lipid rich nature. The matrix itself is made from the mixture of secretion of glands within the eye lids²⁰. Meibomian glands, also referred to as tarsal glands, are long sebaceous glands that are situated in tarsal plates of the eyelid²¹. There are approximately 45 glands per eye (25 in the upper lid, 20 in the lower lid) that maintain a central canal that secrete sebum, a lipid rich secretion. Meibomian glands are holocrine, meaning that lipids are secreted by the action of cell degradation. The purpose of these secretions are to create an oily film over the eye²¹. Tear layers are structured as an inner-mucin enriched mucous layer, a middle aqueous layer, and the outer lipid layer secreted from the meibomian glands²¹.

This layer has a function of stability as it reduces the surface tension controlling evaporation²². This keeps the eye moisture in a relatively stable condition and also prevents the intrusion of bacteria into the eye itself²⁰. The primary phospholipid in meibomian fluid is phosphatidylcholine (Figure 2), however, other phospholipids are important, such as

phosphatidylserine (Figure 3) which is important to formation of polar lipid monolayers^{20,23,24}.

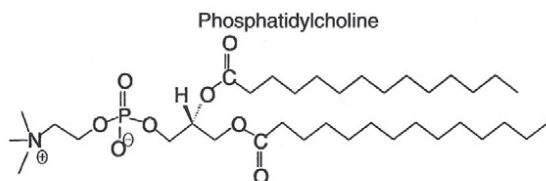


Figure 2. Structure of phosphatidylcholine²⁰

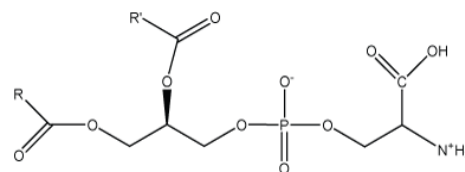


Figure 3. Structure of phosphatidylserine²⁵

There is current evidence that suggests that the meibomian glands are controlled by neuronal, hormonal, and vascular stimulus²⁰.

1.3.2 Proteomics of tear fluid: Relevance to Δ 9-Tetrahydrocannabinol

Pharmacokinetic study shows that THC is absorbed into lipid heavy areas of the body: heart, lung, brain, etc²⁶. THC is transported throughout the body by specific lipoproteins and is activated by cannabinoid receptors CB1 and CB2, located mainly in the brain²⁶. It has been shown, in Elmes et al., that at least three fatty acid binding proteins (FABPs) bind THC and CBD²⁶. In the study, computational data was examined to determine the likelihood of THC and CBD binding to FABPs. Energy scores were consistent with probable binding to FABPs: FABP3, FABP5, and FABP7. These three FABPs are genetic variants of FABPs²⁶. FABP5 is also known as Epidermal-Fatty Acid

Binding Protein thought to aid in the fatty acid uptake transport and metabolism. FABP5 is found in epidermal cells²⁷.

FABP5 has been shown to be efficient in use as a biomarker for diseases such as Dry Eye Disease (DED). In a study conducted by Shinzawa et al., FABP5 was measured in tear fluid by Enzyme Linked Immunosorbent Assay (ELISA) and was found to be 4076 ± 5746 pg/mL in a group of 12 healthy control individuals²⁷. FABP5 can be found in the brain, liver, and kidneys, all tissues that have been known to store THC and metabolites^{14,27}. The use of biomarkers for ophthalmological disease is not currently relevant to this study, however the presence of FABP5 in tear fluid could be related. It is hypothesized that THC and its metabolites would be detectable in meibomian layer of tear composition for its affinity to lipid rich media. This hypothesis is strengthened through the presence of FABP5 within tear fluid and a known interaction between FABP5 with THC and other cannabinoids.

1.4 Legality of Marijuana and DUID

The legalization of Marijuana state by state is complex as there is varying legislation. In bill H.R. 1588, sponsored by Representative Gabbard, there is a current movement to strike Marijuana from the Controlled Substances Act (21. U.S.C. 801 et seq.)²⁸. Marijuana is currently listed on the Controlled Substances Act as a Schedule 1 substance, meaning there is high potential for abuse with no widely accepted medical use²⁹.

With the current surge of Marijuana use, decriminalization of Marijuana would likely continue to lead to another increase in Marijuana usage. An inflation of marijuana use would pose issues in terms of motor vehicle operation. Laboratory studies indicate that

operating a vehicle under the influence of marijuana slows reaction time, reduces the ability to divide attention, and impairs ones concentration and coordination^{30,31}.

There are complications with the analysis of driving under the influence of Marijuana cases such as the fact that those who are involved on auto accidents may be under the influence of multiple substances at the time and therefore the control groups for analysis are difficult³⁰. Regardless, operation of a motor vehicle when one's perception and reaction time is impaired should be prevented.

1.4.1 State by State DUID Prevention

Regardless of Federal legality, Marijuana is treated differently state by state as it is recognized as a psychoactive substance that leads to impairment. State legislation has increasingly made strides to use specific wording to address impaired driving when influenced by substances other than alcohol¹. When under the influence of Marijuana, it is illegal to operate a motor vehicle. Yet, states utilize three different types of legislative terminology when it comes to DUID. The first is the "incapable" terminology, which forces the state to prove that the illicit substance caused the impaired driving¹. The second terminology is "under the influence". Like the term "incapable" the term "under the influence" forces the state to prove to the court that the drug was the reason for the impaired driving¹. These examples of terminology emphasize the burden of proof in cases of DUID. The final terminology is known as "per se." Per se laws are similar to existing measures of blood alcohol concentration (BAC) in which a designated concentration of drug or drug

metabolite will be the zero tolerance threshold in determination of legal operation of a vehicle¹.

Per se laws have inherent issues in their wording and application to legislation and law enforcement. The idea of zero tolerance works for legal substances that have impairing properties. Operating a motor vehicle under the influence of alcohol is considered illegal under the basis that the individual is operating over a designated limit. States have differing legislation surrounding legalization of marijuana. This means if a state has a zero-tolerance policy as well as legislation that criminalizes the possession and intoxication of marijuana, any concentration will be deemed as impairing¹. On the contrary, states with per se laws and legalized marijuana, they then have the ability to create concentration limits³⁰ for impairment.

1.5 Research Objective

Marijuana use is becoming more and more prevalent on the basis of alteration of current legislation. There is a possible need for technology to assist in the assessment of impairment in DUID cases. As Marijuana use adversely effects a person's ability to operate a motor vehicle safely, there is an emerging need for accurate and simplified methods to detect concentrations of drug and drug metabolites in subjects of DUID. As previously stated, there are inherent complications with the current matrices of toxicological analysis, blood and urine. THC and its metabolites, being lipophilic in nature, are hypothesized to be detectable in tear fluid. This hypothesis is strengthened by knowledge of the lipid rich meibomian layer of tears as well as existing proteomic studies of FABP5. The ultimate

purpose of this research is to explore the possibility of using tear fluid as a matrix to support impairment findings in DUID cases.

2. MATERIALS AND METHODS

2.1 Instrumentation Theory

2.1.1 Liquid Chromatography

Chromatography is a method that separates the target analytes of interest from the overall mixture. Any sample that is submitted for detection has either already been through the purification/extraction process or has been deemed ready for analysis. Chromatography separates components of samples through the utilization of a mobile phase and a stationary phase. The mobile phase is a medium that transports the input sample through the system. The stationary phase is a packed column that the mobile phase and sample flow through. Drug analytes have different structures, and therefore variations of polarity and structure, the stationary phase will allow the analytes within the sample to adsorb to its surface with alternate affinities. Sample analytes with varying affinities for the stationary phase will move through the stationary phase at specified rates, resulting in separation³². In other words, differing chemical attractions drive the separation mechanism of Chromatography.

There are two types of chromatography, Gas Chromatography (GC) and Liquid Chromatography (LC). The main difference between the two methods is the type of mobile phase where LC utilizes a liquid mobile phase and GC uses a gaseous mobile phase. However, a benefit to LC, specifically High Pressure Liquid Chromatography (HPLC), is its ease of use with non-volatile compounds³². The mobile phase conditions used in LC can be altered to enhance the resolution of target compounds and results. LC instruments can introduce a combination of mobile phases and washes. Most procedures utilize two paired solvents rather than one, allowing the user to utilize different ratios of mobile phase

introduced at a given time³². Each ratio of mobile phase is indicative of the parameters set by the user in order to obtain specific ratios of aqueous or organic solvents³².

Stationary and mobile phase conditions differ due to the nature of the necessary separation. The two designations of conditions are known as “normal” and “reverse-phase” conditions. Normal conditions are the original condition set in which the mobile phase is a non-polar solution and the column, or stationary phase, consisted of polar components. Reverse phase switches these conditions and utilizes a non-polar stationary phase and a polar mobile phase³². This technology and difference in phase conditions allows the user to be more adaptive to their specific analyte of interest.

2.1.2 Mass Spectrometry

Once separated, the injected sample along with the analytes of interest must be detected. Samples separated from LC or GC are passed through different detectors in order to record elution times. A Mass Spectrometer is a detector that, in order of procedure, follows elution from the LC or GC column. To complete analysis by Mass Spectrometry (MS), analyte molecules must be converted to gas phase ions. Ions can be manipulated by in the mass spectrometer by magnetic and electrostatic fields. Mass Spectrometry completes detection through ionization and separation of mass to charge (m/z) ratio of the produced ions. A m/z ratio will show relative abundance of signals gathered from unique ion fragments³³. The separation is made possible by utilization of the magnetic and electrostatic fields that can separate both fragment molecules and change the path of ions, thus preventing or directing the ions to the detection mechanism³³.

A mass spectrometer is composed of five main parts: ion source, inlet, mass analyzer, detector, and data system³³. The ion source induces a repulsion that ionizes the molecule³³. There exists different versions of ion sources that yield different types of ionization. This procedure utilizes an Electrospray Ionization (ESI) technique. ESI begins by introducing sample in solution form, by either eluent flow or direct infusion, and is then converting the sample into the gas phase. ESI utilizes electromagnetic fields and heated pressurized nitrogen gas to ionize the sample and utilizes electric fields to transfer ions from its solution to the gas phase^{34,35}. ESI is classified as a “soft” ionization technique, meaning there is less fragmentation³⁴. The ionization product in methods that are not “soft” methods will create smaller fragments at a greater multitude³⁴.

The ion plume travels to the mass analyzer which segregates ions based on their m/z ratio. The quadrupole consists of four rods that run parallel to one another fashioned like four points of a square, as seen in Figure 4³⁶. These rods create a route that the ions are moved through by alternating positive and negative frequencies³⁶. By outputting specific frequencies between the rods, the ions either pass through the center of the rods or are diverted out resulting in further separation³³. Some mass spectrometers utilize tandem mass spectrometry (MS/MS) which is a utilize two or more quadrupole mass analyzers to further fragment and separate ions for analysis³⁶.

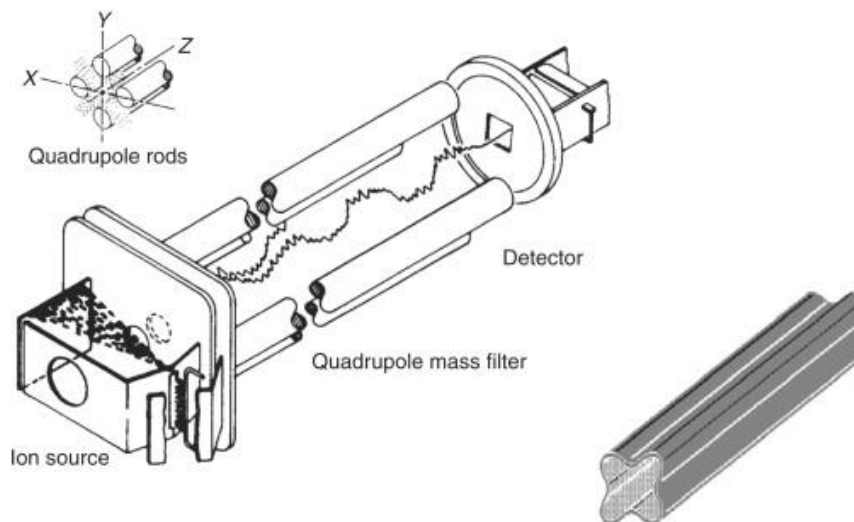


Figure 4. Quadrupole Mass Analyzer pole orientation³⁶

2.1.3 Sample Preparation

Sample preparation is a critical part of analyte analysis and detection. This process occurs before the analytical process to ensure the sample is compatible with the instrumentation or improve its performance. At its core, sample preparation is a chemical or physical modification to a sample. The sample usually consisted of two components: the analyte and matrix³⁷. In the case of this study the analytes of interest are THC, and its primary metabolites THCCOOH and 11-OH-THC. The matrix was the collected tear fluid. A sample preparation method was utilized in this procedure to effectively filter target analyte and matrix from the collection material.

2.2 Instrumentation, Samples, and Reagents

2.2.1 LC-MS/MS Instrumentation

Analysis was conducted by usage of the QSight® 220 CR LC-MS/MS (PerkinElmer, Waltham, Massachusetts, USA). Data was collected and quantified with PerkinElmer Simplicity 3Q™ Software (version 1.6.1903.11142).

2.2.2 Patient Samples

Patient samples were collected from four participants between the ages of 21-40. All samples were collected and donated in accordance with International Review Board (IRB) Standards in a collaborative effort with IMMAD (Quincy, Mass, USA) and Boston University School of Medicine Biomedical Forensic Science Program (Boston, Massachusetts, USA). All participants were required to fill out a marijuana questionnaire to obtain necessary information regarding their current usage. Questionnaire can be found in Appendix B. Participants were responsible for having their own recreational or medicinal Marijuana to achieve detectible levels of THC, adhering to IRB standards. All dosing was completed through the smoking route of administration.

Blood samples, heart rate, and blood pressure were taken as a baseline measurement for both precautionary measures and biomarkers for changes in matrix and physiological response. Patient tear samples were collected with IRB approved, BVI Weck-Cel® Sterile Cellulose strips (Beaver-Visitec International, Waltham, Massachusetts, USA), measuring approximately 2 x 20 mm. The cellulose strip was placed in the lower eyelid, one in each eyelid, for 10 seconds to allow for saturation. The strips were placed in *Thompson Filter*

Vials (Thomson Instrument Company Oceanside, California, USA) containing 300 μ L of Quantisal Buffer solution (Immunoanalysis Corporation Pomona, California, USA).

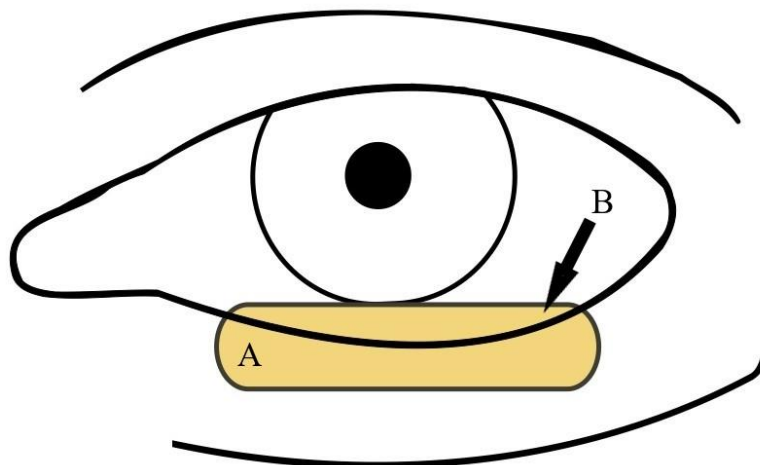


Figure 5. Tear Collection with BVI Weck-Cel® Cellulose strips: A) Cellulose strip covers a majority of the area of the lower eyelid for enhanced saturation B) Location of meibomian glands

2.2.3 Reagents/Standards

Drug standards and deuterated internal standards were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA) and Cerilliant - Certified Reference Materials (Round Rock, Texas, USA). Lot numbers can be seen in Table 1. Lab grade 0.9% Saline solution was used as synthetic tear fluid. NaCl was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Optima grade acetonitrile (ACN), and Optima grade methanol (MeOH) were purchased from Fisher Scientific. Extraction buffer was obtained

from Immunalysis Quantisal™ (Pomona, CA, USA). Deionized (DI) water in this study was from an Ultrapure (Type 1) water system purchased from Millipore Sigma (Burlington, Massachusetts, USA). Formic acid used in mobile phases was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Table 1. Certified Reference Standards

Standard	Lot Number
THC	0581756 - Cayman
11-OH-THC	0548589 - Cayman
THCCOOH	0577400 - Cayman
THC-D3	0583658 - Cayman
11-OH-THC – D3	FE01031903 - Cerilliant
THCCOOH – D3	0571148-4 - Cayman

2.3 Methods

2.3.1 LC-MS/MS Parameters

Separation using the QSight® 220 CR LC-MS/MS was performed with the Halo® C18 3.0x50 mm (MAC-MOD, Chadds Ford, Pennsylvania) (2.7 μm) column. A mobile phase binary gradient of 0.1 Formic Acid in DI Water (Mobile Phase A) and 0.1% Formic Acid in acetonitrile (Mobile Phase B) was utilized through full loop injection. All parameters can be found in Table 2. Parameters were referenced from Gardner, 2020³⁸. ESI in positive ionization mode was utilized for analysis of analytes. All source conditions are outlined in Table 3 and all multiple reaction monitoring (MRM) transition parameters for analytes and internal standards are found in Table 4.

Table 2. LC Method Conditions

Time (Min)	Rate (mL/Min)	A(%)	B%
0.00	0.80	70	30
0.50	0.80	70	30
5.00	0.80	10	90
8.51	0.80	70	30
10.50	0.80	70	30

Column Temperature: 40°C
Injection Volume (Full Loop): 20 µl

Table 3. MS source conditions

Source Parameter	Specified Value
Ionization Mode	Positive
ESI Voltage (V)	5850
HSID™ Temp °C	200
Nebulizer Gas	300
Source Temp (°C)	250
Dwell Time (ms)	10

Table 4. MRM parameters

Compound	Q1	Q2	Type	Internal Standard	CCL 2	CE	EV
THC	315.3	193.4	Qualifier	THC-D3	-56	-31	46
	315..3	123.3	Quantifier	THC-D3	-71	-44	45
11-OH-THC	331.1	313.4	Quantifier	11-OH-THC	-52	-19	43
	331.1	193.1	Qualifier	11-OH-THC	-60	-32	41
THCCOOH	345.2	327.3	Quantifier	11-OH-THC	-53	-21	41
	345.2	299.4	Qualifier	11-OH-THC	-65	-25	47
THC- D3	328.3	123.3	IS		-71	-44	45
11-OH-THC D3	334	196.1	IS		-60	-32	41

2.3.2 Filter Vial Separation

Standards of the analytes of interest at 1 mg/mL , THC, 11-OH-THC, and THCCOOH, were obtained and diluted to 10 µg/mL with methanol. This 10 µg/mL dilution was used to further dilute into both 1 µg/mL and 100 ng/mL solutions to be used as working stock solutions. Internal standard working stock was created with deuterated internal standards. Each internal standard was diluted with methanol to 100 ng/mL. The internal standard solution was ultimately diluted to a concentration of 1µg/mL.

To create calibration standards, saline solution was used to replicate blank tear matrix. 200 µL of saline was spiked with specific concentrations of working stock solution to evaluate different concentrations of drug analyte within tear fluid. This fluid was then absorbed with two BVI Weck-Cel® Sterile Cellulose strips, measuring approximately 2 x 20 mm, and placed in Thompson eXtreme PVDF 0.2 µm, pre-slit, red cap, filter vials containing Quantisal buffer solution. This was left for 30 min to aid in extraction. 30 µL of 1µg/mL internal standard was added and the vials were then compressed forcing the fluid and analyte through the filter as seen in Figure 6.

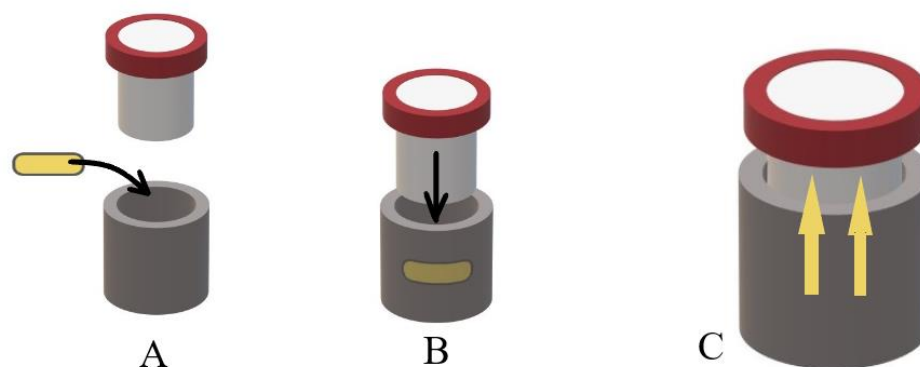


Figure 6. Collection and use of filter vials: A) cellulose strip is placed into the bottom portion of the vial B) Top portion, with the filter, is introduced and compressed with the bottom portion C) Analyte collected in the cellulose is forced through the filter in the top portion as the cellulose is left in the bottom portion.

2.3.3 Patient Sample Collection

Patient baseline samples were collected with cellulose collection material cut small enough to be left in the volunteer's lower eyelid. The cellulose material was left for approximately 10 seconds to induce complete saturation of the material. The material collected was then placed in Thompson filter vials containing extraction buffer and placed in the freezer at -20°C . Volunteers, once produced baseline data was acquired, were asked to dose themselves and return to the testing area within a 30-minute time interval to allow for peak concentrations of THC and metabolites. Upon arrival to the designated testing area, participants were tested again for heart rate and blood pressure, and asked to provide data on physiological changes. Blood was drawn as well. The same cellulose testing material was utilized to collect the evidentiary tear fluid to the same parameters. Similarly, the material was placed in Thompson filter vials containing extraction buffer and stored in a freezer until ready for analysis.

2.3.4 Method Validation

2.3.4.1 Calibration Model

For this procedure, it was important to understand the relationship between available concentration of material and ability to detect the target analytes. Determined from 5 separate runs, the Calibration model used 9 data points of varying concentrations: 0.5, 1, 2, 5, 10, 20 50, 100, and 250 ng/mL. Baseline calibration data was gathered by spiking 200 ul of saline solution for each calibrator on point at these specific concentrations. This style of procedure yields a calibration curve that provides an image of plotted concentration in comparison to analyte peak area to internal standard peak area.

2.3.4.2 Limit of Detection/Limit of Quantitation

Working with a novel matrix it is important to understand the complexities of detecting drug analyte. Five batches of prepared calibrators were run through LC-MS/MS standard procedure to evaluate the ability to detect target analyte at known concentrations. A Limit of Detection (LOD) was determined by examining the 9 data points seen in Section 2.3.4.1. The lowest point in which the method was able to detect a concentration of analyte, reliably and accurately, was the LOD. Limit of Quantitation is the lowest analyte concentration that can be reliably and accurately quantitated. Limit of Quantitation was analyzed using samples from the same 9 data point concentrations.

2.3.4.3 Ion Suppression/Enhancement

When coeluting compounds on the same run, there can exist ion suppression or enhancement that can alter the expected results of the data set. In order to account for ion

suppression and enhancement, a minimum of 10 blank patient samples should be collected and analyzed for any presence of ion signaling. Especially with a novel matrix, it is crucial to the detection of analyte that there is no interference nor unexpected signal. The 10 blank samples consisted of tears are collected in the same fashion of the patient samples. The 10 samples come from non-users, expecting to see no cannabinoid peaks.

2.3.4.4 Carryover

When running samples, high concentrations of analyte may be detectable in subsequent samples. For this reason, carryover can negatively alter qualitative and quantitative analysis. This occurrence is not an intended result and steps were taken to mitigate this response. Vials with no analyte present, or blanks, were strategically placed after the highest concentration of analyte. This serves the purpose of evaluating carryover.

3. RESULTS

Results were processed in two parts: calibration and analysis of patient samples. All calibration data was prepared through the procedure in section 2.3.2. All patient samples were collected according to procedure listed in section 2.3.3. Patient samples were placed in 300 µl of extraction buffer. Calibration standards were stored in the same manner.

3.1 Analyte Detection and Separation

Chromatographic Separation of analytes was completed by using a Halo® C18 3.0 x 50 mm 2.7 µm LC column. Each analyte maintained a specific retention time confirmed by retention time of the internal standards and known retention time of target analytes. This data can be found in Table 5 and seen in Figure 7. THC-D3 was used as the internal standard for THC. The internal standard 11-OH-THC-D3 was used for both 11-OH-THC and THCCOOH due to the extreme similarities in retention time.

Table 5. Average Retention Time: Compounds & Internal Standards

Compound	Average Retention Time (min) (N=5)
THC	4.88
11-OH-THC	3.59
THCCOOH	3.64
THC-D3	4.87
11-OH-THC-D3	3.57

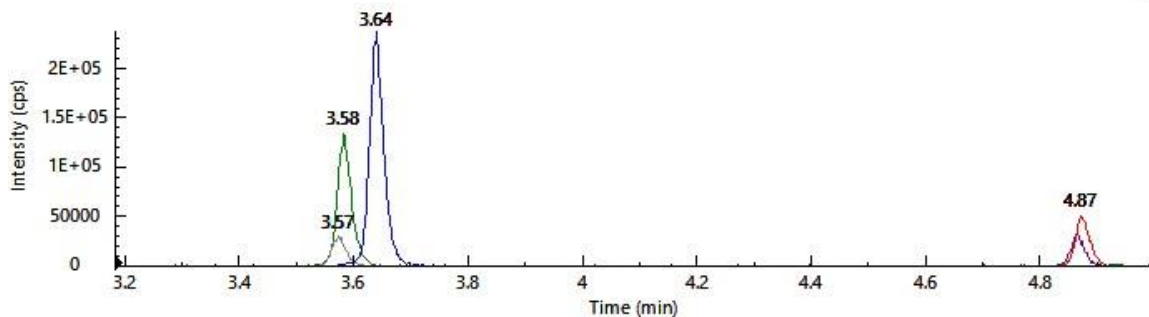


Figure 7: Analyte Peak References

3.2 Method Validation

3.2.1 Dynamic Range

The dynamic range was determined for the model of calibration for the analytes of interest. The purpose of this range was to establish a correlation between the concentration of analyte and signal response. A quality dynamic range for an analysis of matrix should express a direct relationship between signal intensity and analyte. Although a dynamic range can be expressed linearly, a relationship can also be non-linear in nature³⁹. The range was assessed for the procedure to produce a correlation between signal intensity and concentration.

It was determined in previous work, *Detection and quantitation of cannabidiol and delta (9) tetrahydrocannabinol in oral fluid of a therapeutic-use cannabidiol donor using the Qsight 220 CR LC-MS/MS*, by Jenna Gardner, a stable working range for detection of THC in oral fluid ranged between 0.25 – 500 ng/ml in oral fluid³⁸. This was determined by assessing previous studies, workplace drug testing limits, and published data of presence

of analytes in the body³⁸. This working range was altered due to the low sample volume of tear fluid in comparison to the sample volume in collected oral fluid.

Due to the low sample volume, adding more data points to and shortening the range to 0.5 - 250 ng/mL was deemed a benefit to the procedure. A working range for tears was set at 0.5, 1, 2, 5, 10, 20, 50, 100, 250 ng/mL. Sample volume in the collection of tear fluid is an added variable that was addressed. Despite having theory to back up the presence of THC within tear fluid itself, collection of matrix for the purpose of detecting cannabinoid analytes was novel. The cellulose collection strips used collected approximately 200 ul of sample volume. This was used as the baseline sample volume for the calibration curve. Variability in collected sample volume from patients is a hindrance that effects the accuracy of data.

A calibration model was set from five separate runs of calibration standards following the working range. Out of all of the calibrators, five out of the nine must have been of a permissible sample accuracy, $\pm 20\%$ deviation from 100% accuracy, to be an effective working curve. It was determined that analytes followed an exponential fit pattern and all average coefficient of variation (R^2) values were calculated and were > 0.99 . THCCOOH followed an interesting fit pattern as it appeared linear in nature. However, calculated R^2 value was an acceptable 0.9922 when a quadratic fit was applied. All charts can be seen in Figures 5-7 and all R^2 values can be seen in Table 6.

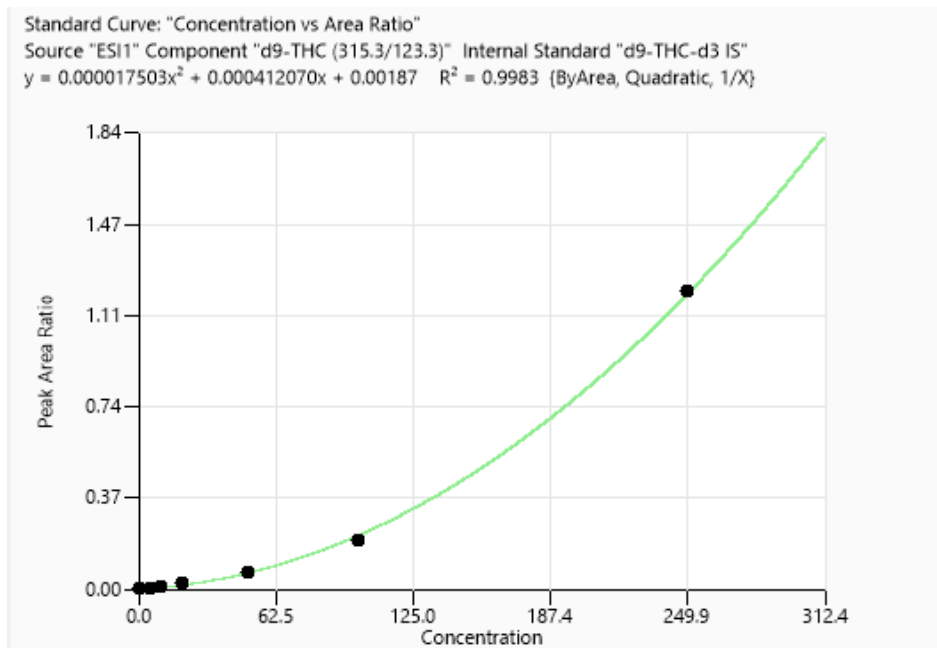


Figure 8: THC Standard Curve

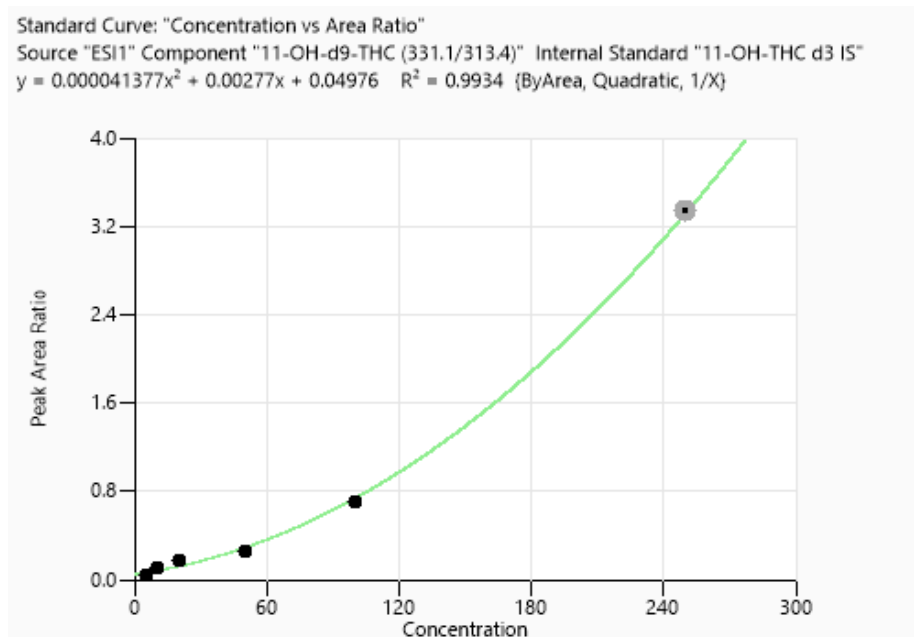


Figure 9: 11-OH-THC Standard Curve

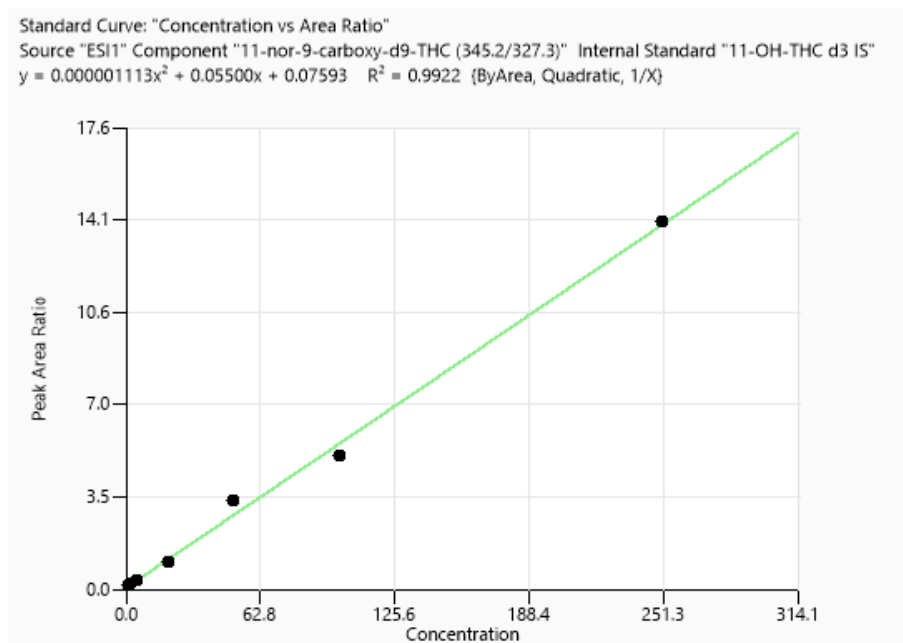


Figure 10: THCCOOH Standard Curve

Table 6: R² values for Analytes of interest

Compound	R ² Value
THC	0.9983
11-OH-THC	0.9934
THCCOOH	0.9922

3.2.2 Limit of Detection & Limit of Quantitation

Limit of detection (LOD) and limit of quantitation (LOQ) are important factors in the analysis of THC and its analytes. Limit of Detection is defined as the lowest concentration that can be accurately depicted in an analyte screen. The limit of detection is the analyte concentration that can be reliably distinguished from zero⁴⁰. In order to determine this limit, matrix samples of decreasing concentration were run in order to determine the lowest point of detection. The calculated LOD and LOQ was determined by analysis of four calibrators (0.25, 0.50, 1, 2 ng/mL) over a minimum of two runs to meet all detection and identification criteria. LOD was completed according to American Standards Board (ASB)⁴¹. LOD values can be found in Table 8.

Limit of quantitation is the lowest concentration in which defined analytes can be detected and quantified in regards to pre-determined criteria. Limit of quantitation can be equal to or greater than the limit of detection⁴². This threshold is the lowest point where the method can operate with acceptable precision⁴¹. Determination of LOQ data began by determining the coefficient of variation (%CV) to outline a quantitated value for precision.

$$\%CV = \frac{\text{Standard Deviation}}{\text{Mean Response}} \times 100$$

According to ASB *Standard Practices for Method Validation in Forensic Toxicology*, the %CV should not exceed 20%. In certain well-established methods %CV should be maintained around 10%. Because the analysis of tear fluid is a novel matrix, the %CV was evaluated using the 20% threshold. CV% can be found in Table 10.

Due to the novelty of use of tear fluid, it was necessary to understand the limitations to what could and could not be detected and quantitated with the existing procedure. It is necessary to be able to quantitate accurately and precisely within a specific matrix. In many states, presence of analyte can be enough to prosecute due to zero tolerance statutes¹. States, such as Colorado, that maintain cutoffs in concentration need accurate measures of quantitation¹. Colorado maintains a 5 ng/ml permissible inference for THC in DUID investigations. Knowledge of quantitative concentrations is a necessary factor for this analysis.

It was of benefit to evaluate LOD and LOQ data at a lower concentration than the lowest calibrator. In order to maintain a consistency to data found in tests of current admissible matrices such as blood and oral fluid, 0.25 ng/mL was added as another point for determination of LOD and LOQ. LOD values were found to be around the 0.25 ng/mL value whereas the LOQ values were determined to be higher as seen in Tables 11 and 12.

Table 7: Recorded Concentrations for LOD/LOQ Calculation (ng/mL) – THC

	0.25 ng/mL	.5 ng/mL	1 ng/mL	2 ng/mL
Batch 1	0.152	N/D	1.684	1.413
Batch 2	0.184	N/D	0.065	1.913

N/D: Non-Detectable

Table 8: Recorded Concentrations for LOD/LOQ Calculation (ng/mL) – 11-OH-THC

	0.25 ng/mL	.5 ng/mL	1 ng/mL	2 ng/mL
Batch 1	N/D	N/D	N/D	N/D
Batch 2	N/D	N/D	N/D	2.000

N/D: Non-Detectable

Table 9: Recorded Concentrations for LOD/LOQ Calculation (ng/mL) – THCCOOH

	0.25 ng/mL	.5 ng/mL	1 ng/mL	2 ng/mL
Batch 1	< 0	1.088	1.421	1.475
Batch 2	0.257	0.484	N/D	2.009

N/D: Non-Detectable

Table 10: %CV for LOD/LOQ Calculation

Analyte	0.25 ng/mL	.5 ng/mL	1 ng/mL	2 ng/mL
THC	9.52%	N/D	92.53%	15.03%
11-OH-THC	N/D	N/D	N/D	N/D
THCCOOH	N/D	38.42%	N/D	15.33%

N/D: Non-Detectable

Table 11: Limit of Detection Results

Compound	Limit of Detection (ng/mL)	Detected Concentration
THC	0.25	0.15
		0.18
11-OH-THC	2.00	2.00
THCCOOH	0.25	0.26

Table 12: Limit of Quantitation Results

Compound	Limit of Quantitation (ng/mL)	%CV
THC	0.25	9.52%
11-OH-THC	N/D	N/D
THCCOOH	2.00	15.33%

3.2.3 Carryover

Evaluation of carryover in detection procedure was completed by analysis of blank matrix prior to the injection of the highest calibrator in triplicate. Per the existence of carryover, the value can be calculated with the following equation:

$$\%Carryover = \frac{\text{Average Peak Area (Blank)}}{\text{Average Peak Area (Calibrator 9)}} \times 100$$

Carryover was examined throughout the five calibration runs evaluating the blank samples following the highest calibrator concentration at 250 ng/mL. Within the blank samples of the five runs there existed no ion detection at any of the analyte's retention times. For example, a presence of carryover for THC would have yielded a peak around 4.88 in the blank sample. No carryover was detected for any of the three analytes, therefore no calculations were made.

3.3 Patient Samples: Detection and Quantitation of Analytes

The primary goal for this procedure was to better understand the novel matrix of tear fluid in detection of the targeted cannabinoids. Understanding that there could be a potential lack of quantitation due to the limited sample volume, detection was the primary goal. However, quantitative results were evaluated considering similarities of existing matrices, such as oral fluid, to tear fluid.

Data was analyzed through two methods of evaluation. Primarily, the charted retention times vs analyte intensities were evaluated for the existence of peaks within the expected range. This expected range came from the calibration data as well as internal

standard comparison. The goal was to observe peaks around the expected retention time for detection and to observe the area of those peaks to gather quantitative data. The data was then plotted on the calibration curves in order to perform quantitative analysis.

Each spectrum was observed individually in the initial examination. Each patient sample had three spectra, one for each target analyte, each measuring retention time and analyte intensity. Although the route of administration, smoking, was the same for every patient, the amount of inhaled drug varied from person to person to reach desired intoxicating levels. In this regard, those who took part as volunteers filled out surveys as to their use of Marijuana. There were obvious recorded differences in consistent general use, some being chronic users where as others having social or sporadic tendencies of use. All of this was considered in analysis.

Once the initial analysis took place, patient sample data was compared to produced calibration curves within the Simplicity software. This helped produce an understanding about quantitative results in tear fluid. From previous LOD and LOQ calculations, quantitation for the main target analytes could hypothetically be observed in patient samples above 1 ng/mL. Although it was not the primary objective, quantitation values are of extreme benefit.

When evaluating the data of patient samples, the procedure was sufficient in detection of THC and THCCOOH. There were complications in the detection of 11-OH-THC due to the existence of a strong peak at approximately 5.05 min retention time. This was not indicative of any target analyte. When conducting an initial examination of the

spectrum, there was a peak that was consistent with the expected retention time range for the THC analyte as well as the THCCOOH analyte. This is outlined in Figures 9-11.

Figure 8 depicts a baseline tear sample of one participant.

Table 13: Blood Cannabinoid Concentration Results – Patient 13

Collected Matrix		Concentration ng/mL	Lab Cutoffs ng/mL
Blood	THC	31	0.50
	11-OH-THC	5.8	1.0
	THCOOH	54	5.0

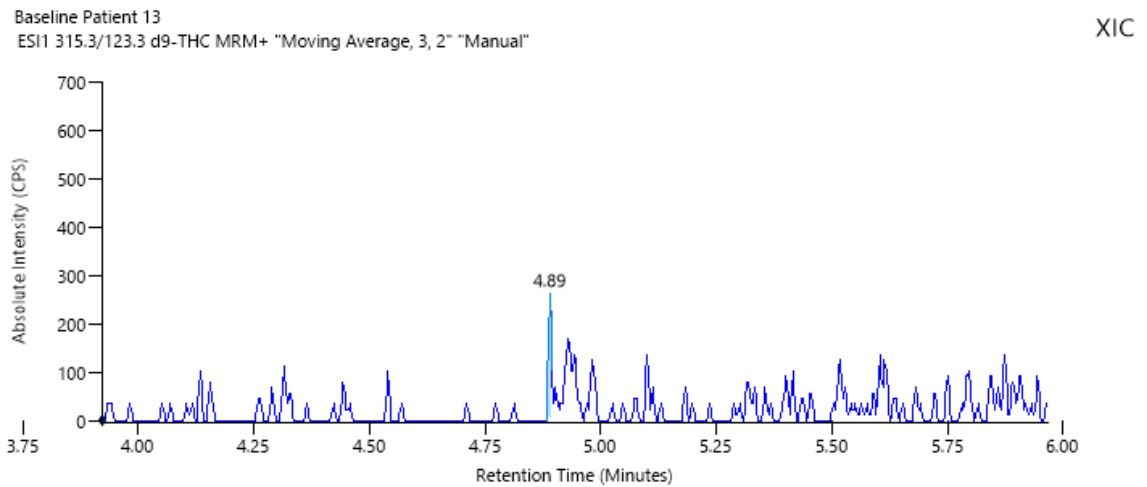


Figure 8: Baseline Data Patient 13 – THC Data Collected After Dose Patient 13

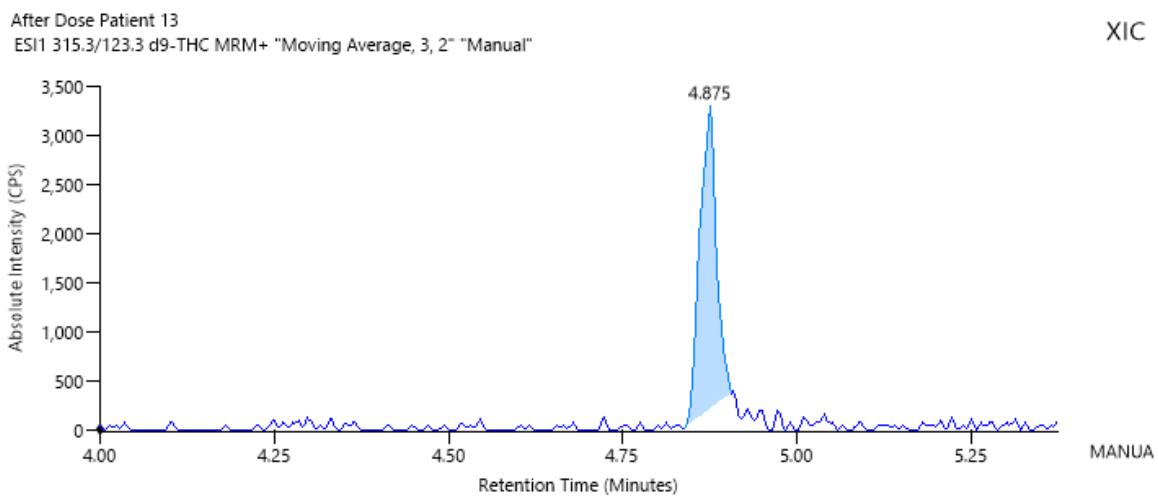


Figure 9: Data Collected After Dose Patient 13 - THC

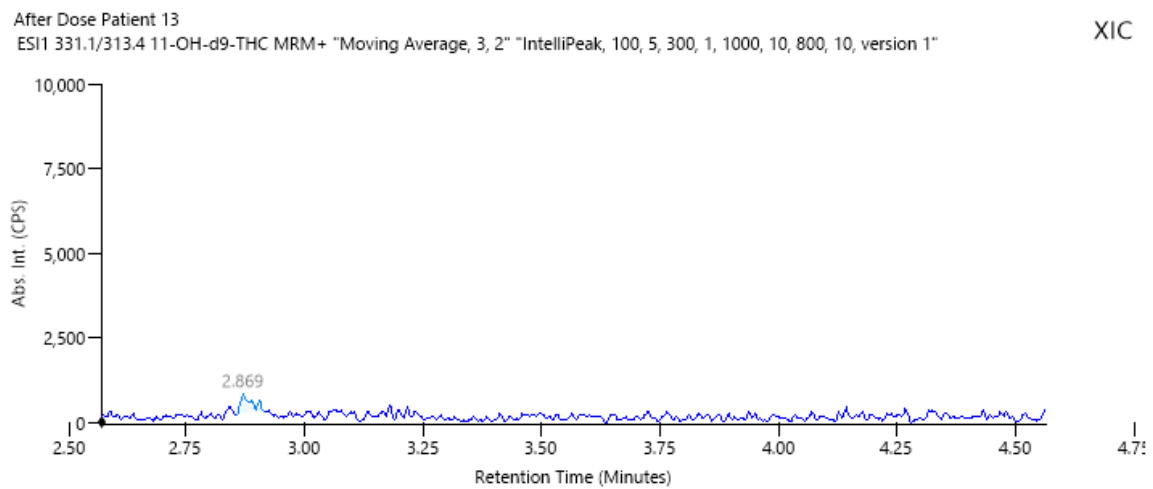


Figure 10: Data Collected After Dose Patient 13 – 11-OH-THC

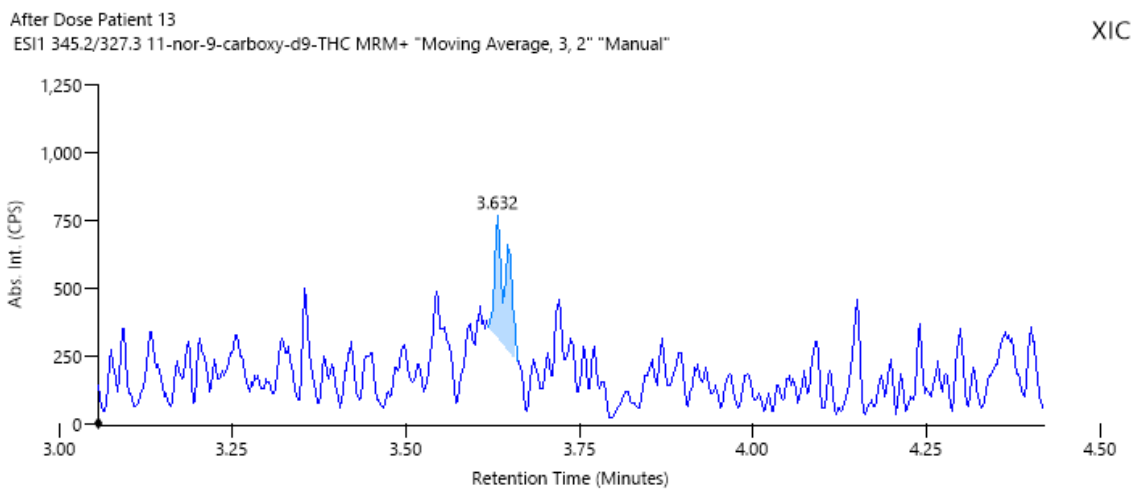


Figure 11: Data Collected After Dose Patient 13 - THCCOOH

4. DISCUSSION

4.1 Complications of a Novel Matrix

Working with a novel matrix poses complications as there is limited information on its application to toxicological testing. When referencing other journals about chromatography/mass spectrometry methods for detecting components of tear fluid, although there was evidence that the fluid could be analyzed with this method, methods for detecting drug analytes in tear fluid was effectively non-existent.

The first obstacle was the collection of tear fluid. There was a common misconception in general understanding that the subject would have to produce an emotional response to produce collectable tears. As stated in Section 1.3, glands are constantly producing fluid, in the form of water and sebum, to maintain moisture in the environment of the eye. The most probative location for collection was determined to be the lower eyelid for its ease of access, concentration of fluid, and location of meibomian glands.

The next question to answer was the method of collection. Schirmer paper strips are a technology used in the ophthalmological community in order to test for DED. A strip will collect tear fluid through capillary action to show the individuals ability to produce tears. Schirmer paper strips were utilized in *Schirmer Paper Noninvasive Microsampling for Direct Mass Spectrometry Analysis of Human Tears* by Yao et al. in congruence with Paper Spray-MS/MS⁴³. It was hypothesized that this collection method would have parallels to a method utilizing LC/MS. The method listed in Yao et al. analyzed environmental exposures and certain drug analytes. Mentioned in the paper, the collection

of tear fluid with the Schirmer paper has complications because of low collected sample volume⁴³. This was found to be the case in this study as well. The Schirmer paper was able to fully saturate at approximately 30-50 ul, an exceptionally low sample volume for detection.

Polyvinyl Alcohol (PVA) and cellulose were considered as alternative collection material. Used in drug matrix collection tests, such as Quantisal® Oral Fluid drug analyte tests, cellulose was proven to be a material with great collection ability and low interference with THC and related cannabinoids³⁸. PVA was used in ophthalmological DED tests, manufactured by Inflammadry® and had shown efficacy in absorption/collection of tear fluid specifically. Both materials were available in isolated strip form.

Both materials were tested on the ability to: collect the fluid at high sample volume, be able to release the analyte in an extraction step and maintain low interference with the target analyte for good peak identification and quantitation. Cellulose, used in congruence with extraction buffer, was the best at filling these three criteria. The cellulose strips cut to the dimensions of the lower eyelid were able to retain approximately 200 ul of sample volume, a significant increase in volume from the Schirmer paper.

4.2 Sample Preparation: Extraction

While building the sample preparation method, past research was referenced to guide the extraction of drug analyte in a matrix. As previously stated, sample preparation for Paper Spray MS was an adequate way of utilizing Schirmer paper strips as a collection mechanism⁴³. The two problems that posed included the unavailability of Paper Spray MS

and the small sample volume collection abilities of Schirmer paper strips. Although it was evident that the strips were not of value in the collection process, it was still questioned whether it was an issue with extraction solvent rather than the sample volume size.

Initially, a standard methanol extraction was used. However, when extracting with methanol, signal intensity within the calibrators was either low or non-existent. This extraction inconsistency was not sufficient enough to retain information. All of the ordered drug standards and working stock are preserved and diluted with methanol in order to reach the desired concentration for calibration. Because of the extraction with methanol, there could have existed a possible dilution. Sample volumes, between 50-200 uL, is low and any dilution could pose issues. Schirmer paper strips were only able to retain 30-50 uL of sample. Therefore, more dilution would limit the amount of analyte that could be detected.

Procedures with matrices that are known for interfering with detection utilize solid phase extraction elution columns that separate analyte from interferences. This method was evaluated with tear fluid collected in Schirmer paper strips, however, like methanol results were either inconsistent or non-existent. There could be a multitude of reasons why the columns were not effective in the recovery of analytes, however it is believed that the issue was once again with dilution of low sample volume. It was found that a stable buffer helped to ensure the proper detection of analytes. Quantisal oral fluid collection devices (Immunoanalysis, Pomona, California, USA) are devices that ready an oral fluid sample for downstream drug detection. The collection material, a wand with a cellulose tip, saturates with oral fluid and is placed in extraction buffer that extracts and stabilizes the drug analyte. It was hypothesized that this action could be replicated in the analysis of tear fluid. Using

the buffer material, analyte was extracted and stabilized to detect adequate levels of primary analyte, THC.

4.3 Filter Vials

There are different methods of sample preparation that can be utilized in LC/MS procedures. Traditional methods include solid phase or liquid/liquid extraction.

These methods act as purifying steps in which unwanted portions of the sample, such as matrix interferences, are removed or reduced from the sample and can be disposed of properly. Matrices, such as blood, have different components in which interferences can be problematic to analysis.

Since collected samples were low in sample volume, a complex sample preparation method posed issues surrounding dilution and loss of analyte. Multi-step processes decreased retention of analyte significantly. This allowed for the pursuit of simplified methods that would maintain sample volume. Thompson Filter Vials with a Quantisal buffer solution were used in the procedure as the technology allowed for separation of analyte from collection material while retaining the original sample. The vial system worked on a compression mechanism where solution was placed in a base vial. The top component worked as a compressible plunger that forced sample into a top chamber through a selected filter. The ability to remove original sample in buffer from collection

material and allowing for filtration was deemed beneficial and allowed for direct analysis by LC-MS/MS.

4.4 Source Polarity: Positive and Negative Ionization

In previous research, the method optimization parameters were set in positive ionization mode. In MS Electrospray Ionization methods positive ionization is usually the preferred method and was utilized in the detection procedure. Research has shown with certain analytes and methods that negative ionization has produced a stronger analysis than positive ionization⁴⁴. In *Think Negative: Finding the Best Electrospray Ionization/MS Mode for Your Analyte* by Piia Liigand et al., it was determined that negative ionization produced better results and higher sensitivity in the majority of compounds that they analyzed⁴⁴. Negative ionization was run for all analytes at median calibrations in order to reach a better specificity and resolution of result. Negative ionization was not effective in the MS method as the data was either low in signal intensity or nonexistent.

4.5 The First Analysis of Patient Sample 13

Patient samples were analyzed one at a time because of the limited number of samples at the time of analysis. The first patient sample analyzed was ran with one of the last calibrations runs and was run after a sample blank to ensure no carryover. When evaluating the data of a patient sample, it was noted that THC could be detected at a peak intensity of approximately 4,000 cps, a peak area of 5,513 counts, and a retention time of 4.875 minutes. These criteria were all indicative of the confirmed presence of THC due to

peak intensity corresponding to specific MRM transitions and quantitative and qualitative ion ratios. When examining the baseline data, which was expected to be no presence of any analyte, there was a significantly smaller peak at a retention time of 4.89 and an intensity of approximately 700. The first thought of seeing the peak for THC in the baseline data would be a product of carryover. However, from the carryover analysis, the procedure did not have any evidence that carryover would pose any problems.

Participants were asked to abstain from partaking in any dosing before baseline data was taken, however it is difficult to enforce these rules when the subject cannot be under supervision. According to the baseline survey data for patient 13, the answers indicated potential chronic use. It is known that THC analyte can be detectable in chronic users for up to 72 hours after use and the peak within the baseline is believed to be a byproduct of this occurrence.

4.6 Sample Volume & LOD/LOQ

In specific after dose patient samples, patients 10-12, it was seen that there was an ability to detect analyte however there was trouble in quantitating data. This patient data can be found in Appendix A. There are a few explanations for this occurrence. The primary explanation is the variation of sample volume in each collection. It was observed that each patient had a variation of tolerance for placing the cellulose under the eyelid. Individuals who regularly use products, such as contacts, are used to the changes in the eye environment when something is placed on or around it. Patient 13, who had the best results/peak intensity, was the sole contact wearer in the group of 4 (patients 10-13). The

other three patients were in discomfort and quickly wanted the cellulose to be removed. Although the two strips collect approximately 200 μ l of matrix, there is variability on saturation of the material. A lower sample volume would ultimately lead to less accurate quantitation.

5. Conclusions

The hypothesis for this procedure was based on the lipid rich nature of the meibomian layer of tear fluid. The lipophilic nature of cannabinoid analytes, THC, 11-OH-THC, and THCCOOH, help facilitate detection of these analytes in fluids and tissues that are lipid rich. The existence of the meibomian layer as well as FABP5 in tear fluid, the novel matrix was a point of exploration that was deemed beneficial to cannabinoid detection. A method that collects this matrix in an extraction buffer for stability was shown to be an efficient and effective way to retain the analyte allowing for direct injection and subsequent analysis by LC-MS/MS. It was made clear that THC analytes could be detected and quantitated at levels as low as 0.25 ng/mL and THCCOOH could be detected at 0.25 ng/mL and quantitated at 1 ng/mL with the developed procedures. There arose complications with the 11-OH-THC analyte in terms of detection and sensitivity at lower levels.

5.1 Future Directions

The detection of target analyte, THC, in tears may be a probative measure that has potential use for toxicological analysis. However, there are aspects of the analysis that can be modified to a degree that would create a more effective method for quantitation. It would be of benefit to attempt different modes of ionization as studies show that there can be increased sensitivity and selectivity. Although negative ionization did not show to be an effective method, with the altered collection methods and MS parameters these factors could have a beneficial outcome. Similarly, it would be interesting to explore different

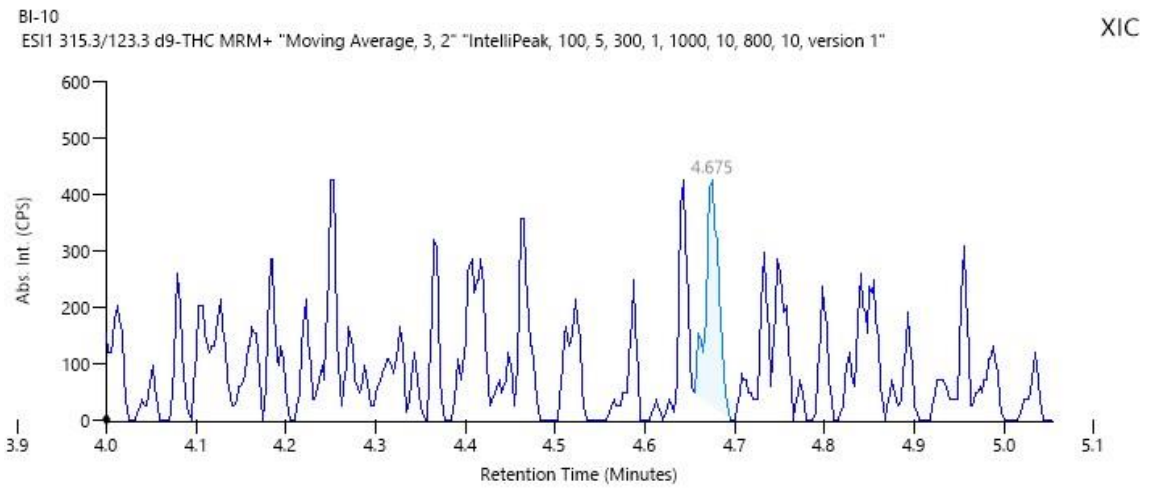
extraction methods. Standard direct injection with the aid of extraction buffer was sufficient to detect and quantify analytes to a degree. It could be of benefit to explore other extraction methods to optimize the procedure.

It would be of benefit to complete tests on a larger group of participants in order to further optimize detection and quantitation. This study was focused on the ability to detect and quantitate; therefore, it was not priority to collect tears at different time points. However, it would be of extreme benefit to do so.

Utilizing the current procedure, it would be of benefit to cultivate and optimize these methods into tools to aid in roadside DUID enforcement. The development of technologies to create roadside detection tools is a current topic in impairment assessment. Because of the issues that arise with current methods of detection, the use of tear fluid may be a simplified and non-invasive collection tool that could be of use in law enforcement agencies. This tool could allow law enforcement to detect drug analyte in a manner as simple as the breathalyzer for alcohol detection.

APPENDIX A: [Results from collected patient data]

Collected Matrix		Concentration ng/mL	Lab Cutoffs ng/mL
Blood	THC	3.4	0.50
	11-OH-THC	N/D	1.0
	THCOOH		

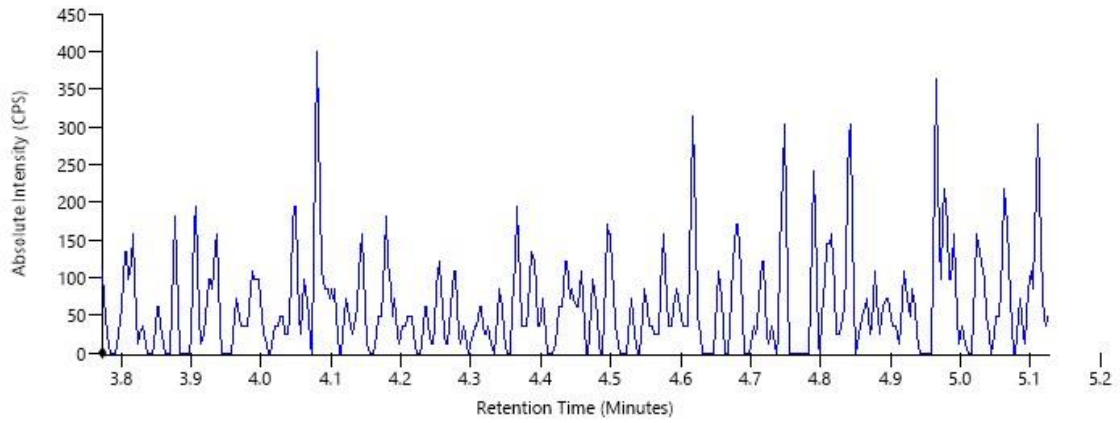


Baseline Data Patient 10 – THC

AT-10

ESI1 315.3/123.3 d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

XIC

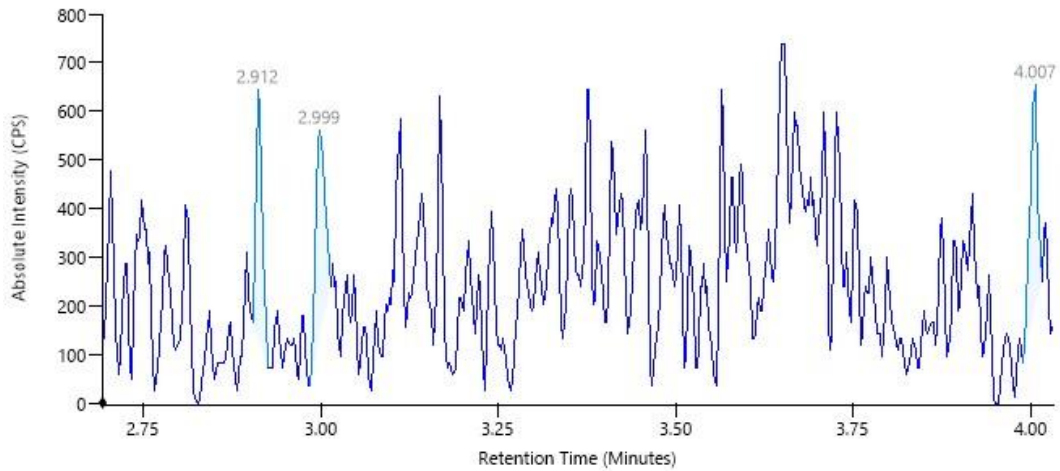


Data Collected After Dose Patient 10 – THC

BI-10

ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

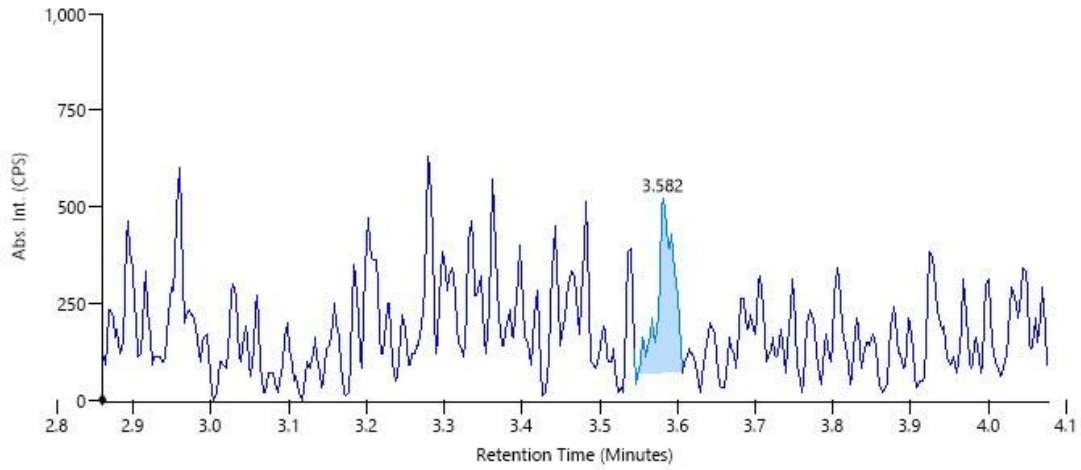
XIC



Baseline Data Patient 10 – 11-OH-THC

AT-10
ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

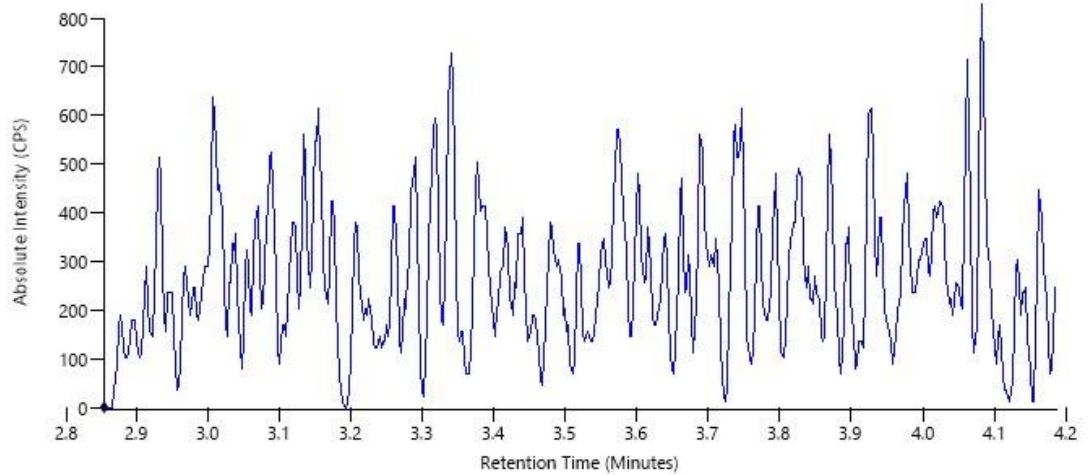
XIC



Collected After Dose Patient 10 – 11-OH-THC

BI-10
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

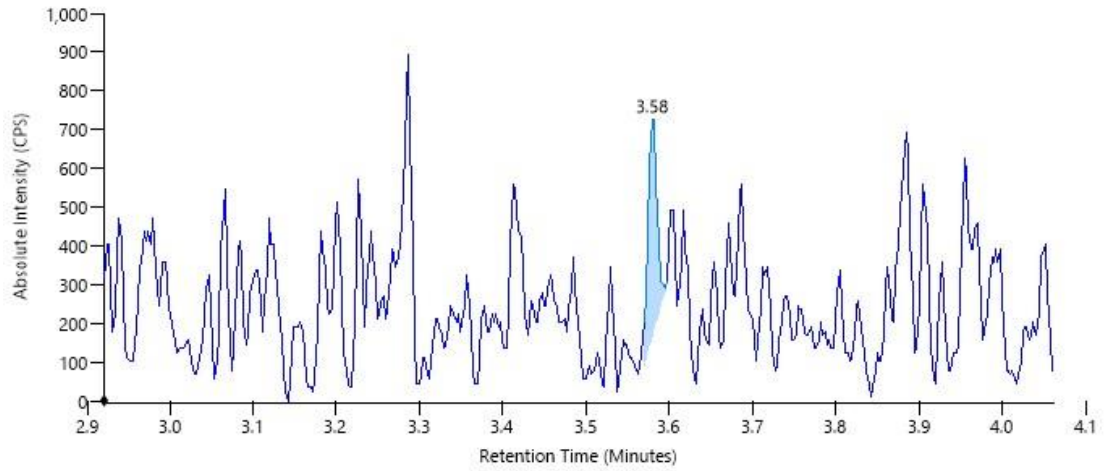
XIC



Baseline Data Patient 10 – THCOOH

AT-10
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

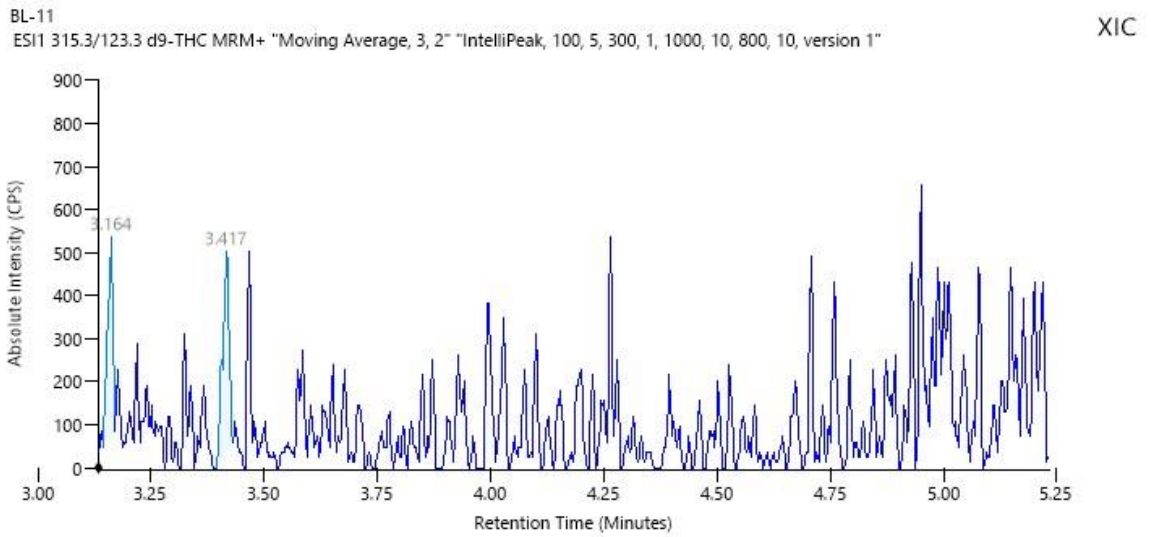
XIC



Data Collected After Dose Patient 10 - THCOOH

Patient 11 Data:

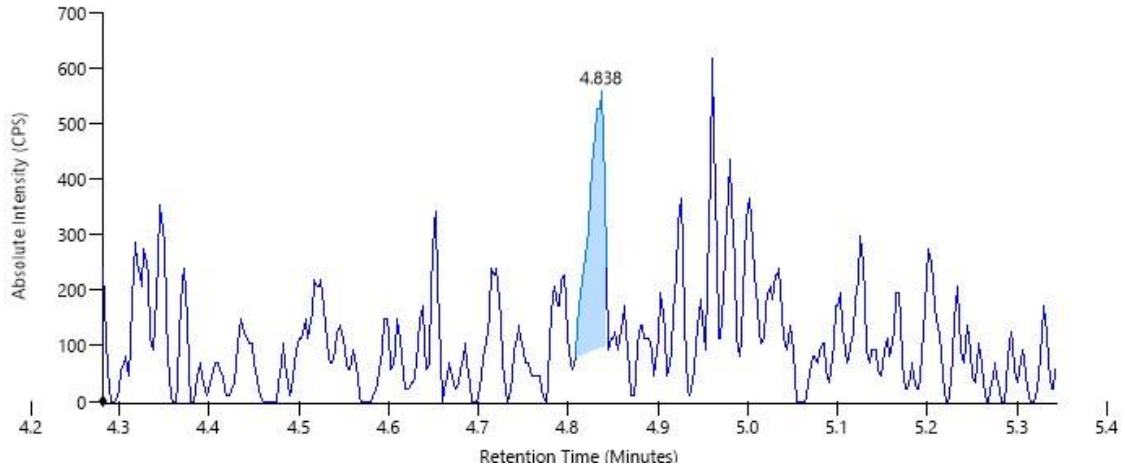
Collected Matrix		Concentration ng/mL	Lab Cutoffs ng/mL
Blood	THC	6.6	0.50
	11-OH-THC	1.5	1.0
	THCOOH		



Baseline Data Patient 11 – THC

AT-11
ESI1 315.3/123.3 d9-THC MRM+ "Moving Average, 3, 2" "Manual"

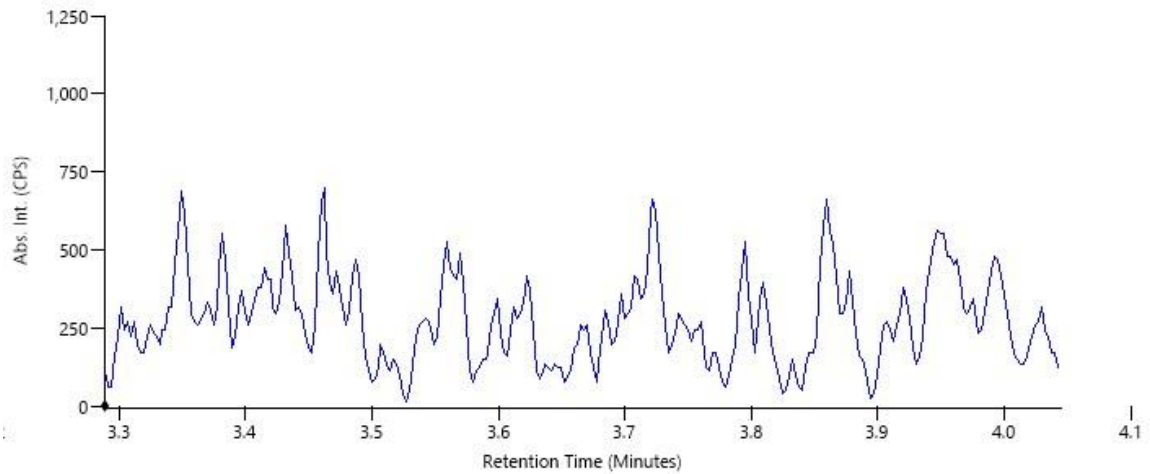
XIC



Data Collected After Dose Patient 11 - THC

BL-11
ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

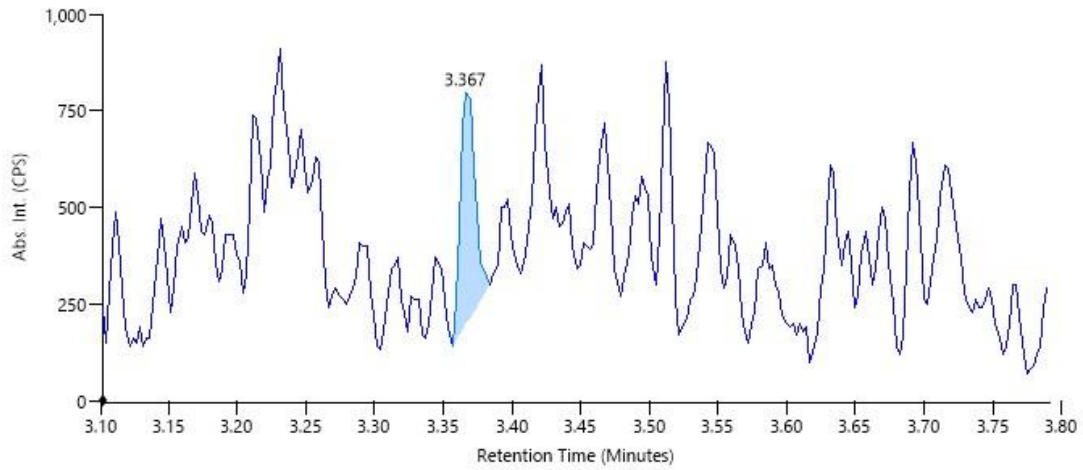
XIC



Baseline Data Patient 11 – 11-OH-THC

AT-11
ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

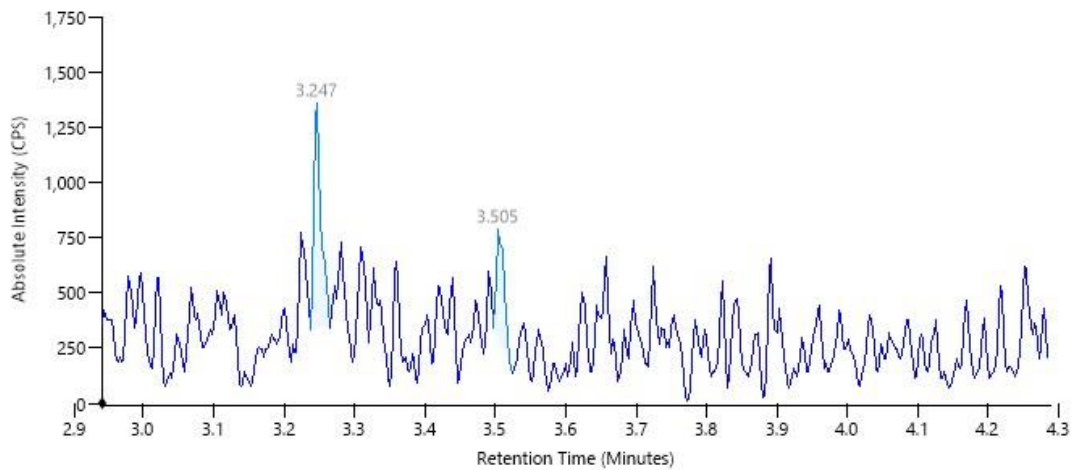
XIC



Data Collected After Dose Patient 11 – 11-OH-THC

BL-11
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

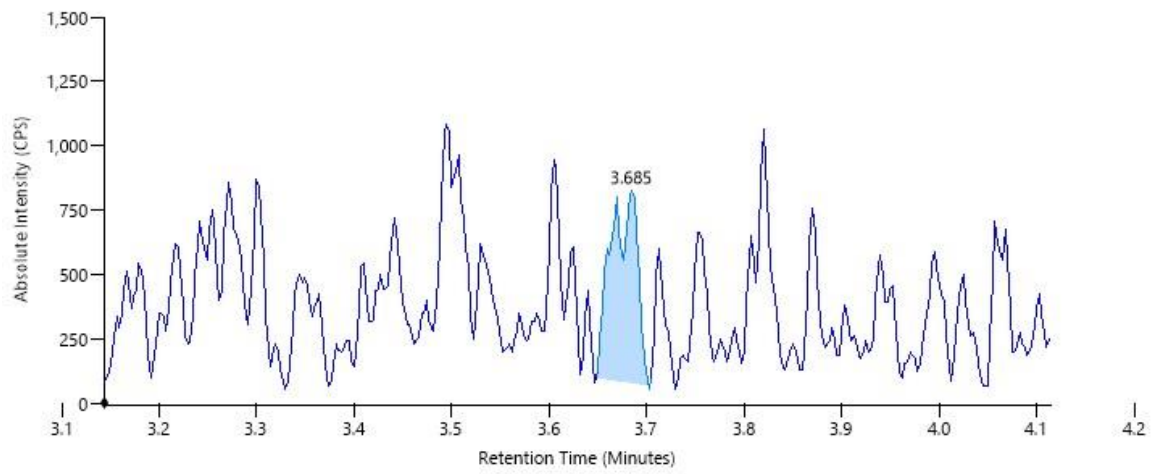
XIC



Baseline Data Patient 11 - THCOOH

AT-11
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

XIC



Data Collected After Dose Patient 11 - THCOOH

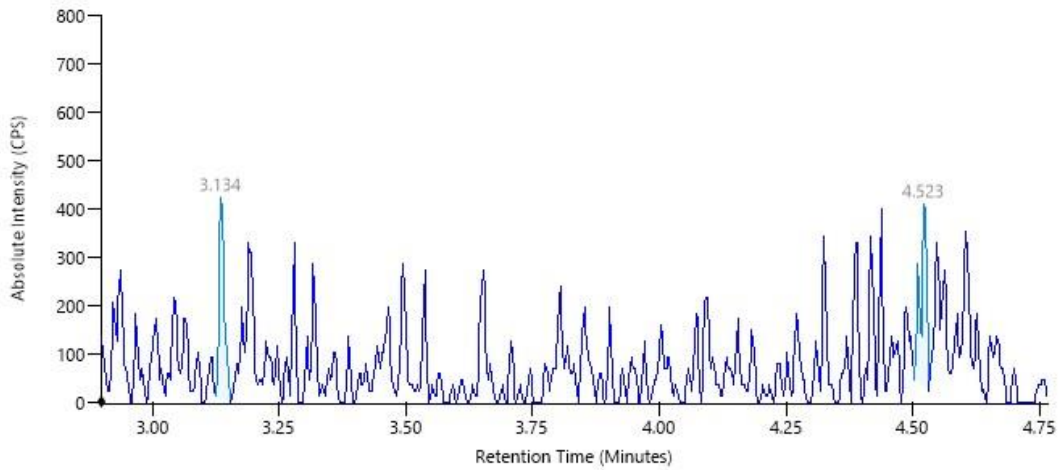
Patient 12 Data:

Collected Matrix		Concentration ng/mL	Lab Cutoffs ng/mL
Blood	THC	2.1	0.50
	11-OH-THC	N/D	1.0
	THCOOH		

BL-12

ESI1 315.3/123.3 d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

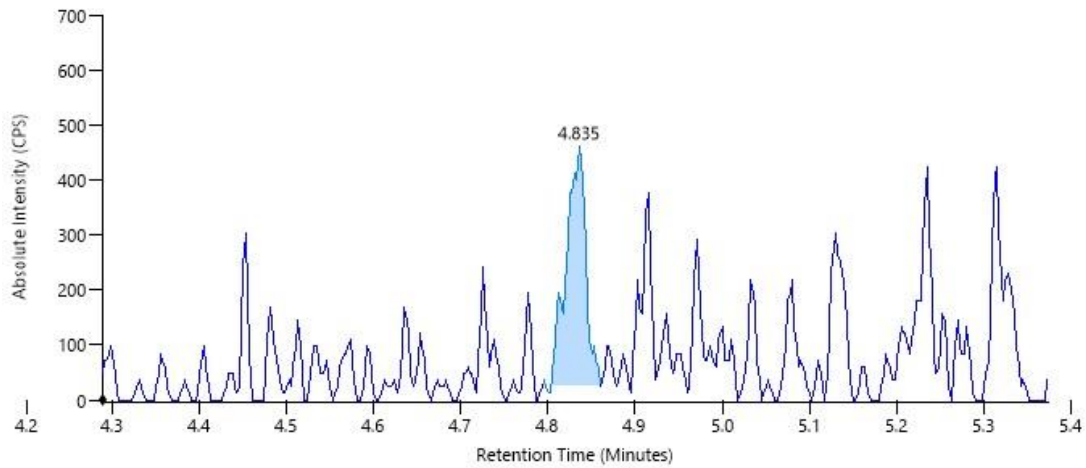
XIC



Baseline Data Patient 12 – THC

AT-12
ESI1 315.3/123.3 d9-THC MRM+ "Moving Average, 3, 2" "Manual"

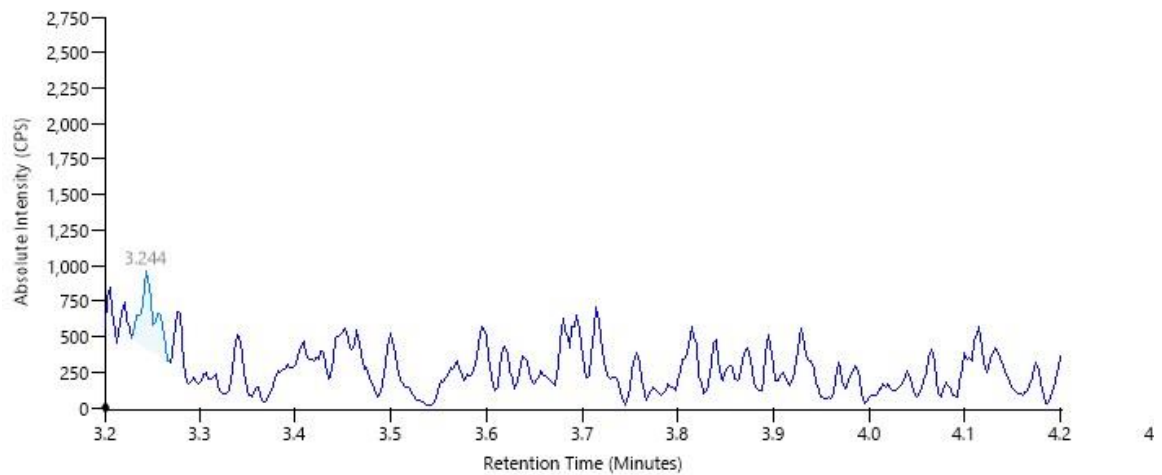
XIC



Data Collected After Dose Patient 12 - THC

BL-12
ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

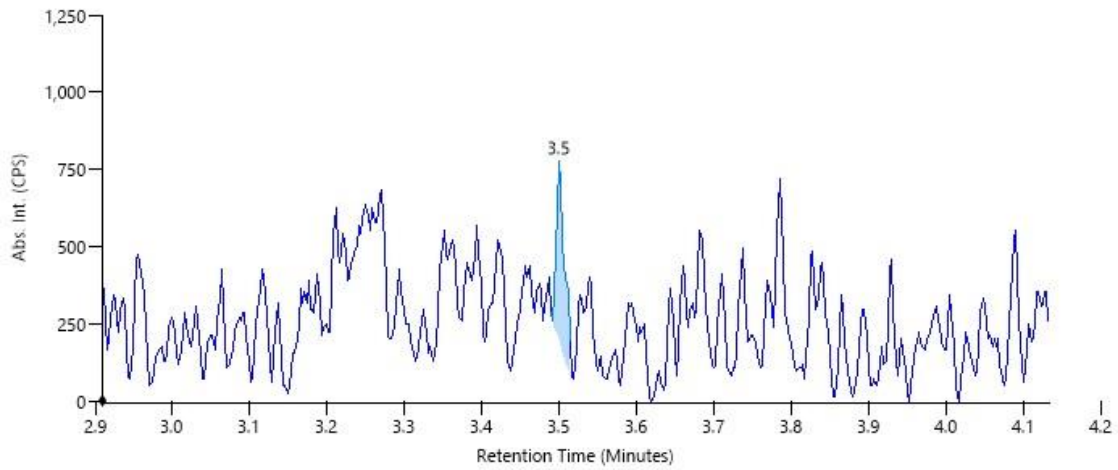
XIC



Baseline Data Patient 12 – 11-OH-THC

AT-12
ESI1 331.1/313.4 11-OH-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

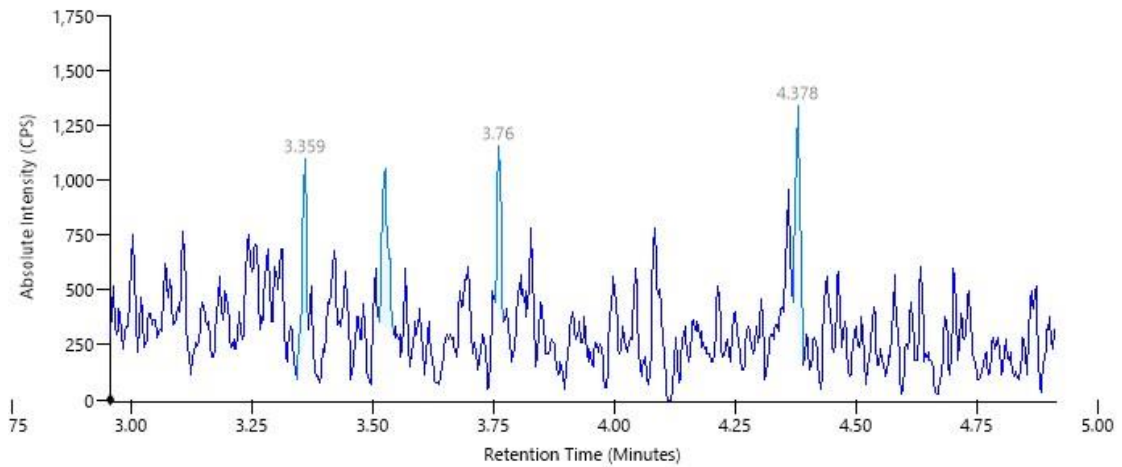
XIC



Data Collected After Dose Patient 12 – 11-OH-THC

BL-12
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "IntelliPeak, 100, 5, 300, 1, 1000, 10, 800, 10, version 1"

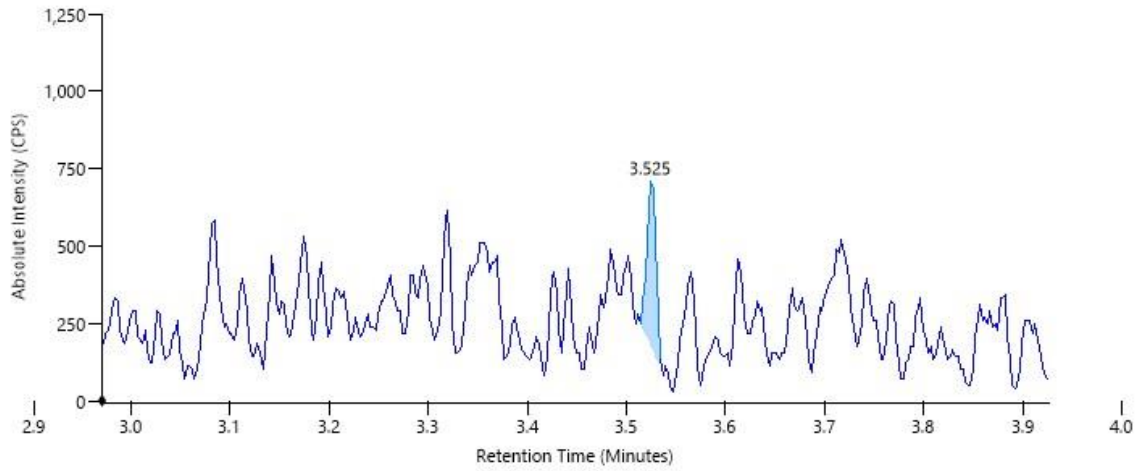
XIC



Baseline Data Patient 12 – THCOOH

AT-12
ESI1 345.2/327.3 11-nor-9-carboxy-d9-THC MRM+ "Moving Average, 3, 2" "Manual"

XIC



Data Collected After Dose Patient 12 – THCOOH

Appendix B: Daily Sessions, Frequency, Age of Onset, and Quantity of Cannabis Use Inventory (DFAQ-CU)

DFAQ-CU Inventory

Instructions: Please read each of the following questions and mark the response alternative that best describes your use of cannabis. *Note that the term cannabis is being used to refer to marijuana, cannabis concentrates, and cannabis-infused edibles.*

1. Have you ever used cannabis?

0 = No

1 = Yes

**If response = 0 then skip to end of questionnaire*

2. Which of the following best captures when you last used cannabis?

1 = over a year ago

2 = 9 – 12 months ago

3 = 6 – 9 months ago

4 = 3 – 6 months ago

5 = 1 – 3 months ago

6 = less than 1 month ago

7 = last week

8 = this week

9 = yesterday

10 = today*

11 = I am currently high*

**If response = 10 (today) or 11 (I am currently high) then answer 2b below*

2b. How high are you right now?

0 = I am not at all high

1 = I am a little bit high

2 = I am moderately high

3 = I am very high

4 = I am extremely high

3. Which of the following best captures the average frequency you currently use cannabis?

0 = I do not use cannabis

1 = less than once a year

2 = once a year

3 = once every 3-6 months (2-4 times/yr)

4 = once every 2 months (6 times/yr)

5 = once a month (12 times/yr)

6 = 2 – 3 times a month

7 = once a week

8 = twice a week

9 = 3 – 4 times a week

10 = 5 – 6 times a week
11 = once a day

12 = more than once a day

4. Which of the following best captures how long you have been using cannabis **at this frequency?**

1 = less than 1 month

7 = 2 – 3 years

2 = 1 – 3 months

8 = 3 – 5 years

3 = 3 – 6 months

9 = 5 – 10 years

4 = 6 – 9 months

10 = 10 – 15 years

5 = 9 – 12 months

11 = 15 – 20 years

6 = 1 – 2 years

12 = more than 20 years

5. Before the period of time you indicated above, how frequently did you use cannabis?

0 = I did not use cannabis

7 = once a week

1 = less than once a year

8 = twice a week

2 = once a year

9 = 3 – 4 times a week

3 = once every 3-6 months (2-4 times/yr.)

10 = 5 – 6 times a week

4 = once every 2 months (6 times/yr.)

11 = once a day

5 = once a month

12 = more than once a day

6 = 2 – 3 times a month

6. How many days of the past week did you use cannabis?

0 = 0 days

4 = 4 days

1 = 1 day

5 = 5 days

2 = 2 days

6 = 6 days

3 = 3 days

7 = 7 days

7. Approximately how many days of the past month did you use cannabis? _____

8. Which of the following best captures the number of times you have used cannabis in your entire life?

1 = 1 – 5 times in my life

4 = 51 – 100 times in my life

2 = 6 – 10 times in my life

5 = 101 – 500 times in my life

3 = 11 – 50 times in my life

6 = 501 – 1000 times in my life

7 = 1001 – 2000 times in my life

9 = 5001 – 10,000 times in my life

8 = 2001 – 5000 times in my life

10 = More than 10,000 times in my life

9. Which of the following best captures your pattern of cannabis use throughout the week?

0 = I do not use cannabis at all

1 = I only use cannabis on weekends

2 = I only use cannabis on weekdays

3 = I use cannabis on weekends and weekdays

10. How many hours after waking up do you typically first use cannabis?

0 = I do not use cannabis at all

5 = 1 – 3 hours after waking up

1 = 12 – 18 hours after waking up

6 = within 1 hour of waking up

2 = 9 – 12 hours after waking up

7 = within ½ hour of waking up

3 = 6 – 9 hours after waking up

8 = immediately upon waking up

4 = 3 – 6 hours after waking up

11. How many times a day, on a typical weekday, do you use cannabis? _____

12. How many times a day, on a typical weekend, do you use cannabis? _____

13. What is the primary method you use to ingest cannabis?

0 = I do not use cannabis

4 = Bong (water pipe)

1 = Joints

5 = Hookah

2 = Blunts (cigar sized joints)

6 = Vaporizer (e.g., Volcano, Vape pen)

3 = Hand pipe

7 = Edibles

8 = Other _____

14. Which of the following other methods to ingest cannabis do you use **regularly** (at least 25% of the time use you cannabis)? [Mark all that apply]

- | | |
|---------------------------------|---|
| 0 = None | 5 = Hookah |
| 1 = Joints | 6 = Vaporizer (e.g., Volcano, Vape pen) |
| 2 = Blunts (cigar sized joints) | 7 = Edibles |
| 3 = Hand pipe | 8 = Other _____ |
| 4 = Bong (water pipe) | |

15. What is the primary form of cannabis you use?

- 0 = None****
- A = Marijuana***
- B = Concentrates (e.g., Oil, Wax, Shatter, Butane Hash Oil, Dabs)**
- C = Edibles*
- D = Other _____

16. What other forms of cannabis do you use **regularly** (at least 25% of the time you use cannabis)? [Mark all that apply]

- 0 = None****
- A = Marijuana***
- B = Concentrates (e.g., Oil, Wax, Shatter, Butane Hash Oil, Dabs)**
- C = Edibles*
- D = Other _____

*****If response to questions 15 and 16 = 0 (None) then skip to question 29*

****If responses to questions 15 or 16 = A (Marijuana) then answer questions 17-21*

***If responses to question 15 or 16 = B (Concentrates) then answer questions 22-26*

**If responses to question 15 or 16 = C (Edibles) then answer question 27*

Note: If you use more than one form of cannabis then complete all of the associated questions listed above.

****If responses to questions 15 or 16 = A (Marijuana) then answer questions 17-21 below.*

Please use the image below to refer to various quantities of marijuana. The image is not to scale; the dollar bill is included to help provide size perspective.



For questions 17 to 19 below, clearly indicate the number of grams of marijuana you use with a number between 0 – 100. Do NOT include other forms of cannabis you may use (such as concentrates). You may use up to 3 decimals to indicate amounts under 1 gram.

Note: 1/8 of a gram = 0.125 grams, 1/4 of a gram = 0.25 grams, 1/2 of a gram = 0.5 grams, 3/4 of a gram = 0.75 grams. 1/8 of an ounce = 3.5 grams, 1/4 of an ounce = 7 grams, 1/2 ounce = 14 grams, 1 ounce = 28 grams

17. In a typical session, how much marijuana do you personally use? _____

18. On a typical day you use marijuana, how much do you personally use? _____

19. In a typical week you use marijuana, how much marijuana do you personally use? _____

20. On a typical day you use marijuana, how many sessions do you have? _____

21. What is the average THC content of the marijuana you typically use? Leave blank if you do not know.

1 = 0 – 4%

5 = 20 – 24%

2 = 5 – 9%

6 = 25 – 30%

3 = 10 – 14%

7 = greater than 30%

4 = 15 – 19%

***If response to questions 15 or 16 = B (Concentrates) then answer questions 22-26 below*

22. In a typical session you use cannabis concentrates, how many hits do you personally take? ____

23. On a typical day you use cannabis concentrates, how many hits do you personally take? _____

24. How many hits of cannabis concentrates did you personally take yesterday? _____

25. On a typical day you use cannabis concentrates, how many sessions do you have? _____

26. What is the average THC content of the concentrates you typically use? Leave blank if you do not know.

1 = 0 – 9%

4 = 30 – 39%

2 = 10 – 19%

5 = 40 – 49%

3 = 20 – 29%

6 = 50 – 59%

7 = 60 – 69%

9 = 80 – 90%

8 = 70 – 79%

10 = greater than 90%

***If response to questions 15 or 16 = C (Edibles) then answer question 27 below*

27. When you eat edibles how many milligrams of THC do you personally ingest in a typical session? _____

28. What is your current age? _____

29. How many years in total have you used cannabis? _____

30. How old were you when you FIRST tried cannabis? _____

31. Has there been any time in your life when you used cannabis regularly (2 or more times per month for 6 months or longer)?

0 = No

1 = Yes*

**If response = 1 (Yes) then answer questions 31b and 31c below*

31b. How old were you when you FIRST STARTED using cannabis regularly (2 or more times/month)? _____

31c. Has there been any time in your life when you used cannabis on a daily or near daily basis for 6 months or longer?

0 = No

1 = Yes*

**If response = 1 (Yes) then answer question 31ci below*

31ci. How old were you when you FIRST STARTED using cannabis on a daily or

near daily basis? _____

32. Which of the following best captures the average frequency that you used cannabis before the age of 16?

0 = more than once a day

1 = once a day

2 = 5 – 6 times a week

3 = 3 – 4 times a week

4 = twice a week

5 = once a week

6 = 2 – 3 times a month

7 = once a month

8 = once every 2 months (6 times/yr.)

9 = once every 3-6 months (2-4 times/yr.)

10 = once a year

11 = less than once a year

12 = never

33. Do you have a physician's recommendation to use cannabis for medicinal purposes?

0 = No

1 = Yes*

2 = Yes, but I use it for both medicinal and recreational purposes*

**If response = 1 or 2 (Yes) then answer questions 33b and 33c*

33b. Which medical condition(s) do you use cannabis for?

33c. What percentage of the time do you use cannabis for recreational (rather than

medicinal) purposes? _____

DFAQ-CU Scoring

Daily Sessions Items: 20, 25

Frequency Items: 2, 3, 6, 7, 8, 9, 10, 11, 12

Age of Onset Items: 30, 31b, 31ci, 32

Marijuana Quantity Items: 17, 18, 19

Concentrate Quantity Items: 22, 23, 24,

Edibles Quantity Item: 27

Note: Standardize (z-transform) scores prior to calculating the mean of each of the 6 factors (daily sessions, frequency, age of onset, marijuana quantity, concentrate quantity, edibles quantity).

Screening/Characterization Items: 1, 2b, 4, 5, 13, 14, 15, 16, 21, 26, 28, 29, 31, 31c, 33, 33b, 33c

LIST OF JOURNAL/ORGANIZATION ABBREVIATIONS

AAFS	American Academy of Forensic Science
Anal Chem	Analytical Chemistry
ASB	American Standards Board
Chem Biodivers	Chemistry & Biodiversity
Clin Biochem	Clinical Biochemistry
Curr Eye Res	Current Eye Research
DOT	Department of Transportation
Int J Mol Sci	International Journal of Molecular Sciences
IRB	International Review Board
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
JAMA	The Journal of the American Medical Association
J Biol Chem	Journal of Biological Chemistry
J Nat Prod	Journal of Natural Products
J Pharmacol Exp Ther	Journal of Pharmacology and Experimental Therapeutics
Methods Enzymol	Methods in Enzymology
Mol Pharmacol	Molecular Pharmacology

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