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ISBN

9798189229176

Author

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Publication Date

2026-06-25

Peer reviewed|Thesis/dissertation

Development of LC-MS/MS Confirmation Method for the Effective Separation of Common
Cannabinoid Isomers and their Metabolites in Whole Blood

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Forensic Science

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2026

ABSTRACT

Since the passage of the 2018 Farm Bill, which based the legality of cannabis products on the concentration of delta-9-THC, the market for hemp-derived cannabinoid compounds has greatly expanded. Other psychoactive cannabinoids, such as the isomer delta-8-tetrahydrocannabinol (Δ 8-THC), are commonly being used in cannabis products but have previously been difficult to differentiate in both a qualitative and quantitative manner in blood samples sent to forensic toxicology laboratories. The cannabinoid market also continues to change and expand, with different isomers and new semi-synthetic derivatives being produced and becoming available for purchase. In this study, a chromatographic method was created to detect and quantify the separate presence of the five most commonly seen cannabinoid compounds in whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as well as indicate the possible presence of 12 less common cannabinoids, such as exo-THC, tetrahydrocannabiphorol (THCP), and Δ 10-THC. This method was then tested using the American Academy of Forensic Sciences-Academy Standards Board (ASB)-approved standard practices for method validation in forensic toxicology. The method was created to be effective to the level of scrutiny required for forensic testing while also being as time and cost effective as possible.

ACKNOWLEDGEMENT

A big thank you to everyone at the Sacramento County District Attorney's Crime Lab for not only allowing me to witness the reality of my dream job through this internship but also for being so welcoming and allowing me to not feel out of place in a professional environment I have never encountered before. A really big thanks to my supervisor Craig Triebold for helping me through every step of this project, for allowing me to learn through every mistake, and for encouraging me to ask a (seemingly) unlimited number of questions to get to a place of understanding. The same goes to the other members of the toxicology unit, who were always willing to step in and help or even just be encouraging when I needed some support.

I would also like to thank my committee members, Dr. Wilson Rumbhia and Dr. Karyn Bischoff, for their very helpful input along the way, and especially to my committee chair, Dr. Benjamin Moeller. He kept me on track during each step along the way and also managed to keep me calm through it all. Thank you for helping me realize that it was possible, and the work would get done in time.

Thanks also go out to our director, Dr. Ashley Hall, and program coordinator, Aliyah Middleton. They both gave me so much direction along the way, from starting with no idea how to start writing a thesis or get involved in an internship to now having accomplished so much is thanks to their help in so many ways. Also thank you to my cohort for being the best group of like-minded peers of which I have ever been a part. I always know I have a support system with you guys.

Lastly, a massive thank you to my family (housemate included) for the never-ending support. I would not have made it this far without your encouragement to achieve my dreams, understanding, and ability to keep me sane through the craziness.

Introduction

Cannabinoids are the second most common impairment-causing substances found in the toxicology reports of DUI toxicology casework, following only alcohol¹. Many of these compounds commonly originate from the *Cannabis sativa* plant². Delta-9-tetrahydrocannabinol (Δ 9-THC) is the main psychoactive component of this plant. Cannabis containing Δ 9-THC above 0.3% on a dry-weight basis is considered a Schedule III federally controlled substance by the US Drug Enforcement Agency (DEA) if it exists in a US Food and Drug Administration (FDA)-approved product or in a product under a medical marijuana license. This means it has accepted medical use but has some potential for physical and psychological dependence³. If found in any other product, this cannabis is considered a Schedule I controlled substance. This means the substance has no accepted medical use and has high potential for abuse³. The Farm Bill passed in 2018 defines “hemp” as Cannabis containing no more than 0.3% of this Δ 9-THC⁴, which is legal to produce and sell. Delta-8-tetrahydrocannabinol (Δ 8-THC) is an isomer of Δ 9-THC (Figure 1) that has psychoactive effects very similar to those of Δ 9-THC, but is only about two-thirds as potent⁵. Despite Δ 8-THC's ability to cause impairment, it is not currently considered a controlled substance because it is produced from legal hemp-derived cannabidiol (CBD). The current cannabis market continues to produce product with Δ 9-THC as well as very concentrated Δ 8-THC to satisfy customers looking for an expected “high”. The presence of both of these impairing compounds, along with an expanding array of other psychoactive cannabinoids and derivatives in the drug market, makes differentiating between Δ 9-THC, Δ 8-THC, and other cannabinoids in a sample greatly important in a forensic toxicology laboratory setting.

At the molecular level, THC acts as a partial agonist for both the cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) receptors. The CB1 receptor is linked to the commonly attributed effects of using THC, including stress response, memory, and reward behavior⁶. CB2 receptors can be linked to neurons that are tied to addiction behaviors, leading to the addictive nature of the drug⁶. In naturally grown cannabis, nearly all of the THC is found in the form of Δ^9 -THC, while Δ^8 -THC has been commonly reported to make up less than 1%⁷ of the THC content. It has also been observed that Δ^8 -THC has lower potency than Δ^9 -THC. In a number of current hemp-based products, though, the concentration of Δ^8 -THC is commonly increased through a chemical reaction that converts the existing cannabidiol (CBD) in those samples into Δ^8 -THC⁸. With higher concentrations of Δ^8 -THC, an increase in intoxicating psychoactive effects is expected⁸. Even with this increased prevalence of Δ^8 -THC, the physiological effects have not been as widely studied. Other intoxicating psychoactive substances in cannabis have also been isolated and are being concentrated in laboratory settings. This may pose a danger to cannabinoid consumers, since these compounds have not been tested for safety and long-term effects. These products are, like Δ^8 -THC, found in naturally grown cannabis in very small amounts and are isolated and concentrated by sellers. Some of these cannabis-derived products are nonintoxicating and have possible health benefits⁹. Of these, cannabidiol (CBD), cannabigerol (CBG), cannabinol (CBN), cannabidivarin (CBDV), tetrahydrocannabivarin (THCV), and cannabichromene (CBC) are included in this study. These products mainly affect the same CB1 and CB2 receptors but have more targeted effects on managing sleep and appetite, rather than the intoxicating effects⁹. Some other cannabis-derived products are intoxicating, when isolated, and could pose a risk to users¹⁰, such as *exo*-THC and Δ^{10} -THC. All of these new substances require more research to determine their potential to produce adverse effects, whether

short-term or long-term, before legal judgement can be passed on their use. Nevertheless, being able to identify and address the presence of any of these substances in toxicological samples could be significant and could aid toxicologists in providing opinions regarding possible impairment or toxicity.

When THC enters the body, through any form of ingestion, it starts to undergo the process of metabolism. THC is first converted into 11-hydroxy-THC (OH-THC). This metabolite is still psychoactive within the body, so, even if all traces of THC have been converted, there can still be an active substance in the system⁸. OH-THC is then further oxidized into 11-nor-9-carboxy-THC (COOH-THC), a nonpsychoactive compound⁹. The concentration of THC increases and peaks more quickly than either of the major metabolites. When inhaled, the maximum concentration in plasma has been observed at 8 minutes for THC, 15 minutes for OH-THC, and 81 minutes for COOH-THC in a random group of non-chronic users¹¹. When ingested orally, the absorption is much slower, resulting in a maximum concentration of THC within 1-2 hours, and the peak OH-THC relative to the peak delta-9-THC is much higher¹¹. In any case, $\Delta 8$ -THC has been observed to have a slower absorption and formation of OH-THC metabolite than $\Delta 9$ -THC¹². A higher concentration and longer persistence of COOH-THC can be an indicator of chronic use of THC. The concentration will be at a much higher value if the subject is a frequent user due to accumulation in the body from each instance of use¹³. With chronic use, COOH-THC was found to be detectable up to more than 30 days¹⁴. Both OH-THC and COOH-THC will be present in the body in the same isomeric form, $\Delta 8$ - or $\Delta 9$ -, as the THC that was ingested. Because of this, being able to differentiate the isomers of these metabolites could also help draw conclusions about a subject's use of THC and is, therefore, included in the scope of this study.

Since the isomeric compounds share the same chemical formula and are nearly identical in structure, their detection and correct identification in a biological sample has proven to be difficult using chromatographic approaches commonly utilized in forensic laboratories¹⁵. For example, the only structural difference between Δ 9-THC and Δ 8-THC is the position of a single double bond within the ring structure of the molecule (**Table 1**). This structural similarity also means that the chemical behavior of these compounds is difficult to differentiate. Here, a quantitative method was developed to accurately separate the Δ 8 and Δ 9 isomers of THC and its most common metabolites. 13 other cannabinoid compounds (**Figure 1**) were tested within the method to confirm their inability to interfere with the essential cannabinoids and to provide a presumptive assessment of semi-synthetic cannabinoids present in a sample when needed. This method was created for use at the Sacramento County District Attorney's Office Laboratory of Forensic Services (SACDA Crime Lab), so some decisions were made for greatest efficiency at this location but may be relatable concerns to other laboratories. The greatest concern here was the issue of quantitating Δ 9-THC when Δ 8-THC is present, since they co-elute within the laboratory's current method. Being able to accurately separate these compounds and improve laboratory efficiency is a great advantage, as the use of THC-containing products continues to rise in popularity.

Table 1. List of Cannabinoid Compounds

Compound Name	Molecular Formula
Included in Validated Confirmatory Method	
Delta-8-THC*	C ₂₁ H ₃₀ O ₂
Delta-9-THC*	C ₂₁ H ₃₀ O ₂
11-hydroxy-delta-8-THC	C ₂₁ H ₃₀ O ₃
11-hydroxy-delta-9-THC	C ₂₁ H ₃₀ O ₃
11-nor-9-carboxy-delta-8-THC	C ₂₀ H ₂₈ O ₄
11-nor-9-carboxy-delta-9-THC	C ₂₀ H ₂₈ O ₄
Tested for Interference and Presumptive Identification	
Delta-8-tetrahydrocannabiphorol (delta-8-THCP)	C ₂₃ H ₃₄ O ₂
Delta-9-tetrahydrocannabiphorol (delta-9-THCP)	C ₂₃ H ₃₄ O ₂
(6aR, 9R)-delta-10-THC*	C ₂₁ H ₃₀ O ₂
(6aR, 9S)-delta-10-THC*	C ₂₁ H ₃₀ O ₂
Exo-THC*	C ₂₁ H ₃₀ O ₂
Cannabidiol (CBD)	C ₂₁ H ₃₀ O ₂
Cannabigerol (CBG)	C ₂₁ H ₃₂ O ₂
Cannabinol (CBN)	C ₂₁ H ₂₆ O ₂
Cannabidivarin (CBDV)	C ₁₉ H ₂₆ O ₂
Tetrahydrocannabivarin (THCV)	C ₁₉ H ₂₆ O ₂
Cannabichromene (CBC)	C ₂₁ H ₃₀ O ₂
Hexahydrocannabinol (HHC)	C ₂₁ H ₃₂ O ₂

The five starred (*) compounds are isomers of the same THC compound. The other 13 are common cannabinoids also found within the *Cannabis sativa* plant or are metabolites of THC detected in the body after ingestion.

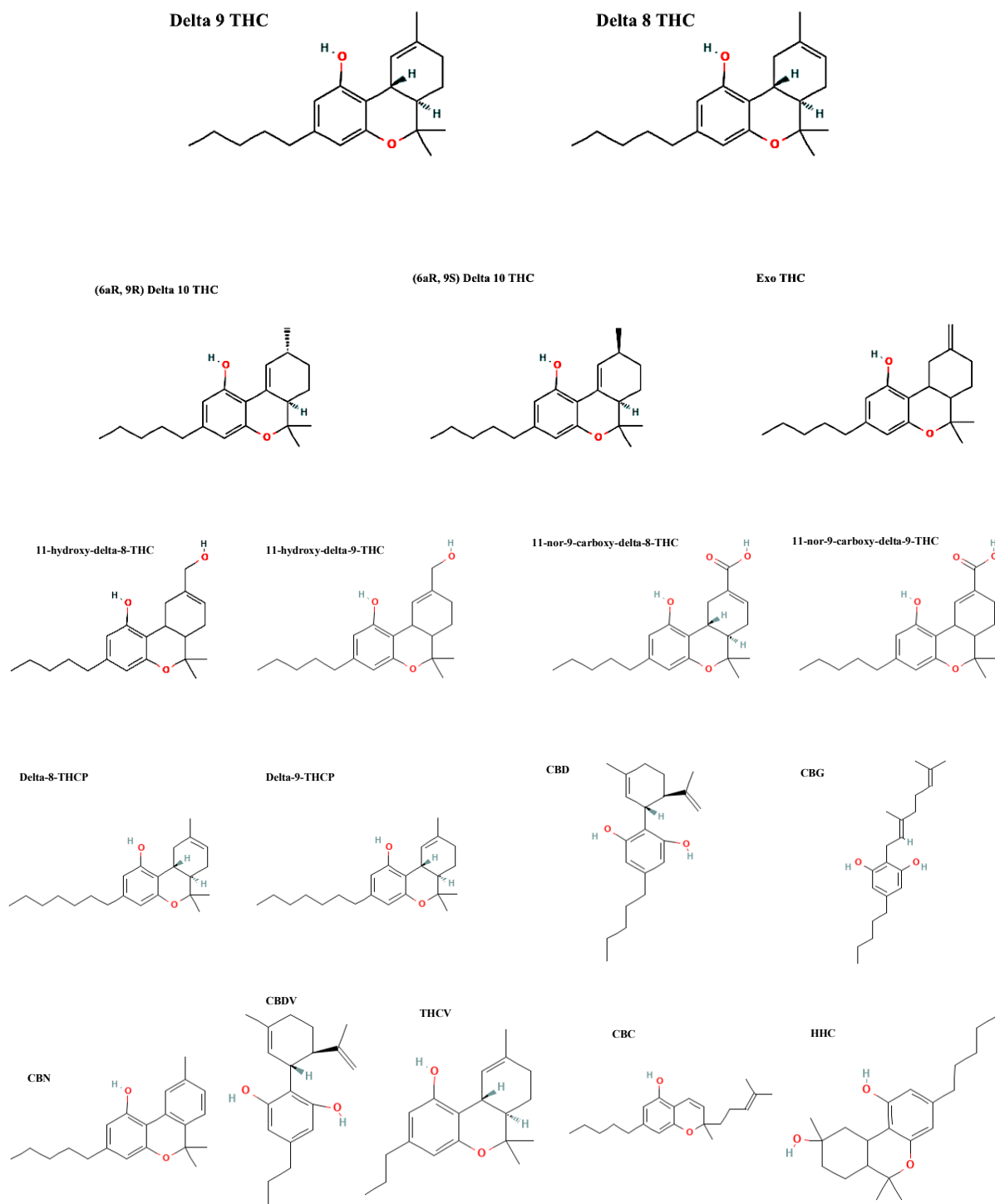


Figure 1. Molecular Structures of the 18 cannabinoids tested¹⁶. Isomers of Δ^9 -THC are listed first, followed by the rest of the 18 analytes.

Understanding the potential pharmacological effects of the targeted compounds is important when interpreting potential findings detected in the laboratory. Cannabinoids have a diverse array of effects on humans and have a range in their potential impairment, which influences how detections of substances are addressed within potential legal proceedings.

Δ 8-THC and Δ 9-THC have been discussed at length as the most common psychoactive cannabinoids found in toxicology casework. These and their metabolites of OH-THC and COOH-THC have been widely researched and are greatly understood in their physiological effects and use. The other cannabinoids in this study are lesser-known and lesser-used but are important compounds for monitoring if they could be causing impairment. It is also important to ensure that the presence of these drugs would not interfere with the quantitative results of the widely known compounds.

Tetrahydrocannabiphorol (THCP) is a cannabinoid that is produced in extremely small amounts in the natural plant. If it is present at a significant concentration in a sample, it is suggestive of laboratory synthesis rather than natural production. THCP exists in both delta-8 and delta-9 forms, but insufficient research has been accomplished to determine if these isomers have different pharmacological profiles. THCP is not yet commonly used, but its potency raises concern. Testing has shown THCP to have a higher affinity toward the CB1 receptor than THC, making it more active¹⁷. THCP has demonstrated activity at half the concentration than the minimum for THC. This higher potency makes THCP a substance of great concern if it becomes more commonly used. It has been suggested that THCP may be present in some medicinal cannabis and responsible for the wide-ranging effects that patients experience¹⁷.

Delta-10-THC is another semi-synthetic cannabinoid that exists in very trace amounts naturally. It is generally produced from a chemical reaction with cannabidiol (CBD). It exists in

two structural forms, but it seems there is little difference in effect from them. There has been little research done on its psychoactivity, but it is suggested to have similar effects to THC, just less potent¹⁸. It is often included within products produced by unauthorized drug manufacturers alongside Δ 8-THC marketed as a “legal alternative” to Δ 9-THC.

The research on exo-THC is even more limited than the other compounds, but it is known by the other names of delta-9,11-THC or just delta-11-THC. It is used very often on labels of THC products both to advertise an “exotic” component and as an incorrect label of 11-hydroxy-THC¹⁹. Advertisements will occasionally claim that exo-THC is significantly more potent than Δ 9-THC, but there is no data to support these claims. Since the claim of exo-THC is often due to a misunderstanding, it is important to be able to identify the actual compound in the rare occasion it is actually present.

Cannabidiol (CBD) is the most abundant cannabinoid in hemp. It is a psychoactive but non-intoxicating substance that can have positive therapeutic effects on anxiety, depression, pain, metabolism, and other body systems²⁰. It is reported to reduce the negative side effects of Δ 9-THC, so it is often used in high doses within Δ 9-THC products to give a better experience. It is also the base compound from which most semi-synthetic psychoactive cannabinoids are derived.

Cannabigerol (CBG), cannabinol (CBN), and cannabichromene (CBC) are the three other most common cannabinoids in hemp. They are all non-psychoactive, but have individual therapeutic uses. CBG, in its acid form, is the precursor compound to all other cannabinoids grown naturally²¹. CBN is very slightly active at the same receptors as Δ 9-THC but it is at such low affinity that it does not pose any intoxicating effects²². CBC is currently being studied as a promising anti-inflammatory or anti-convulsant agent²³. Tetrahydrocannabivarin (THCV) and cannabidivarin (CBDV) are similar in that they are both non-psychoactive and are being studied

for therapeutic uses. CBDV is the natural precursor of THCV, and both have a similar effect profile currently being applied to possible use in the management of obesity and diabetes²⁴. These are included in this study to demonstrate the absence of interference with the other compounds in a combined method.

The last cannabinoid included in this study is hexahydrocannabinol (HHC). HHC actually exists as two different forms (9R and 9S), but very little research has been done to identify any significant difference between the activity of the forms²⁵. HHC is a relatively new cannabinoid to the market and is generally formed through a derivatization of Δ^9 -THC. There is not much data to support the physiological response of HHC, but it has been seen to demonstrate very similar effects to Δ^9 -THC. The activity of HHC, though, can greatly vary based on the exact process that was used to create it. As it becomes more popular, it will become increasingly important to learn more about HHC and how it affects a subject.

With continuing development of semi-synthetic cannabinoids and increasing prevalence of hemp-derived substances, the need to accurately detect and monitor a range of cannabinoids is increasingly important. Many THC confirmation methods in forensic laboratories continue to be directed toward the detection of Δ^9 -THC. This is effective because Δ^9 -THC is the most common cannabinoid causing impairment, but, since the semi-synthetic cannabinoids are very similar in structure and are newer developments, there is a possibility that those confirmation methods in use are encountering interferences from these other cannabinoids. In this study, a method is developed that introduces Δ^8 -THC into the quantitative confirmation and provides sufficient evidence that a wide range of other cannabinoids will not interfere with that confirmation.

Materials and Methods

I. Materials

Reference standards for 11-hydroxy-delta-8-THC and 11-nor-9-carboxy-delta-8-THC were purchased from Cayman Chemicals. Reference standards for delta-8-Tetrahydrocannabiphorol (delta-8-THCP), delta9-Tetrahydrocannabiphorol (delta-9-THCP), (6aR, 9R)-delta10-THC, (6aR, 9S)-delta10-THC, Cannabichromene (CBC), Cannabidol, exo-THC, Cannabidivarin (CBDV), Tetrahydrocannabivarin (THCV), Cannabigerol (CBG), Cannabinol, (±)-11-hydroxy-delta-9-THC, (-)-delta-9-THC, (-)-11-nor-9-carboxy-delta-9-THC, (-)-delta-8-THC, and hexahydrocannabinol (HHC) were purchased from Cerilliant Corporation. Internal standards of delta-9-THC-D3, (±)-11-hydroxy-delta-9-THC-D3, and (-)-11-nor-9-carboxy-delta-9-THC-D3 were also purchased from Cerilliant Corporation.

All solvents and solvent additives were purchased from Fisher Scientific at LC-MS quality. These included methanol, acetonitrile, water, 2-propanol, and formic acid. These same solvents were used in extraction experimentation.

For the extraction experiments, the 6mL ISOLUTE® SLE+ columns were purchased from Biotage. 6mL CereX® THC solid phase extraction columns were purchased from Fischer Scientific. Ammonium hydroxide (NH₄OH) and acetic acid (HOAc) were also purchased from Fischer Scientific. Zinc sulfate heptahydrate was purchased from Aldrich Chemical Company. Ethyl acetate (EtAc), hexane, and 2-propanol (IPA) were purchased from Sigma-Aldrich Corporation. Methyl t-Butyl Ether (MTBE) was purchased from BDH Chemicals.

Whole blood matrix used for development, calibration, and QC was porcine blood sodium fluoride and potassium oxalate (preservative and anticoagulant) purchased from the Meat Laboratory at the UC Davis Department of Animal Science. Validation tests were completed

using 10 human whole blood samples containing sodium fluoride and potassium oxalate (preservative and anticoagulant) from previous casework at the SACDA Crime Lab that had tested negative for any presence of cannabinoids.

Other materials used include: plastic snap-cap microcentrifuge tubes, disposable glass culture tubes, disposable glass centrifuge tubes, LC-MS autosampler vials, and *Biohit* micropipettes and disposable pipette tips.

II. Solutions

Methanol mobile phase was made by adding 1mL of formic acid to 999mL of methanol. Water mobile phase was made by adding 1mL of formic acid to 999mL of water. The strong wash used for LC-MS/MS was made by combining 200mL ACN, 200mL methanol, 75mL 2-propanol, 25mL water, and 0.5mL formic acid. The water mobile phase was used as the LC-MS/MS weak wash. The seal wash was made with 50mL of methanol and 450mL water. The sample wash used was made from mixing equal parts of methanol, 2-propanol, and ACN.

THC internal standard was made to include 200ng/mL of Δ^9 -THC-D3 and OH- Δ^9 -THC-D3 and 2000ng/mL of COOH- Δ^9 -THC-D3 in methanol. For chromatographic method development, a full mixture solution of 20 μ L of each of the 17 cannabinoids was used (588ng/mL of each). Tests with HHC were done separately, as that standard was obtained later. For extraction and validation tests, a mixture containing 500:2500ng/mL of Δ^8 -THC, Δ^9 -THC, OH-THC:COOH- Δ^8 -THC, COOH- Δ^9 -THC was used to spike the 50 μ L of blank blood to the desired concentration during the first step of the extraction procedure.

III. Extraction

Three extraction procedures were tested to evaluate the most efficient option. The first extraction method tested was the method currently used by the SAC DA crime lab. First, 1mL of

sample was added to a culture tube. 100µL of 200:2000ng/mL (Δ^9 -THC-D3, OH- Δ^9 -THC-D3:COOH- Δ^9 -THC-D3) internal standard was added in and vortexed. Then, 500µL of 0.1M zinc sulfate was added to each of these tubes and vortexed. Cold ACN was added to these, and vortexed for 1 minute, then centrifuged for 5 minutes. The supernatant was transferred to a second set of culture tubes. 2mL of water was added, and then it again was vortexed for 1 minute and centrifuged for 5 minutes. The samples were then decanted into Cerex[®] THC solid phase extraction columns on the positive pressure manifold (with no need for column conditioning) and eluted by gravity or pressure at a rate of one drop per second. 1mL of a wash of H₂O:ACN:NH₄OH (85:15:1 prepared daily) was added and eluted to dryness under nitrogen flow for 15 minutes. An elution solvent of hexane:ethyl acetate:acetic acid (90:10:3 prepared daily) was added, and the elution was collected at a rate of one drop per second into a culture tube. This eluent was transferred to centrifuge tubes and evaporated to dryness under a nitrogen stream at approximately 40°C. 100µL of reconstitution solvent of methanol:water (75:25) was added, vortexed for 1 minute, then centrifuged for 5 minutes. 90µL of this sample was transferred to LC/MS vials for analysis.

The second extraction method tested the use of another solid phase extraction (SPE) column. 400µL of each sample was added to a microcentrifuge tube. Then, 200µL of 0.1% formic acid in water was added to each tube. 60µL of 200:2000ng/mL (Δ^9 -THC-D3, OH- Δ^9 -THC-D3:COOH- Δ^9 -THC-D3) internal standard was added to each sample then vortexed for 2 minutes. Each mixture was then transferred to ISOLUTE[®] SLE+ extraction columns, and light pressure was applied for the liquid to absorb onto the column for 5 minutes. 1.5mL of methyl t-butyl ether (MTBE) was added to the column and eluted at a rate of 1 drop per second. After fully eluted, a second aliquot of 1.5mL of MTBE was then added and eluted at 1 drop per

second. This eluent was then transferred to centrifuge tubes and evaporated under a nitrogen stream at approximately 40°C. A reconstitution solvent of 150µL methanol was added, vortexed for 1 minute, and centrifuged for 5 minutes. 50µL of water was added to match starting LC conditions and transferred to LC vials for analysis.

The final extraction method used a protein precipitation. First, a 1:1 ACN:methanol solution was prepared with 5:50ng/mL (Δ^9 -THC, OH- Δ^9 -D3:COOH- Δ^9 -THC-D3) internal standard, diluting from the 200:2000ng/mL internal standard stock. 50µL of sample was added into pop-cap microcentrifuge tubes and 150µL of the prepared solution was added to each. All samples were vortexed for 2 minutes and then centrifuged for 5 minutes at 14000rpm. The supernatant was transferred to centrifuge tubes and evaporated to dryness under a nitrogen stream at approximately 40°C. A reconstitution solvent of 60µL methanol was added and vortexed for 1 minute. 20µL of water was added to match starting LC conditions of 75:25 methanol:water, and this was transferred to LC vials for analysis.

The extraction method from previous research²⁶ tested samples after the first centrifuge step. Tests were done to evaluate the best extraction solvent and procedure. These solvents were ACN, ACN:MeOH (1:1), and cold ACN. The addition of an evaporation and reconstitution step was also tested with a reconstitution of only methanol, with a reconstitution of 75:25 methanol:water, and with comparison to an extraction without the step.

IV. Instrument Details

The method was created using a Waters Acquity UPLC I-Class system with Xevo TQ-S Triple Quadrupole Mass Spectrometer using an electrospray ionization (ESI) source. The column was a Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 100 mm column, and MassLynx V4.1 software was used in this method development.

Specific mass spectroscopy transitions were specified for each of 17 cannabinoids (**Table 2**). Of these, only $\Delta 8$ -THC, $\Delta 9$ -THC, OH-THC, COOH- $\Delta 8$ -THC, and COOH- $\Delta 9$ -THC (the first five listed) were included in the final MS method.

During method development, the aqueous mobile phase (A) of water with 0.1% formic acid and the organic mobile phase (B) of methanol with 0.1% formic acid was used. An injection volume of 5 μ L and flow rate of 0.4 μ L/min was used. The sample temperature was maintained at 10°C within the holder. The column was held at a constant 40°C throughout the run. All of these parameters were held constant as the method was adjusted through the solvent gradient.

The final solvent gradient started at 75% B and held for 3.5 minutes. The gradient then immediately increased to 80% B and held there for 1.5 minutes. Then the MPB was gradually increased to 90% over another 1.5 minutes, followed by an immediate increase to 95% B. This was held for 1.5 minutes. Finally, the B was lowered to 75% to match the starting conditions and held for 1 minute to allow for re-equilibration. The total run time was 9 minutes.

Table 2: Mass Spectroscopy Method Transitions

Transition (Daughter) Mass	Cone Voltage	Collision Energy (V)
Δ8-THC*		
93.15	4	22
123.09	4	36
135.17	4	20
193.18	4	22
Δ9-THC*		
93.15	54	22
123.09	54	34
135.09	54	20
193.18	54	22
OH-THC*		
123.08	2	34
331.23	2	22
COOH-Δ8-THC*		
165.11	54	18
187.12	54	28
193.16	54	26
257.21	54	22
COOH-Δ9-THC*		
119.12	34	30
187.12	34	28
193.16	34	26
245.21	34	34
Delta-8-Tetrahydrocannabinophorol (delta-8-THCP)		
93.14	66	24
123.09	66	34
135.17	66	20
221.23	66	24
Delta-9-Tetrahydrocannabinophorol (delta-9-THCP)		
93.14	38	26
123.09	38	34
135.17	38	22
221.23	38	26
(6aR, 9R)-delta-10-THC		
93.15	66	26
123.09	66	32
135.17	66	22
193.18	66	24
(6aR, 9S)-delta-10-THC		
93.15	46	24
123.09	46	34
135.17	46	22
193.18	46	26
Exo-THC		
93.15	42	24
123.09	42	32
135.17	42	20
193.18	42	26

Cannabidol (CBD)		
93.25	42	22
123.09	42	18
135.11	42	34
193.18	42	22
Cannabigerol (CBG)		
67.11	6	46
78.6	6	42
123.09	6	32
193.17	6	16
Cannabinol (CBN)		
195.18	2	26
208.17	2	32
223.11	2	22
241.17	2	18
Cannabidivarin (CBDV)		
93.14	6	22
123.08	6	32
135.16	6	20
165.16	6	26
Tetrahydrocannabivarin (THCV)		
123.08	44	30
135.16	44	20
165.16	44	24
231.16	44	18
Cannabichromene (CBC)		
81.13	22	14
123.09	22	32
193.18	22	20
259.25	22	12
Hexahydrocannabinol (HHC)		
81.13	32	22
123.08	32	34
137.11	32	20
193.16	32	22

*The five compounds included in the confirmatory method.

V. Statistical Analysis

In order to judge the validity of this new method, the American National Standards Institute/American Academy of Forensic Sciences Standards Board (ANSI/ASB)-approved standard practices for method validation²⁷ were followed. These standards include tests for interference, calibration, limits of detection and quantitation, bias and precision, and carryover.

a. Interference

The standards require multiple different interference studies to be performed. An interference study should show no signal in any of the target analytes under different circumstances. For blank matrix tests, blank samples were extracted as expected without the addition of the internal standard. The next interference test was with the internal standards used. The validation requirements specify that, when stable-isotope internal standards are used, impurities of the target analyte can be present, and there can be some identical MS fragmentation that would cause interference. For this requirement, a blank sample of human blood was extracted exactly according to the method. Another set of blank human blood samples was spiked by adding a mixture of the drug standards for the five quantitative compounds to the 50 μ L of blood used in the first step of the extraction to reach a concentration of 80:400ng/mL (Δ 8-THC, Δ 9-THC, OH-THC:COOH- Δ 8-THC, COOH- Δ 9-THC). These samples were extracted without the addition of the internal standard.

The final interference study was with other commonly encountered analytes. Quite often, casework blood samples will contain multiple drug categories. It is important to make sure that the presence of any other drug will not interfere with the results of this method. This test was done using the performance mix for the LC-Time-Of-Flight (TOF)-MS screening at the SACDA Crime Lab, which includes a wide range of all commonly encountered drug classes, as well as

the performance mixes for benzodiazepines, opiates, carisoprodol and meprobamate (SOMA), stimulants, acid-neutral drugs, and strong-base drugs. To do this, a set of samples at the 50 μ L first step of extraction was spiked with the addition of 5 μ L of one concentrated standard mix, and the extraction was completed as intended.

b. Calibration

The validation standards require a calibration model to be used for all quantitative methods. This model is created by determining an appropriate calibration range and using relative responses between each analyte and its internal standard to create a mathematical model to match the pattern with increasing concentration. This model should be created and tested using blank matrix samples spiked to different concentrations evenly spread across the range to establish the curve. The best model should be chosen from these results, the most common being a simple linear regression. Then, the match to the calibration curve should be assessed through both its correlation coefficient (r) and a visual inspection of the residual plots for any evidence of outliers or recurring patterns in the variance.

The concentrations chosen for this calibration were 1:5ng/mL, 5:25ng/mL, 10:50ng/mL, 50:250ng/mL, and 100:500ng/mL (THC, OH-THC:COOH-THC).

c. Limit of Detection and Quantitation

A lower limit of detection and quantitation is needed to determine the clear cutoff point between a readable peak and background noise. The validation standards require the determined limit of detection (LOD) to be tested multiple times through spiking blank matrix samples to ensure the analytes at the LOD will always be read by the instrument. One option for determining both the LOD and the limit of quantitation (LOQ) is to choose the lower limit of the calibration curve. Several tests were run to determine the lowest concentration the method would

be able to read through spiking the blank blood at decreasing concentrations, and the chosen lower limit of the calibration curve was adjusted to lowest reliable concentration. This was only done for the five compounds included in the final method.

d. Ion Suppression and Enhancement

When the response of an analyte is higher or lower than expected due to other compounds present, this is called ion enhancement or ion suppression. These other compounds present could come from the matrix during extraction or from interactions with the internal standards. The validation standards require testing to show suppression or enhancement to be below 25% or prove that there is no negative impact on the other aspects of validation, such as the LOD or calibration curve. To test for suppression or enhancement, a post-extraction addition technique was used at three concentrations, low, medium, and high, within the calibration range. With this, two sets of samples were created to be tested and compared. The first set was neat standards. These standards were made in a 75:25 (MeOH: Water) solvent to match the end result of the extraction method. Each of these standards was tested six times to calculate a mean response area. The second set of samples was blank human blood from 10 different sources taken through the extraction method and then, in the final reconstitution step, spiked to the same concentration as the neat standards. Each of these 10 samples was tested twice at each concentration level. To calculate the ion suppression or enhancement, the mean peak area of each set was calculated, and then the percent difference was calculated according to the equation:

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{\overline{X} \text{Area of Set 2}}{\overline{X} \text{Area of Set 1}} - 1 \right) \times 100$$

in which Set 2 was the spiked extraction samples and Set 1 was the neat standards.

The high concentration level was 80:400ng/mL (THC, OH-THC:COOH-THC), close to the high end of the calibration curve, which is 100:500ng/mL. The mid-range concentration level

was 40:200ng/mL, and the low concentration was 4:20ng/mL, close to the limit of detection and quantification at 1:5ng/mL.

e. Bias and Precision

Bias refers to how close the calculated concentration of an analyte is to the actual concentration in the sample. Precision refers to the ability of the method to produce the same result when run multiple times. To assess this, the validation standards require a test of three different samples at each of three concentrations through five runs. The percentage of bias was calculated using the percent error equation in which the experimental value was the grand mean of the results at a single concentration, and the theoretical value was the nominal concentration. The coefficient of variation, %CV, (measure of percent against precision) was assessed both within a single run and between runs. The within-run calculation divides the standard deviation of the single run by the mean results of that run. The between-run calculation uses the same equation but with all results from each of the chosen concentrations.

The concentrations chosen for the bias and precision tests were the same as the high, medium, and low concentrations used for ion suppression and enhancement. These were 80:400ng/mL, 40:200ng/mL, and 4:20ng/mL .

f. Carryover

Carryover refers to an analyte remaining on the column after a sample is run that then elutes with the next sample. This could cause unwanted interfering peaks or greater peak areas, decreasing the accuracy of the calculated concentrations. To test for carryover, the validation standards require a triplicate analysis of a blank extracted sample run immediately after a high concentration sample or neat standard. The high concentration used in the test will become the

confirmed concentration at which the method is free from carryover. To be able to use the full calibration range, then, the concentration tested for carryover must be the maximum of the range.

For this experiment, an extracted sample at 100:500ng/mL was run and immediately followed by an extracted blank human blood sample.

Results

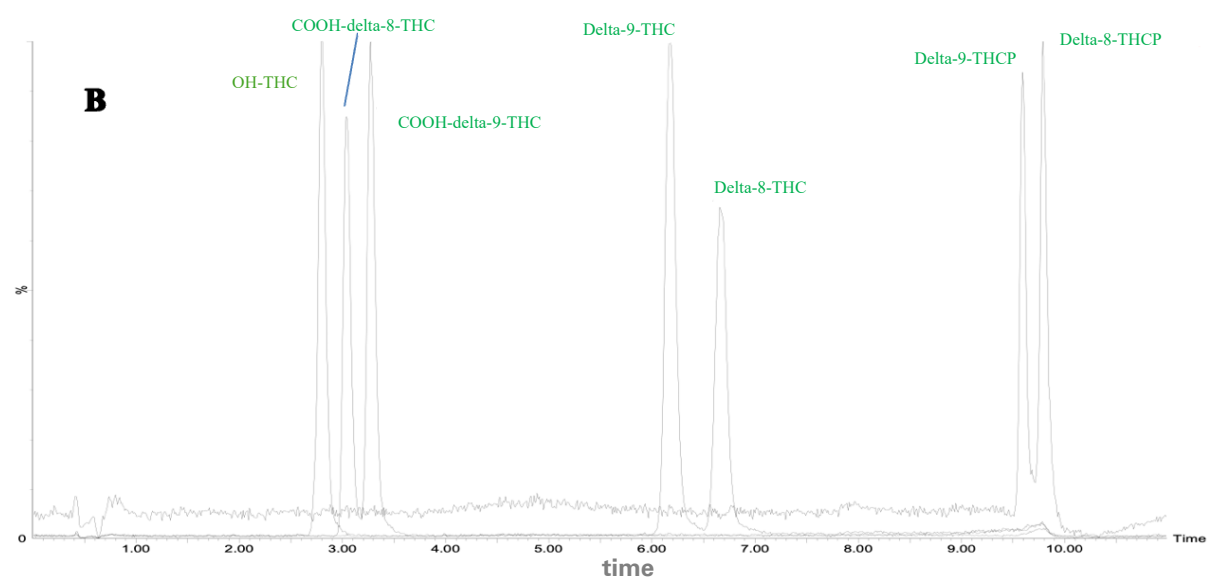
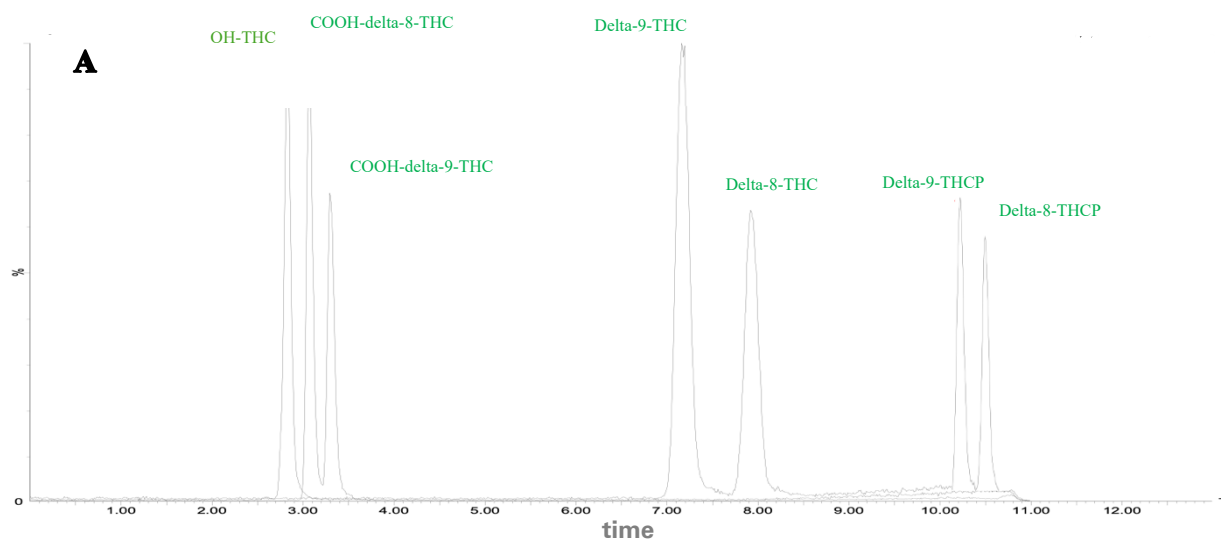
I. Method Development: Mass Spectrometry

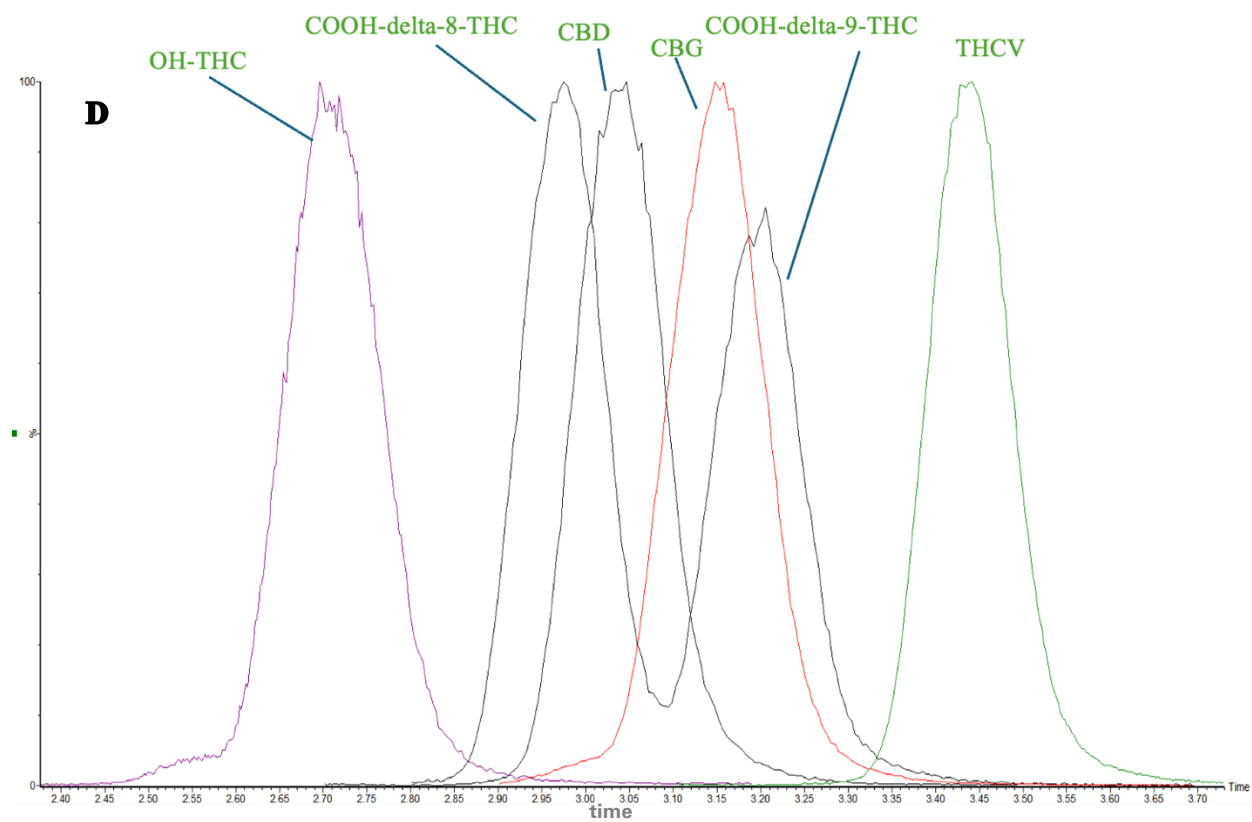
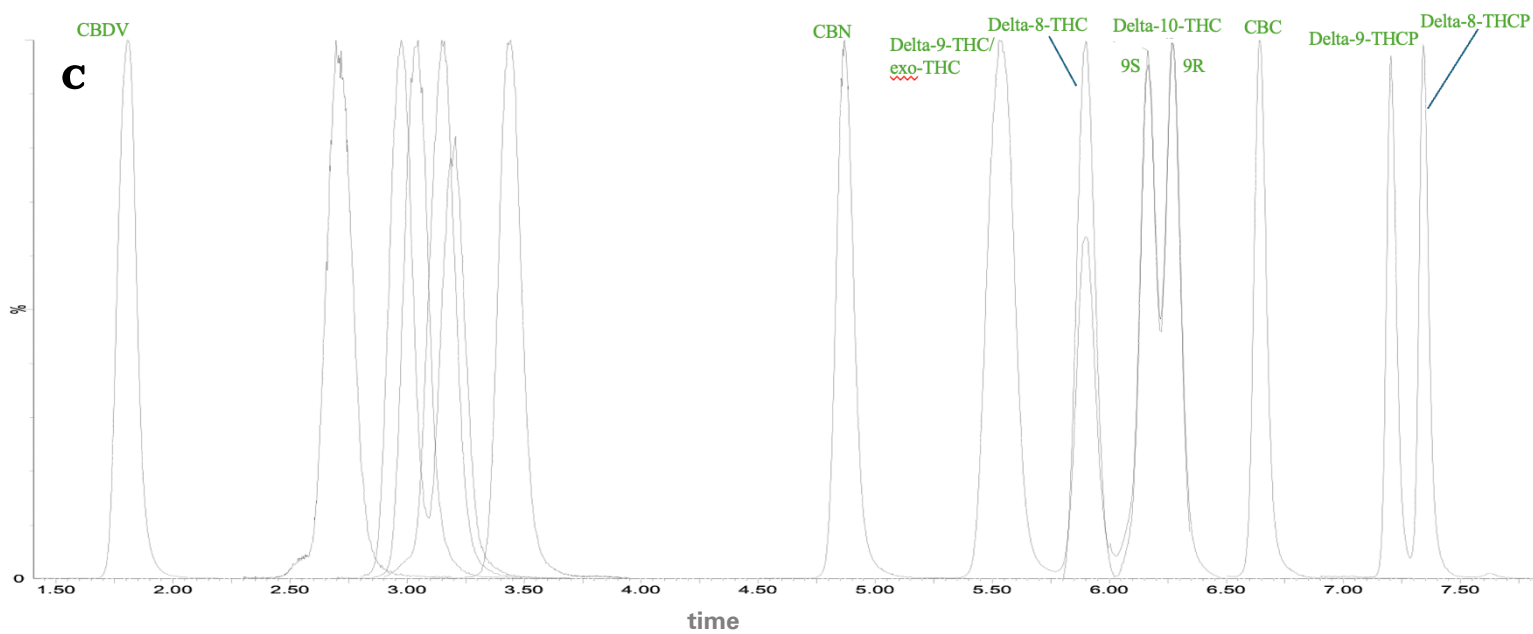
To create the mass spectrometry (MS) method, each analyte was directly infused into the electrospray ionization (ESI) source. The Intellistart™ software through the MassLynx® system was run to find the top four most abundant product ions (“fragments”) of each analyte. Every couple of samples, the results of Intellistart™ were confirmed through manual searching for fragments on the live mass spectrum output. A possible major fragment was selected, and the collision energy was changed to maximize the abundance of that fragment. The Intellistart™ was run in both positive and negative mode to determine which would be most effective for all analytes. The signal and fragmentation patterns were stronger in positive mode for nearly all of the compounds, so only positive ionization was used in the continuing development. **Table 2** shows the selected ion transitions of each compound, the cone voltage, and the collision energy used to maximize that fragment.

The MS method supported the reasoning for further development within the LC gradient. All of the isomers and cannabinoids with very similar structures had the same best fragmentation ions. These cannabinoids are Δ 8-THC, Δ 9-THC, delta-8-THCP, delta-9-THCP, delta-10-THC (both forms), exo-THC, CBD, CBDV, and (all but one transition for) THCV (**Table 2**).

II. Method Development: LC-MS Parameters and Solvent Gradient

The development of the chromatographic gradient started with a scouting method that increased in %B from 50-100% to determine at what approximate percentage the first compound eluted. That led to a starting isocratic method using a concentration of 75% B. This method showed good separation of the quantification compounds, but the final eluting compounds of delta-8 and delta-9-THCP did not elute until nearly 11 minutes (**Figure 2A**). Including a gradient increase in solvent B% (hold 75% B 3 min, ramp to 90% to 10 min, equilibrate at 75% to end at 11 min) decreased that dead-space by a minute (**Figure 2B**). Further experimentation of solvent gradient was executed to minimize the run-time until the method was running at 9 minutes with effective separation for all 18 analytes (**Figures 2C-D**). One main goal was to achieve baseline separation of $\Delta 8$ and $\Delta 9$ -THC, which can be seen in **Figure 2E**.





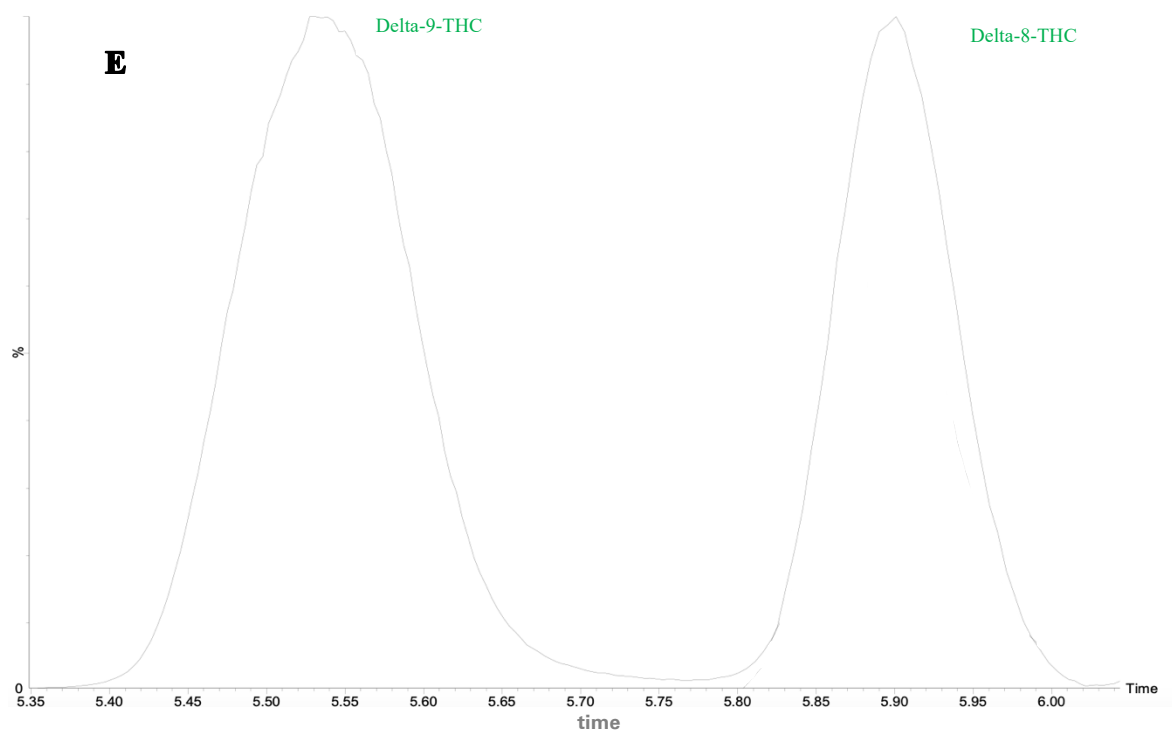


Figure 2. Chromatograms of quantitative analytes and last eluting analyte in the 11-minute method (A), 10-minute method (B), of all analytes in the 9-minute method (C) with an enlarged section from 2.4 to 3.7 minutes (D), and of Δ 8-THC and Δ 9-THC in the 9-minute method. (E)

III. Method Development: Extraction Method

Protein precipitation-based extraction was chosen after comparison to the time required with solid phase extraction (SPE) column extraction techniques. As shown in **Figure 3**, six different protein precipitation extraction approaches were evaluated for each compound separately. 1:1 ACN:MeOH extraction solvent with a reconstitution using methanol before adding water showed the best results.

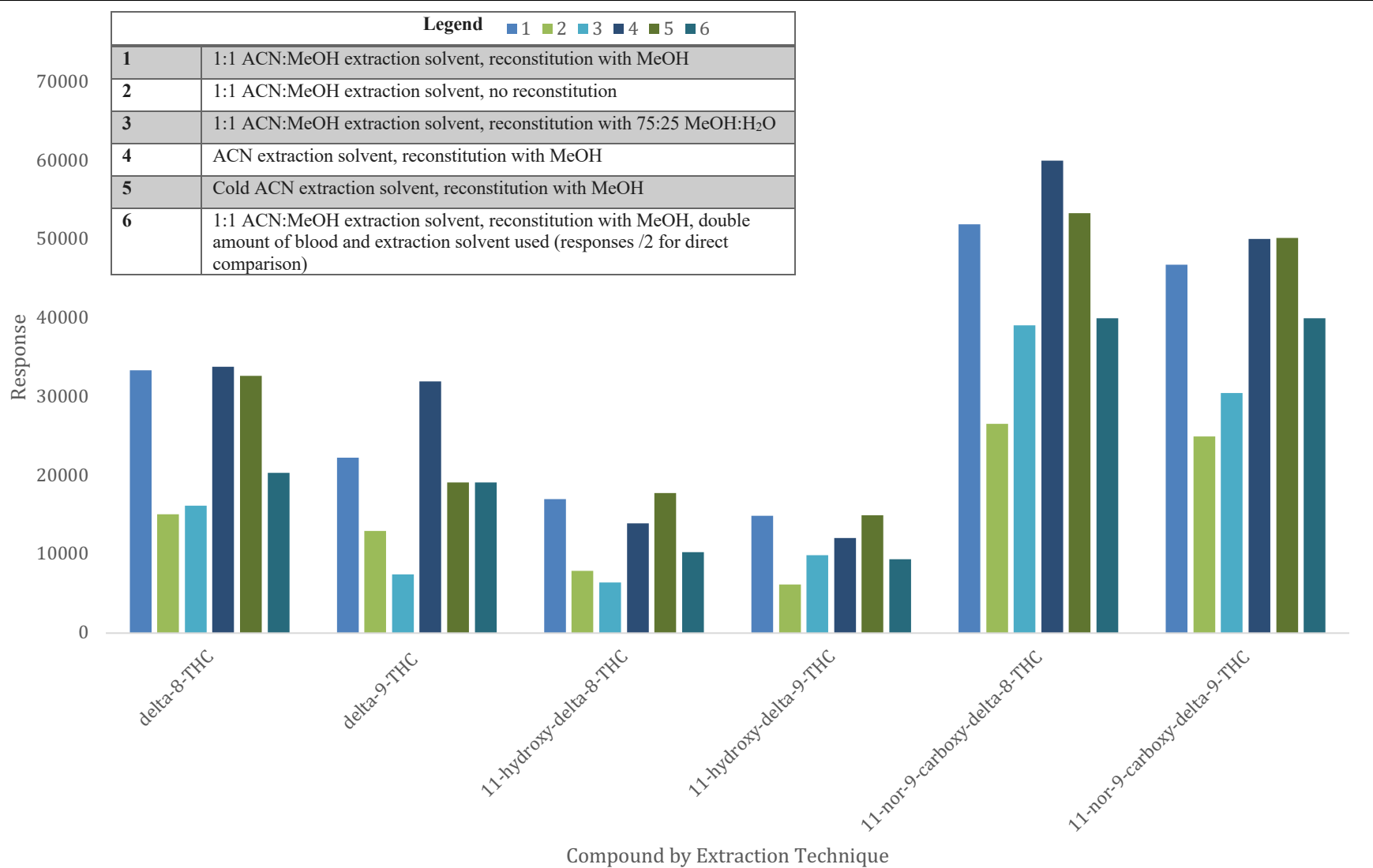


Figure 3. Extraction Method Test

IV. Validation Studies

In order to judge the validity of this new method, the American National Standards Institute/American Academy of Forensic Sciences Standards Board (ANSI/ASB)-approved standard practices for method validation²⁷ were followed for the five cannabinoids included in the confirmatory method. The other cannabinoid compounds are not included in the confirmatory method and are considered presumptive in the non-validated method.

a. Interference

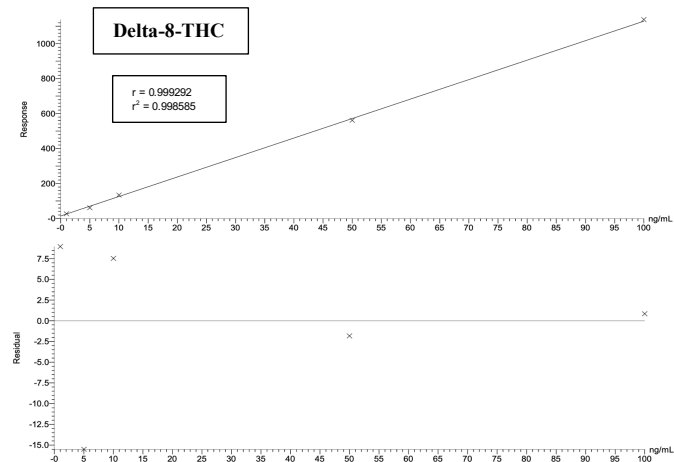
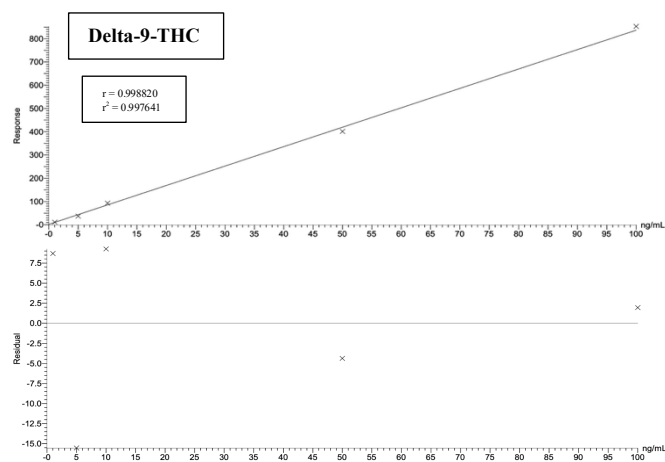
The first test was with blank matrix, in this case, human whole blood. 10 samples of human whole blood that previously tested negative for THC were extracted and tested using the new procedures. In these samples, no response was found for any of the target analytes.

Following addition of only the stable isotope labelled internal standards, the blank sample showed no signal for any of the quantitation analytes and very low signal for both isomers of THCP.

Following the addition of the six drug classes to a set of negative samples, no signal was found for any of the cannabinoid target analytes.

b. Calibration Model

The calibration model showed a linear relationship (**Figure 4**). For each of the five analytes, the correlation coefficient was very close to 1, and the residual plots showed random variance that was not too high. This calibration model was determined to be acceptable.



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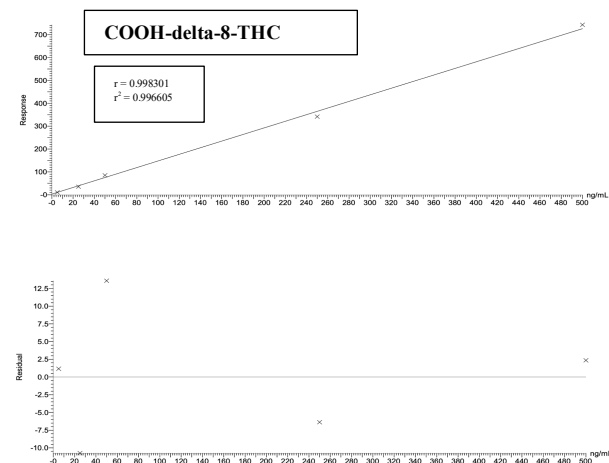
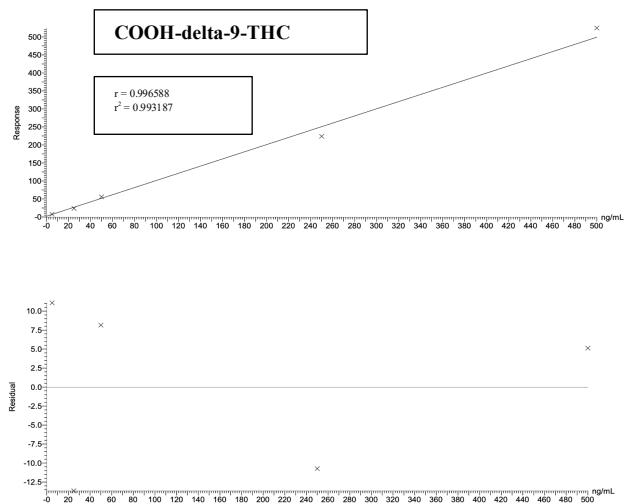
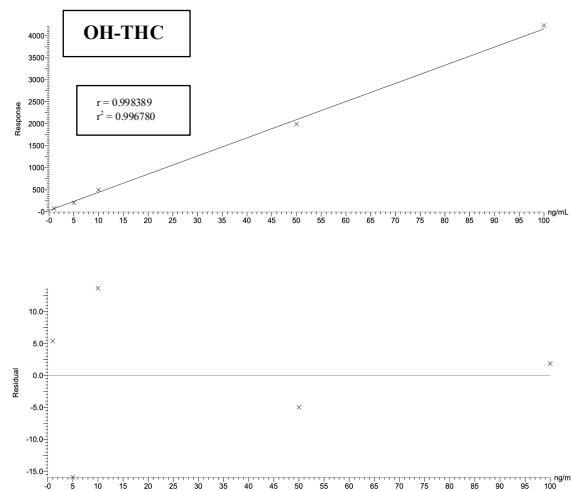


Figure 4. Calibration Curve Model Results.

c. Limits of Detection and Quantitation

For $\Delta 8$ -THC, $\Delta 9$ -THC, and OH-THC, the LOD and LOQ were found to be 1ng/mL. For COOH- $\Delta 8$ -THC and COOH- $\Delta 9$ -THC, the LOD and LOQ were found to be 5ng/mL.

d. Ion Suppression/Enhancement

The results of the ion suppression/enhancement are shown in **Table 3**. Several of the values in this test landed above the 25% target range. In this test, though, the internal standards were also measured through the same means, and in the samples that had greater amount of ion suppression, such as the 37.56% for OH- $\Delta 8$ -THC, the relative internal standard was similarly suppressed, at 37.03%. This means that, even with suppression present, the calculated concentrations will not be affected by this suppression.

Table 3. Ion Suppression/Enhancement Study Results

Concentration (ng/mL)	Ion suppression/Enhancement (%)								
	Compound								
	Delta-9- THC	Delta-8- THC	Delta-9- THC-D3	OH- Delta-9- THC	OH- Delta-8- THC	OH- Delta-9- THC-D3	COOH- Delta-9- THC	COOH- Delta-8- THC	COOH- Delta-9- THC-D3
80:400	-17.57	-12.18	-18.80	-4.11	-14.45	-19.76	1.74	3.48	2.97
40:200	-26.86	-23.83	-29.54	-22.10	-26.19	-28.48	-8.80	-12.11	-13.76
4:20	-24.47	-19.56	-26.25	-27.58	-37.56	-37.03	-10.89	-14.25	-11.55

e. Bias and Precision

The bias and precision results for each of the five analytes were assessed, shown in **Table 4**. The validation standards specify the target bias and precision as below 20%. All of the results found here are within 26%, which is very close to that ideal. Both the within-run precision and bias results have some points above the ideal 20%. For the within-run results, only the 40ng/mL

concentration from $\Delta 8$ -THC was slightly over 20%, but, when compared to the between-run precision, the results are much better. The bias results over 20% were almost all with the 4ng/mL samples.

Table 4. Bias and Precision Results.

	Delta-9-THC	Delta-8-THC	OH-THC	COOH-delta-9-THC	COOH-delta-8-THC
Average Within-Run Precision (% CV)					
80ng/mL	4.12	4.29	4.19	5.75	2.71
40ng/mL	17.89	21.01	16.38	19.34	18.53
4ng/mL	16.88	18.51	13.01	13.21	14.08
Between-Run Precision (% CV)					
80ng/mL	3.59	4.07	3.79	5.80	3.05
40ng/mL	15.27	18.40	13.93	17.28	16.02
4ng/mL	14.43	16.10	11.05	12.67	12.19
Bias (%)					
80ng/mL	9.20	0.66	7.19	8.14	1.08
40ng/mL	19.58	8.18	17.86	21.21	12.17
4ng/mL	25.58	13.58	21.53	25.85	17.99

f. Carryover

Carryover was assessed three times, and no response was found for any of the five target analytes.

Discussion

I. LC-MS/MS Parameter Development

For this method, the LC-MS parameters were chosen within the context of what would be practical and efficient for the laboratory. Because of this, a C18 column was used. All other confirmatory methods in use at the SACDA Crime Lab use a C18 column, so there would have to be good reason to invest in a new column type for a single confirmatory test. A test was conducted using a Raptor™ Fluorophenyl 1.7um, 2.1 x 100 mm column from Restek Corporation for comparison. Other studies¹⁵ have been able to achieve better separation, specifically of the 11-hydroxy-THC isomers, using this type of column. The resulting chromatographic separation looked similar to the results obtained from the C18 column and are not shown here since only one test was run before changing back to the C18 column. More work would have been required to achieve the separation of OH-THC isomers, and the added separation of one set of isomers did not prove to be significant enough to switch to this new column. Therefore, the C18 column was chosen for use in this development, so there would be no need to introduce a new column type to the laboratory.

The LC-MS solvents chosen for the chromatographic separation were the aqueous solvent (A) of water with 0.1% formic acid and the organic solvent (B) of methanol with 0.1% formic acid. Methanol (MeOH) was chosen for this method development as a possible improvement on the THC method currently in use at the SACDA Crime lab. The current method uses acetonitrile (ACN) as the organic solvent. In the bulk amounts needed to run the LC-MS, ACN is much more expensive than MeOH. The updated method aimed to convert the confirmation of THC to using MeOH as the organic solvent, as long as effective separation was still possible.

During the development of the gradient, the isomers with the same mass were the most difficult to separate, since they commonly exhibit similar properties within the column. These analytes are the Δ 8-THC, Δ 9-THC, exo-THC, and delta-10-THC compounds. Both forms of THCP are similar to each other but have a longer carbon chain than the THC compounds, so they proved to elute later than the other cannabinoids. Conversely, CBDV and THCV have a shorter carbon chain so may be similar to each other, but they elute earlier than the other cannabinoids with a similar fragmentation pattern. HHC is not included in the results images because it was added into the method at a later time, but it has a unique mass spectrum and retention time, so it was proved to not interfere with any of the other compounds.

The isocratic method that was first tested had a substantially long run-time of 12 minutes compared to the other methods in use at the SACDA Crime Lab, many at 9 minutes or below, and there was dead-space present between the elution of COOH- Δ 9-THC and Δ 9-THC. This prompted further testing to remove the dead-space and decrease the total run time. The next method reduced that to 10 minutes (**Figure 2B**). Focusing on that same dead-space, increasing the concentration ramp during that section, the final method was reduced to 9 minutes. All analytes were effectively eluted by 8 minutes, but an extra minute was added to the end of the gradient at the original 75% B to equilibrate before the next sample run started.

In the development of the MS method and the chromatographic gradient, it was discovered that OH-THC does not separate between delta-8 and delta-9 forms with the column type used here. The MS fragmentation ions were identical, and the peaks showed no indication of separating. Previous research supports this expectation. When a C18 column is used¹⁷, it is impossible to separate the OH-THC metabolites, but separation can be achieved with a phenyl-based column⁴. As stated earlier, though, changing column types would be a burden to the lab.

Therefore, only one 11-hydroxy-THC compound is included within the method. Whether OH- Δ 8-THC or OH- Δ 9-THC is present, the method will show the same peak. This is acceptable because OH-THC is rarely found in a sample without the original pre-metabolized THC or COOH-THC present. Because of this, it can be assumed that whichever form (Δ 8 or Δ 9) of the other cannabinoids is present, the OH-THC would match. This made the results less confusing, removing the overlapping peaks.

No separation was achieved between Δ 9-THC and exo-THC, either. Exo-THC has been studied very little and is not a common topic of questioning in impairment cases. The co-elution should not be an issue with the quantification of Δ 9-THC because of exo-THC's rarity, but the possible presence of exo-THC is an important note to keep in mind with continuing use of this method.

After the MS and chromatographic methods were finalized, it was decided that the method would only be quantitative for Δ 8-THC, Δ 9-THC and their metabolites, COOH- Δ 8-THC, COOH- Δ 9-THC, and OH-THC. The method was developed using all 18 compounds to prove that they will never pose interference to the quantification of the confirmed compounds in this and future analyses. The expanded method with all 18 compounds exists as a non-verified presumptive test for analysts to use if there is indication of intoxicating cannabinoids other than Δ 8- or Δ 9-THC. This was decided because it is very rare to see any of the other novel cannabinoids in use currently, and determining concentrations of those compounds would not add much value to the conclusions made of impairment. Also, including all 18 compounds in the working method would reduce accuracy by decreasing the maximum dwell-time possible for each analyte, increase cost needed to maintain reference standards, and reduce the efficiency of analysts during the data review process.

When deciding on an extraction method to use, the protein precipitation proved to be the most efficient while maintaining sensitivity. The extraction requires a lower amount of sample (50µL compared to 1mL and 400µL for the other methods tested), which is a significant benefit for saving sample volume for future testing. The protein precipitation also removes the need for purchasing columns and reduces the time each analyst must spend with each sample.

The tests to improve the protein precipitation were evaluated for overall performance. Because of this, even though the extraction with only ACN showed an increase in signal for most of the analytes, the decrease for OH-THC was significant enough for the 1:1 methanol:ACN to still be favored.

II. Validations

The validations completed here, following the ANSI/ASB guidelines, are the standard for method acceptance in accredited forensic laboratories. For this method, the calibration range was selected to be different between two groups of the analytes. $\Delta 9$ -THC, $\Delta 8$ -THC, and OH-THC are often found in lower concentrations within the blood and can be detected to lower concentrations more easily, so their range was selected to be from 1ng/mL to 100ng/mL. These concentrations usually do not exceed 100ng/mL, and, if they do, a dilution can be completed to retroactively obtain results for the high concentration. COOH- $\Delta 8$ -THC and COOH- $\Delta 9$ -THC are often found at higher concentrations within the blood samples because they remain in the body and can build up over time, so their range was selected to be higher at 5ng/mL to 500ng/mL. Due to this, the relationship between the concentrations will always be 1:5 ($\Delta 8$ -THC, $\Delta 9$ -THC, OH-THC:COOH- $\Delta 8$ -THC, COOH- $\Delta 9$ -THC) throughout the rest of the testing of this method.

The calibration model chosen here (1:5ng/mL to 100:500ng/mL) has a much higher upper limit than other recent studies^{4,8,17} and has minimal bias and suppression/enhancement at that high end. This is useful in cases with very recent use or very high dosages taken.

During the assessment of matrix effects, the ion suppression and enhancement tests, it was shown that the suppression of the internal standards aligned with the suppression of the analytes. Since the quantitative calculations are based on the relative response to the internal standard and calibrators run through the same extraction, all suppression should never impact the quantitative results. This trend of high matrix effects is seen in other studies working with cannabinoids in whole blood^{8,26}. Since they can be normalized to the similarly effected internal standard, the matrix effects are shown to have no effect on the reliability of the method.

The increased bias seen could be due to human error in the spiking or extraction process because one sample had a consistently higher concentration for all analytes. Since all of the results were very close to the target of 20%, the further validation studies planned by the SACDA Crime Lab analysts to complete the validation process are expected to demonstrate acceptable bias and precision. Other studies have achieved bias values under 15%^{4,26}, while others⁸ had similar issues with elevated bias. Further testing should demonstrate whether this elevated bias is from inaccuracy in the method or from error in the study.

III. Application of New Method

The results show that the main goals of developing this improved method were achieved. The separation of Δ 8-THC and Δ 9-THC is very reliable with this new method. The comparison of the separation from the original THC method used at the SACDA Crime Lab and the improved method can be seen in **Figure 5**.

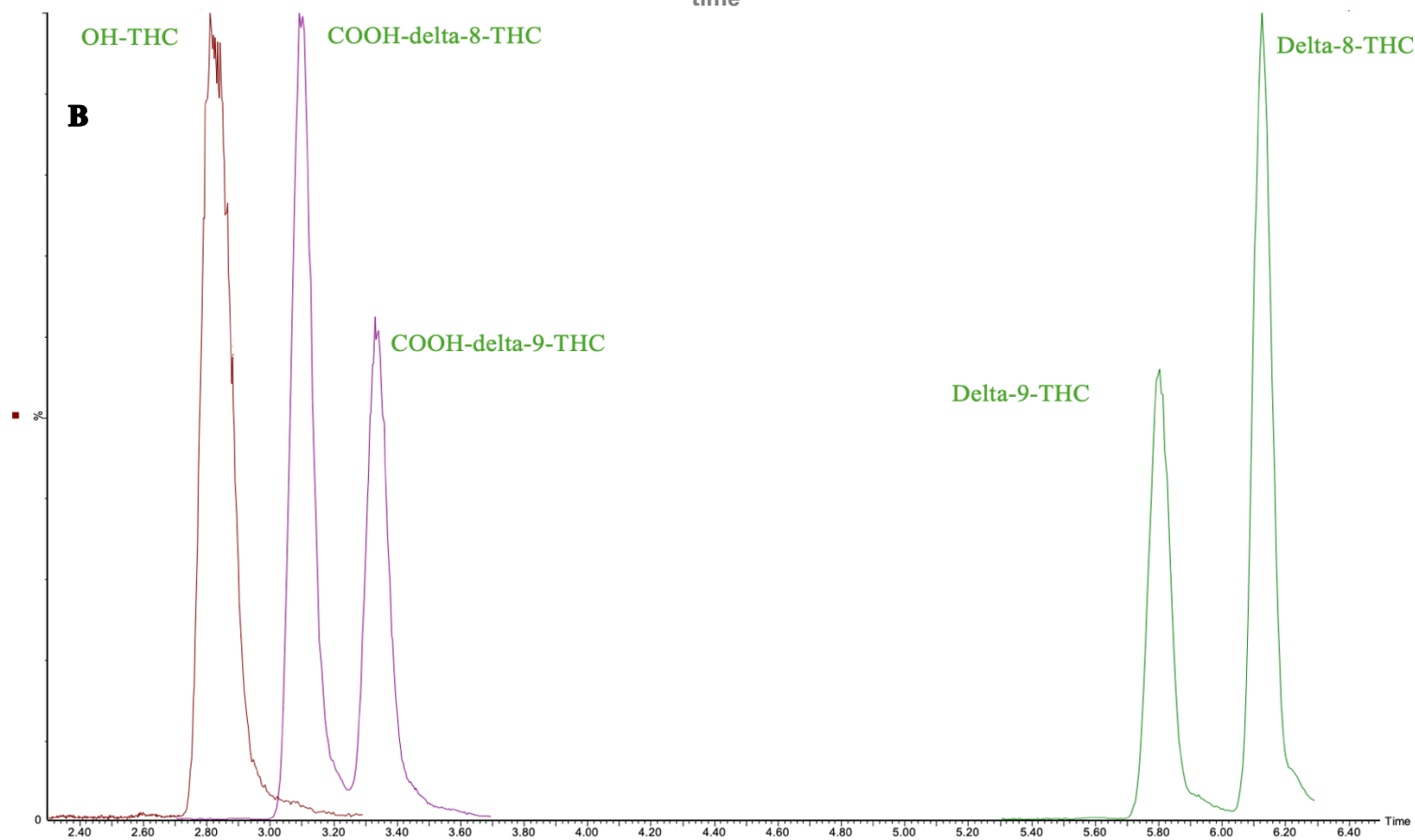
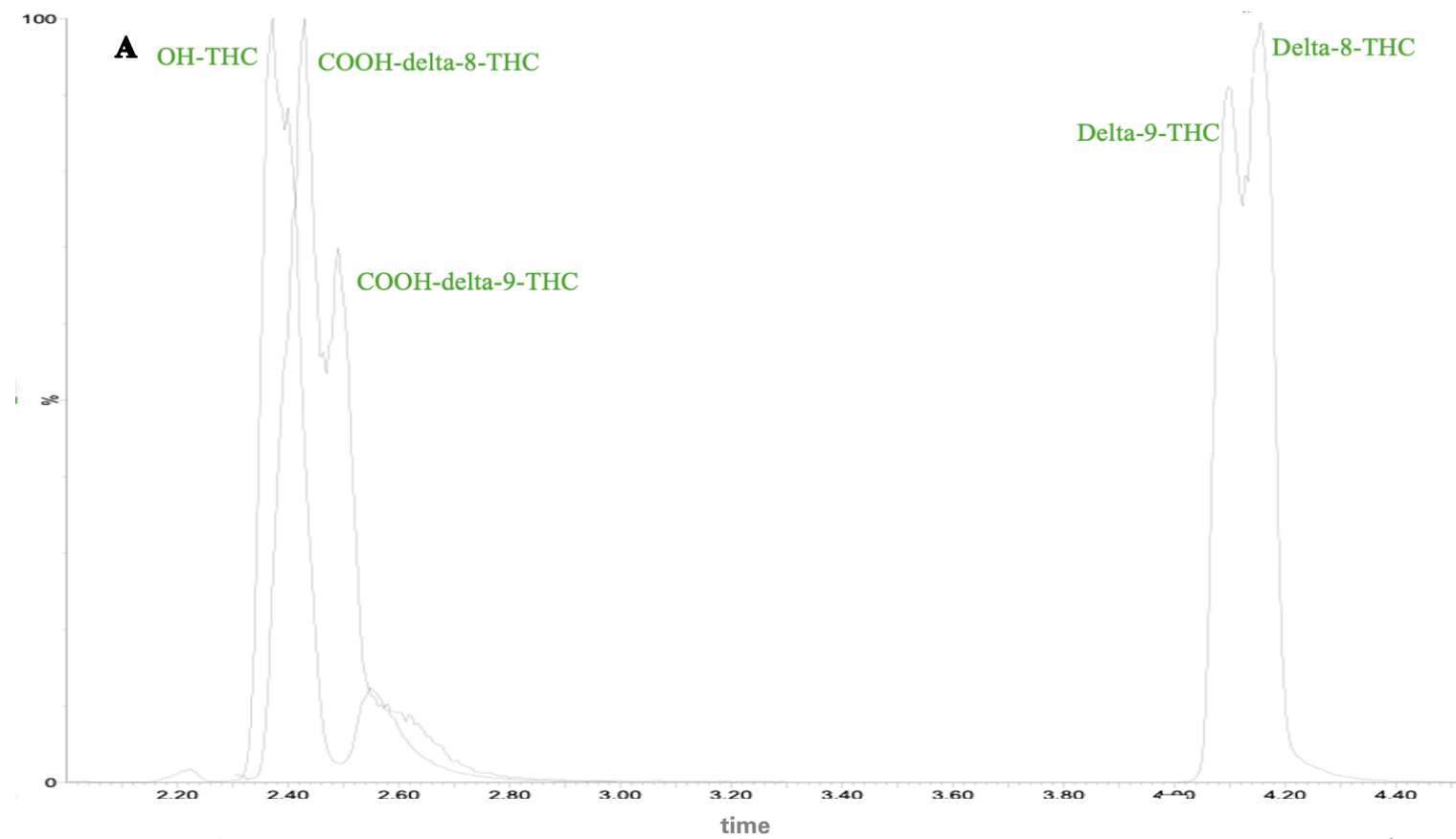


Figure 5. Comparison of Original THC Method (A) to the Updated Method (B).

Even though new potent cannabinoids are being synthesized and sold, this ability to quantify $\Delta 9$ -THC remains the most important application of this method. Multiple studies over the past couple of years have shown that $\Delta 9$ -THC is the most commonly used cannabinoid by a large margin. In most areas of the US, the average content of cannabis products sold contains 10% $\Delta 9$ -THC or more²⁸. The highest content below $\Delta 9$ -THC was found to be CBN, at an average of about 0.75%. This is a major difference from the $\Delta 9$ -THC content. The study that found this was conducted both in states with legal use and without, and that did not show a significant impact on $\Delta 9$ -THC content²⁸. Even in a population more susceptible to dangerous new experimental drugs, in the vaping habits of youths, studies of anonymous surveys show a much higher prevalence of “THC vaping” over “synthetic cannabinoid vaping”²⁹. This could be because of ignorance about the product being used or because it genuinely is much more rare for cannabis products to contain anything other than $\Delta 9$ -THC in high amounts. Frequent users of THC have also demonstrated a great preference toward natural THC products over synthetic. In one study, 93% of users reported preference for natural cannabis over synthetic³⁰. Because of this, the demand for natural $\Delta 9$ -THC is expected to continue to dominate. Therefore, there was a stronger focus on the accurate quantification of $\Delta 9$ -THC in the created method. No interference from $\Delta 8$ -THC should occur now that they are fully separated.

The results also show that this method is free from possible interferences from other cannabinoids. Even though the other cannabinoids are proven to be less common than $\Delta 9$ -THC, they may be present in samples. The presumptive method created allows for the possible indication of cannabinoid compounds if the question arises. If a person demonstrates significant signs of THC intoxication, but the results show no quantification analytes, the test can be run through the MS and processing method that includes the full list of cannabinoids. Since several

of the cannabinoids are less studied, this aspect of the method can allow for confident responses if the analysts are ever asked about interferences of other cannabinoids that have a different legal standing than Δ^9 -THC.

The other main goal of this study was to maximize efficiency and minimize cost. The results prove that this goal was achieved with trustworthy results. Selecting methanol as the organic mobile phase solvent makes a big difference in cost when samples are going to be run in batches that last for hours with a constant flow. On average, the cost of LC-MS grade ACN is three times the cost of the same amount of methanol³¹. The extraction procedure also reduces cost with no need for extraction columns and reduced time required of the analysts. The solvents used are that same lower-cost methanol and small amounts of ACN, which is very manageable. The time saved is also very important. With no extra drying steps and a simplified procedure, each batch will take less time to analyze. This will help with turnaround times in rush cases and with reducing backlog when many cases are coming in to the lab. This new procedure is fast and cost reductive, and the validation procedures prove that it works effectively. There is confidence that no accuracy or sensitivity is lost when transferring to the new method. The original method had the LOD and LOQ set at 1:10ng/mL, which the new method was able to match for Δ^8 -THC, Δ^9 -THC, OH-THC and reduce to 5ng/mL for COOH- Δ^8 -THC and COOH- Δ^9 -THC.

The efficiency was also increased through the requirement of less sample for extraction. The current method at the SACDA Crime Lab requires 1mL of sample to be extracted, with the new method only using 50 μ L. When blood samples are taken by unwilling arrestees, it is often difficult to obtain sufficient volume. In casework testing, it is also important to have sample volume available for retesting in case of error, retrial, or testing requested by the opposing

attorney. Reducing the sample requirement allows for more accuracy and security in the ability to retest as well as more opportunity to run a test even if the sample volume is very low.

The method created here, once validated by the criminalists at the SACDA Crime Lab, falls under the requirements of the 2021 ANSI/ASB “Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Impaired Driving Investigations”³². This lists the requirements to be the testing of THC, COOH-THC, and OH-THC down to concentrations of 1, 5, and 1 ng/mL, respectively. The created method matches those requirements, an improvement from the 10ng/mL lower limit for COOH-THC of the method currently in use at the SACDA crime lab. The standards for “Suspected Toxicological Cause of Death Determination”³³ has less sensitive requirements for THC reporting, at 2ng/mL for THC and 10ng/mL for COOH-THC (this standard does not list OH-THC as a requirement). The lower limit of quantitation found here meets or exceeds the requirements for all types of investigations encountered in toxicology casework.

The method developed here is very comparable to the method presented in Monti et al.’s study²⁶. The researchers developed a protein precipitation extraction and used the same solvents and column type as this study. Their results were comparable with separation of all intended analytes except for OH-THC isomers. The limits of quantitation they found were half the concentration found in this experiment, at 0.5ng/mL for THC and OH-THC and 2.5 ng/mL for COOH-THC, but the method presented here was only tested to the required limits of forensic toxicology testing. Monti et al.’s method had a similar chromatographic gradient but with a final run time of 10 minutes, compared to the 9 minutes presented here. Their extraction method did not include an evaporation and reconstitution step, but when it was assessed within this study, the signal was significantly stronger with the inclusion of the step. The extraction method with

this extra step requires more time by analysts, but the increase in signal was determined to be more important than saving that time.

When compared to other recent studies, this method is a great improvement on efficiency. One study by Muir et al.⁴ was able to reach the same sensitivity as the developed method (1ng/mL and 5ng/mL) with a final run-time of 16 minutes. The high run-time would be a great hinderance to the cost and workflow in a laboratory setting. They used a different type of column, poly-fluoro phenyl (PFP), so were able to achieve separation of OH-THC isomers, but the separation of the other cannabinoids was very similar to the separation achieved in this study⁴. Muir et al.'s study also required the use of 1mL of sample, which is improved with this study's 50µL. Another study, by Reber et al.⁸, reached the same sensitivity and separation but used ACN as a mobile phase solvent and had a total run-time of 12 minutes. Their method also required the use of 1mL of sample. They were able to achieve good separation of Δ8- and Δ9-THC and COOH-THC isomers, but the method created here is shown to be more efficient due to the decreased cost of using methanol mobile phase and having a low run-time .

IV. Limitations

The method has yet to be tested with any unknown samples or by other criminalists, but that work is continuing at the SACDA Crime Lab after this research finished. The method is being further validated and will likely be put into use with incoming DUI casework soon.

The greatest limitation from this study is the elimination of quantitative results from 12 of the cannabinoids. This was not due to the parameters of the method, though. This decision was made during the process of development in order to maximize the sensitivity and accuracy for the most integral analytes. A quantitative method for all 18 analytes could be created and used using the experimental parameters described here, but all instrumentation has limits. The MS

detector would have to be scanning for more transitions while multiple compounds are eluting, and that would decrease its dwell-time for analytes with overlapping detection windows. Also, the inclusion of all 18 analytes would add costs related to maintaining reference materials and would decrease analyst efficiency during the data review process. So, the decision was made to exclude the non-psychoactive and novel cannabinoid isomers and analogs from the quantitation.

Another limitation of this study was the lack of research surrounding many of the novel cannabinoids. It is possible that these analytes are present in many of the cannabis products sold today, whether legally or illegally. This is especially true of the synthetically enhanced hemp-based products with high $\Delta 8$ -THC concentrations. When these products are created with the purpose of evading the legal requirements, it would not be surprising if unintended or not-reported cannabinoids are present. Even when the intention is to create a novel cannabinoid product, the results can vary greatly. As seen in this study³⁴, in products intended to contain HHC, the concentrations of that component are prone to extreme fluctuations. Since the lesser-researched cannabinoids are not included in most analytical techniques, their presence may be much more common than reported, even if in small quantities. Since this is unknown, though, this study was conducted under the assumption that these novel cannabinoids are still rare and are not a significant factor in current impairment and DUI toxicology casework.

Conclusion

The goal of this study was to develop an improved method for the confirmation and quantification of cannabinoids found in forensic toxicology casework. This method was intended to reduce issues of co-elution between the active THC isomers of Δ 8-THC and Δ 9-THC as well as assess the presence of other novel cannabinoids. The method sufficiently separated those key isomers. A baseline separation was achieved that will eliminate any possibility of interference between Δ 8-THC and Δ 9-THC. The method was also proven to have no interference in the presence of other semi-synthetic cannabinoids. Now that the method has been created and is proven to follow the validation standards, the next step is for it to be tested by the Criminalists at the SACDA Crime Lab. They will create their own standards and run the validation tests to ensure that the results align with those found in this study. This process allows for another round of validation and acceptance of this updated method as the official THC confirmation method used in the lab. The validation will include stability studies to analyze how soon after extraction a sample must be tested in order for the results to still be reliable. As new cannabinoids are further researched and they continue to rise in popularity, improvements on this method should continually be made. If any of the cannabinoids included in this study become more popular, or continuing research reveals clinical effects that would change the interpretation of results, and quantification is required, the presumptive method that includes a wider range cannabinoids can be efficiently applied to the quantitative method. Continuing research could include testing previous positive THC samples, especially those that clearly contain Δ 8-THC, with the full method to assess if the other cannabinoids are present. Further research could create a much better understanding of the prevalence of these understudied compounds.

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