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Validation of a novel LC–MS-MS method for the separation and differentiation of Δ^8- and Δ^9-tetrahydrocannabinol isomers and their major metabolites in antemortem whole blood

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Abstract

The 2018 Farm Bill legalized hemp and defined it as cannabis plant material having not more than 0.3% Δ^9-tetrahydrocannabinol (Δ^9-THC) by dry weight. This has opened the door for the sale of hemp-derived Δ^8-tetrahydrocannabinol (Δ^8-THC), a psychoactive isomer of Δ^9-THC. Hemp has minimal amounts of naturally occurring Δ^8-THC; however, the cannabidiol found in hemp can be chemically converted into Δ^8-THC. Unfortunately, depending on the method of conversion, the amount of Δ^8-THC, Δ^9-THC, and other by-products can vary widely. For many laboratories, the emergence of Δ^8-THC products resulted in analytical challenges because of the structural similarity of the isomers resulting in coelution. In response, a novel liquid chromatography–tandem mass spectrometry method was developed to separate the two isomers, with an improved limit of detection (LOD) and lower limit of quantification (LLOQ). With this method, clear separation was achieved between Δ^9-THC and Δ^8-THC and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH) and 11-nor-9-carboxy-Δ^8-tetrahydrocannabinol (Δ^8-THC-COOH) and a partial separation of 11-hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-OH) and 11-hydroxy-Δ^8-tetrahydrocannabinol (Δ^8-THC-OH). While Δ^9-THC-OH and Δ^8-THC-OH did not achieve baseline separation, sufficient separation was achieved to confidently identify and differentiate the two compounds. LOD and LLOQ were the same for quantitative compounds. A quantitative range of 0.5–100 ng/mL was achieved for Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH and 2.5–250 ng/mL for Δ^9-THC-COOH. Qualitative analysis with an LOD of 0.5 ng/mL was achieved for Δ^8-THC-OH and 2.5 ng/mL for Δ^8-THC-COOH. To achieve the desired LODs and LLOQs, alternate multiple reaction monitoring transitions were also explored in addition to those utilized in the laboratory's prior method and other published methods. The method was validated following the American National Standards Institute/Academy Standards Board Standard 036, Standard Practices for Method Validation in Forensic Toxicology with minor exceptions, and was proven to be reliable and robust.

Introduction

Cannabinoids are frequently detected compounds in driving under the influence cases. With the passing of the Farm Bill [1] and the increase in popularity of Δ^8 and other tetrahydrocannabinol (THC) isomers [2, 3], many laboratories started experiencing issues with coelution and interferences caused by the increased presence of Δ^8-tetrahydrocannabinol (Δ^8-THC) and metabolites in samples [4]. The need to separate the Δ^8-THC and Δ^9-tetrahydrocannabinol (Δ^9-THC) isomers and their metabolites arose. At the Ventura County Sheriff's Office (VCSO) Forensic Services Bureau (FSB) Toxicology Laboratory, initially, the designation Δ^9 isomers was removed from the laboratory's reports and "tetrahydrocannabinol" was reported to address the inability to differentiate between the THC isomers. In addition, depending on the amount of the Δ^8 isomer present in the sample, reporting was not possible due to unacceptable chromatographic peak shape or failed ion ratios due to analyte coelution. While separating the two isomers was important to be able to confidently report Δ^9-THC and its metabolites, due to the psychoactive properties of Δ^8-THC [4, 5], determining the presence of and quantity of Δ^8-THC was also relevant in evaluating driving impairment. With the legalization of hemp and the decriminalization of THC use, a wide variety of products have become available for purchase in stores and online, such as plant material, vape pens, edibles, and oils; however, the cannabis contents and impurities can greatly vary from product to product [5, 6]. At the time this validation project began in late 2022, limited published work was available on the subject. Reber *et al.* [7] presented a method of separation of Δ^8- and Δ^9-THC and 11-nor-9-carboxy-Δ^8-tetrahydrocannabinol (Δ^8-THC-COOH) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH) on a 100-mm C18 column; however, due to lack of reference standard availability at the time, THC-OH separation was not evaluated. While this method achieved separation of Δ^8-THC and Δ^9-THC and Δ^8-THC-COOH and Δ^9-THC-COOH, the VCSO FSB Toxicology laboratory's goal was to achieve greater separation of the compounds to prevent larger concentrations of the compounds from

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coeluting, also to be able to distinguish between 11-hydroxy-Δ^8-tetrahydrocannabinol (Δ^8-THC-OH) and 11-hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-OH), as well as improve on the laboratory's limit of detection (LOD) and lower limit of quantification (LLOQ) and aim for LOD/LLOQ of 0.5 ng/mL for THC and THC-OH compounds and 2.5 ng/mL for THC-COOH compounds. Chan-Hosokawa *et al.* [8] achieved greater separation; however, a two-dimensional liquid chromatography (2D-LC) system was not available at the FSB Toxicology laboratory to attempt this method. The application note from Agilent Technologies [9] showed promising separation of Δ^8-THC and Δ^9-THC on a 150-mm C18 column; however, the analysis was for beverages, not whole blood, and therefore metabolites were not evaluated. These methods provided a great starting point for this project. In mid-2023, several methods became available, providing great separation of several isomers and cannabinoid compounds on pentafluorophenylpropyl stationary phase columns [10, 11]. At the end of 2023, Mantinieks *et al.* [12] published a multianalyte liquid chromatography–tandem mass spectrometry (LC–MS-MS) method including separation of Δ^8-THC and Δ^9-THC and evaluation of Δ^8-THC-COOH and Δ^9-THC-COOH on a C18 column as well but did not include evaluation of Δ^8-THC-OH and Δ^9-THC-OH.

The purpose of this project was to achieve greater separation of the Δ^8 and Δ^9 isomers of THC and metabolites utilizing column chemistry and reagents already in use at the laboratory and additionally to improve detection limits of all the compounds from the customary 1 ng/mL for THC and THC-OH and 5 ng/mL for THC-COOH.

Materials

Certified reference standards for calibrators and controls and deuterated standards for internal standards (ISTDs) were obtained from Cerilliant Corporation (Round Rock, TX), Lipomed Inc. (Cambridge, MA), and Cayman Chemical (Ann Arbor, MI). Additional standards were also obtained from the above companies for interference study. See Supplemental Tables S1 and S2 for full list of standards used in the validation.

Drug-free blood was obtained in-house from volunteers from FSB staff, porcine blood was obtained from Old Fashioned Country Butcher (Santa Paula, CA), and UTAK Blank Whole Blood (Product 44600 WB(F)), which is frozen authentic human whole blood, was obtained from UTAK (Valencia, CA). LC–MS grade methanol, water, isopropanol, and acetonitrile were purchased from Proteochem (Hurricane, UT) and Sigma-Aldrich, Inc. (Darmstadt, Germany). Formic acid ampoules (Proteomics Grade) were purchased from Proteochem. Glacial acetic acid, hexane, and ethyl acetate were purchased from VWR (Radnor, Pennsylvania, PA) and were of American Chemical Society grade. Working calibrator, control, and ISTD solutions were prepared in methanol and stored frozen at −20°C.

Method

Calibrators and control preparation

Calibrators and quality control (QC) samples were fortified day of analysis in 1 mL of diluted human blank blood in saline (1:1). Calibrators were fortified at final concentrations of 0.5, 1, 2, 5, 10, 20, 50, and 100 ng/mL for Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH, and 2.5, 5, 10, 25, 50, 250, and 500 ng/mL for Δ^9-THC-COOH and Δ^8-THC-COOH.

Due to the coelution of the THC-OH isomers, a separate calibrator (L9) was fortified with Δ^8-THC-OH at a final concentration of 0.5 ng/mL. See Supplemental Tables S3 and S4 for calibrator set up and calibrators used for each analyte.

Low and high QC samples were fortified at final concentrations of 1.5/7.5 and 80/200 ng/mL (Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH/Δ^9-THC-COOH and Δ^8-THC-COOH).

Low, medium, and high pools were prepared in whole blood at low concentration of 1.5/7.5 ng/mL, medium concentration of 50/120 ng/mL, and high concentration of 80/200 ng/mL, respectively (Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH/Δ^9-THC-COOH and Δ^8-THC-COOH). Initially, Δ^8-THC-COOH was also evaluated for quantitative analysis; therefore, it was included in the pools, calibrators, and QCs.

Extraction

Samples were extracted using a liquid/liquid extraction method as described in an Agilent application note [13], with minor modifications. Briefly, 1 mL of fortified blood was acidified with 800 µL of 10% acetic acid and extracted with 8 mL of 9:1 hexane/ethyl acetate, rocking the tubes for 30 min. After that, samples were centrifuged at 3500–4000 rpm, and organic upper layer was transferred to appropriately labeled tubes and evaporated to dryness at 40°C. Samples were reconstituted in 100 µL reconstitution solvent consisting of 28% mobile phase A (MPA) and 72% mobile phase B (MPB). The modifications to the referenced method include that samples were rocked instead of rotated, samples were centrifuged at 3500–4000 rpm instead of 2800 rpm, and the final reconstitution solution composition was 28:72 MPA/MPB instead of 50:50 acetonitrile/water. To minimize unwanted particles introduced into the LC system, after reconstitution, samples were ultracentrifuged at 13 000 rpm for 5 min in microcentrifuge tubes before transfer to autosampler vials for analysis.

LC–MS-MS parameters

Chromatographic separation and detection were accomplished using an Agilent 1200 Series high-performance liquid chromatography system combined with an Agilent 6470 triple quadrupole detector operating in multiple reaction monitoring (MRM) and positive electrospray ionization mode. Mobile phases (MPs) were (i) 0.1% formic acid in LC–MS water and (ii) 0.01% formic acid in LC–MS methanol. The automatic liquid sampler compartment was set at 4°C and the column compartment was set at 40°C. An Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 × 150 mm ultra-high-performance liquid chromatography column was used together with a gradient program (see Table 1) at a 0.75 mL/min flowrate and 10 µL injection volume. A standard needle wash program was employed, consisting of a 10-s wash of the flush port using a needle wash solution composed of 1:1:1 LC–MS water/LC–MS isopropanol/LC–MS acetonitrile with 0.01% formic acid. The run-time was 17.5 min, which included a 15-min gradient and a 2.5-min post time for re-equilibration. Data acquisition was performed with Agilent MassHunter Data Acquisition software version 10.1 and data analysis was performed using Agilent MassHunter Data Analysis software version 10.2. Agilent MassHunter Optimizer software version 10.1 was used to determine the precursor and product ion transitions

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Table 1. Timetable

Time (min)	A (%)	B (%)	Diverter valve position
0	28	72	Waste
5	28	72	MS
8	24	76	MS
8.7	20	80	MS
12.6	10	90	MS
12.7	0	100	MS
13.7	0	100	Waste
15	28	72	Waste

for each analyte. Source conditions were optimized using the Agilent MassHunter Source Optimizer software version 10.1. The selection of transitions was based on the evaluation of previously used transitions, transitions with the highest sensitivity, and transitions with no coeluting or interfering compounds (see Table 2 for final MRM Parameters and Supplemental Table S5 for source settings).

Method validation

Method development and evaluation of the assay were conducted using recommendations from the American National Standards Institute/Academy Standards Board (ANSI/ASB) Standard 036, Standard Practices for Method Validation in Forensic Toxicology [14, 15]. The individual studies performed were linearity and calibration model fit, bias and precision (within- and between-run), sensitivity (LOD/LLOQ), selectivity (matrix interferences), specificity (compound interferences), carryover, ion suppression/enhancement from matrix and interfering compounds, and processed sample stability.

The overall acceptance criteria for peaks across all studies were Gaussian peak shape, retention time (RT) within 2% of the set calibrator, and MRM transition ion ratios within 20% of the set calibrator. For qualitative compounds, the calibrator used to set the target RT and MRM transition ion ratios was L1, and L4 was used for quantitative compounds. Additionally, LLOQ, bias, and precision studies required quantitative values to be within 20% of their target values.

Matrix interference and suppression/enhancement studies were performed with 10 different matrix sources. For Δ^9-THC, Δ^8-THC, Δ^9-THC-OH, Δ^9-THC-COOH, and Δ^8-THC-COOH, eight sources of blood were obtained from FSB staff members, one source of blood was porcine blood diluted with saline (1:1), and one source of blood was obtained from UTAK.

Δ^8-THC-OH matrix study was performed at a different time, at which point different sources of blood were available, therefore six authentic human blood sources, two porcine blood sources diluted with saline (1:1), and two different UTAK blood lot numbers were used.

For the matrix interference study, the 10 sources of blank blood samples were extracted and evaluated to ensure that no endogenous compounds from the matrix interfered with any of the compounds studied or their ISTDs.

Matrix suppression/enhancement studies were evaluated by post-extraction addition approach at a low concentration of 1.5/7.5 ng/mL and a high concentration of 80/200 ng/mL of (Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH/Δ^9-THC-COOH and Δ^8-THC-COOH). Ten blood sources, as described above, were extracted in duplicate and fortified post-extraction at the low and high concentrations and with ISTD at a final concentration of 25 ng/mL of Δ^9-THC-d_3, Δ^8-THC-d_3, and Δ^9-THC-OH-d_3 and 50 ng/mL of Δ^9-THC-COOH-d_3. Average area counts of six injections of the low and high neat standards were compared with the average area counts of the extracted whole blood samples that were fortified post-extraction, and suppression/enhancement % was calculated as described in the validation guidelines.

Compound interference and suppression/enhancement were evaluated on ~100 compounds at therapeutic to lethal concentrations, including related compounds and commonly encountered drugs of abuse, over-the-counter medicines, and prescription drugs. It has been observed in prior LC–MS-MS casework and validation studies that coeluting unrelated compounds can cause suppression or enhancement of target analytes, even if they do not show interference in the MRM window. The effects of different compounds or compound mixes were evaluated on the target analytes at low and high concentrations, where instead of the extracted matrix samples, neat reference materials of the potential interfering compounds were used. Additionally, all the standards and mixes were analyzed in scan mode to corroborate or rule out any interference or suppression, as some standards may have impurities or may break down during analysis.

LOD and LLOQ (where applicable) were based on a decision point concentration and verified by analyzing samples fortified at LOD concentrations in three different matrix sources, analyzed over 3 days. The LLOQ of quantitative compounds was the same as the LOD. The validation guidelines recommend tripling the amount of matrix sources evaluated at the LOD level if matrix suppression or enhancement is observed to be over 25%. An additional six sources of blood (four human whole blood, one UTAK whole blood, and one porcine blood diluted in saline) were fortified and evaluated at the LOD level for compounds showing suppression/enhancement > 25%.

Bias and precision were evaluated by analyzing three replicates of each of the low, medium, and high prepared blood pools over five different runs, with low pool at 1.5/7.5 ng/mL, medium pool at 50/120 ng/mL, and 80/200 ng/mL of Δ^9-THC, Δ^8-THC, and Δ^9-THC-OH/Δ^9-THC-COOH and Δ^8-THC-COOH.

Carryover was evaluated by analyzing neat standard mixes with each compound at a concentration of at least 1000 ng/mL, followed by analyzing extracted negative samples to monitor for any carryover. The acceptance criteria were for negative samples not to exceed 10% of the signal of the lowest calibrator. Based on concentrations previously observed in casework, the selected calibration range for each compound should encompass most cases analyzed. Of over 1000 cases analyzed in recent years, <1% approached 1000 ng/mL for Δ^9-THC-COOH.

Processed sample stability was evaluated by keeping extracted fortified samples in autosampler vials in the instrument under refrigerated conditions and reinjected for up to 7 days. Instead of the recommended procedure by the ANSI/ASB 036 guidelines, a regularly processed batch such as one of the LOD analyses or a bias and precision batch was kept on the instrument and reinjected. This approach reflects a common situation in which a batch needs to be re-injected at

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Table 2. MRM parameters

Compound	Precursor ion	Product ion	Frag (V)	CE (V)
Δ^9-THC-OH	331.2	193.1[a]	100	28
Δ^9-THC-OH	331.2	123.1	100	44
Δ^8-THC-OH	331.2	193.1[a]	100	28
Δ^8-THC-OH	331.2	201.1	100	28
Δ^9-THC-OH-d_3	334.2	196.1	95	18
Δ^9-THC-COOH	345.2	193.1[a]	108	28
Δ^9-THC-COOH	345.2	119.1	108	32
Δ^8-THC-COOH	345.2	299.2[a]	108	20
Δ^8-THC-COOH	345.2	193.1	108	28
Δ^9-THC-COOH-d_3	348.2	302.2	90	14
Δ^9-THC	315.2	193.1[a]	125	24
Δ^9-THC	315.2	123.1	125	36
Δ^9-THC-d_3	318.2	196.1	95	18
Δ^8-THC	315.2	193.1[a]	125	24
Δ^8-THC	315.2	259.1	125	20
Δ^8-THC-d_3	318.2	196.1	95	18

Abbreviations: Frag, fragmentor; CE, collision energy.
[a]Quantifier MRM transition.

a later time due to an unanticipated event such as instrument failure, and the analyst uses the same autosampler vials that were kept on the instrument. Per the validation guidelines, analytes are considered stable until the average signal (e.g. peak area or ratios of peak area of analyte to ISTD) compared to the time zero average signal falls outside of the method's acceptable bias. Since the analysis was not performed in duplicates, all the calibrators and controls analyzed were evaluated individually.

The calibration model was evaluated by R-script statistical software using the methodology described by Desharnais *et al.* [16, 17], and the appropriateness of the recommended curve fit and maximum % residuals were confirmed by MassHunter curve fit assistant. All calibrators were to be within 20% of target concentrations, and r^2 value > 0.99 for all quantitative calibration curves.

Dilution integrity was not performed. The validated concentration range captures most concentrations encountered in casework. In the off chance, if a compound confirms above the highest calibrator, it would be reported as "Positive (>250 ng/mL)," for example.

Results

Table 3 summarizes the validation criteria and results. Based on the five analyses evaluated, the quadratic curve fit with $1/x^2$ weighting showed the best fit for all quantitative compounds using the aforementioned R-script statistical software. Maximum % residuals were confirmed to be <10% using the MassHunter curve fit assistant.

With the exception of UTAK blood results, the data for all other blood sources across the various studies demonstrated all detection, identification, bias, and precision to be within an acceptable range for LLOQ and all detection and identification criteria to be acceptable for LOD for all compounds. UTAK blood quantitative values were outside the 20% range for multiple compounds on the expanded LOD study, and the blood source overall showed higher matrix suppression than the other sources. When compared to average suppression or enhancement, UTAK blood resulted in 20–30% higher suppression/enhancement in accordance with the average matrix effect calculated across the 10 blood sources. UTAK and diluted porcine blood were included in the matrix and LOD/LLOQ studies and evaluated alongside with fresh human sources of blood to determine whether either source was a viable alternative to use for calibrators and controls. Based on these results, UTAK blood was determined not suitable for this assay. Figure 1 shows an example of all the compounds in an extracted LOD calibrator (a–f) and an authentic positive specimen (g–l). The vertical line in each window shows the expected RTs of compounds relative to their ISTDs. In Window d, only Δ^8-THC-OH is present, while in Window j, both isomers of THC-OH are present and Δ^8-THC-OH is shaded; however, the relative retention time (RRT) for these two windows is marked, where Δ^9-THC-OH would be expected to demonstrate the two isomers are clearly distinguishable, especially using this feature.

Bias and precision were well within the 20% acceptance criteria, with the percent coefficient of variation (%CV) being under 5% for all quantitative compounds.

The negative samples analyzed following high concentration (at least 1000 ng/mL) neat standards resulted in acceptable results, and no further studies were necessary. This quantity has not been previously observed for THC or THC-OH in casework, and <1% of cases showed THC-COOH concentrations close to this amount as observed in prior years of casework.

Matrix studies showed >25% suppression for most compounds; however, the expanded LOD and LLOQ study showed satisfactory results for Δ^9-THC, Δ^9-THC-OH, Δ^9-THC-COOH and Δ^8-THC for quantitative requirements. Initially, Δ^8-THC-COOH was evaluated for quantitative analysis; however, one value was outside of 20% bias. Δ^8-THC-COOH and Δ^8-THC-OH met all qualitative LOD requirements.

The stability study showed acceptable results on Day 7. During earlier phase of the validation, a stability study was performed with injections spread over multiple days, which indicated acceptable stability for at least 7 days. When the

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Table 3. Summary of validation results

	Acceptance criteria	Δ^8-THC	Δ^9-THC	Δ^9-THC-OH	Δ^9-THC-COOH	Δ^8-THC-OH[a]	Δ^8-THC-COOH[a]
Selectivity	≤10% LLOQ or LOD	CBL	Exo-THC	No interferences	No interferences	No interferences	No interferences
Calibration model		Quadratic, $1/x^2$	Quadratic, $1/x^2$	Quadratic, $1/x^2$	Quadratic, $1/x^2$	Single point	Single point
r^2	≥0.9900	≥0.9979	≥0.9967	≥0.9980	≥0.9977	N/A	N/A
LLOQ/LOD (ng/mL)[b]		0.5	0.5	0.5	2.5	0.5	2.5
%Bias	±20%	6	12	6	14		
%CV	≤20%	4	5	5	3		
ULOQ (ng/mL)		100	100	100	250		
Low pool target (ng/mL)		1.5	1.5	1.5	7.5		
MEAN (ng/mL)		1.32	1.30	1.44	7.17		
SD		0.05	0.05	0.07	0.20		
%CV	≤20%	4.0	3.7	4.6	2.7		
%BIAS	±20%	–11.8	–13.2	–4.1	–4.4		
Medium pool target (ng/mL)		50	50	50	120		
MEAN (ng/mL)		46.87	45.91	51.25	117.45		
SD		1.52	1.74	2.36	4.15		
%CV	≤20%	3.2	3.8	4.6	3.5		
%BIAS	±20%	–6.3	–8.2	2.5	–2.1		
High pool target (ng/mL)		80	80	80	200		
MEAN (ng/mL)		73.30	71.90	80.21	205.38		
SD		2.58	1.90	3.35	8.85		
%CV	≤20%	3.5	2.6	4.2	4.3		
%BIAS	±20%	–8.4	–10.1	0.3	2.7		
Low QC matrix effect (compound/ISTD)	25%	–43%/–42%	–46%/–43%	–21%/–19%	–30%/–25%	–11%/–10%	–18%/–25%
%CV	20%	≤22%	≤23%	≤18%	≤20%	≤9%	≤15%
High QC matrix effect (compound/ISTD)	25%	–44%/–49%	–44%/–48%	–21%/–27%	–27%/–31%	–10%/–8%	–19%/–31%
%CV	20%	≤20%	≤21%	≤10%	≤14%	≤9%	≤11%
Carryover	≤10% of LOD	No Carryover	No Carryover	No Carryover	No Carryover	No Carryover	No Carryover
Processed sample stability	≤20% ratio of peak area/ISTD change	7 days (<6%)	7 days (<13%)	7 days (< 5%)	7 days (< 9%)	7 days (< 5%)	7 days (<15%)

[a]Qualitative only compound.
[b]LOD and LLOQ are the same for quantitative compounds. LLOQ is not applicable for Δ^8-THC-OH and Δ^8-THC-COOH.

stability study was repeated at a later date using final method parameters, due to unforeseen circumstances, injections on multiple days were not possible and only a Day 7 comparison was made. At Day 7, the ratios of the peak areas of analytes to their respective ISTDs were evaluated and compared with the ratios of peak areas obtained at time zero. Results for all analytes were well under the method's 20% bias limit (Table 3).

Approximately 100 compounds were evaluated for interference. Several different isomers were looked at, and of the compounds evaluated, only a few showed interference. Cannabicyclol (CBL) and $\Delta^{9,11}$-tetrahydrocannabinol (exo-THC) showed interference with Δ^8-THC and Δ^9-THC, respectively. Cannabinol (CBN) standard contained a small level of Δ^8-THC which was also noted on its certificate of analysis. In scan mode, the RT of CBN was determined, which was over a minute earlier than that of Δ^8-THC. Therefore, CBN was deemed not responsible for the interference.

Discussion

Several methods were reviewed and evaluated for this project [7, 9]; however, none of them proved practical for the laboratory's purposes, and a new method was developed. The laboratory's prior method was validated to quantify Δ^9-THC, Δ^9-THC-OH, and Δ^9-THC-COOH; therefore, the goal was to maintain the ability to quantify these compounds while also separating their Δ^8 and Δ^9 isomers. The prior method used 0.1% formic acid in water for the aqueous MP and 0.1% formic acid in acetonitrile for the organic phase; therefore, initially, different gradients, both published [7] and in-house, were evaluated using those MPs. Similar gradients were also evaluated with methanol. Acetonitrile eluted the compounds quicker than methanol at the same flowrate and gradient, and it had an ideal lower backpressure compared to methanol; however, it lacked the desired separation of the isomers, especially Δ^9- and Δ^8-THC-OH, even at extended run-times. The

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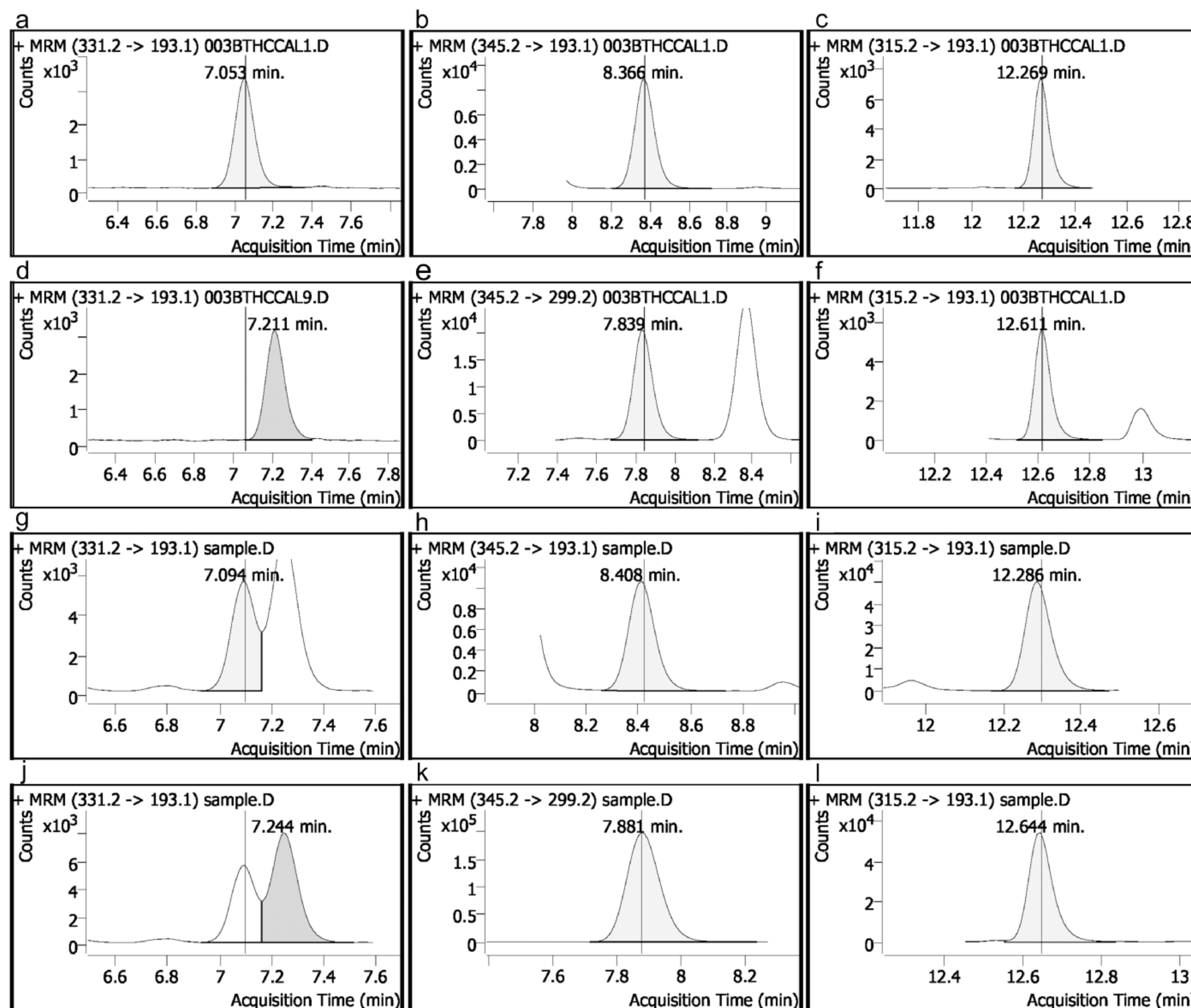

Figure 1. Chromatograms (a-f) showing authentic negative blood specimen fortified at method LOD, containing (a) 0.5 ng/mL Δ^9-THC-OH; (b) 2.5 ng/mL Δ^9-THC-COOH; (c) 0.5 ng/mL Δ^9-THC; (d) 0.5 ng/mL Δ^8-THC-OH (viewed in Δ^9-THC-OH window, RRT line for Δ^9-THC-OH); (e) 2.5 ng/mL Δ^8-THC-COOH; (f) 0.5 ng/mL Δ^8-THC and chromatograms of compounds detected in an authentic positive specimen, containing (g) ∼1.5[a] ng/mL Δ^9-THC-OH; (h) 13.0 ng/mL Δ^9-THC-COOH; (i) ∼8.0[b] ng/mL Δ^9-THC; (j) ∼3.5[a] ng/mL Δ^9-THC-OH viewed in Δ^9-THC-OH window, RRT line for Δ^9-THC-OH; (k) 130[a] ng/mL Δ^8-THC-COOH; (l) Δ^8-THC 40 ng/mL.

[a]Compounds reported qualitative only. Approximate concentration provided for comparison purposes only.

[b]Slight RRT shift and elevated FWMH showing possible presence of an interference; therefore, Δ^9-THC was reported qualitative only.

laboratory aimed to achieve better separation for Δ^9-THC and Δ^8-THC as well as Δ^9-THC-COOH and Δ^8-THC-COOH than previously published on C18 columns to make sure that larger concentrations still retained baseline separation. As the next step, similar to a previously published method [9], a mixture of methanol and acetonitrile was evaluated for the organic MP and showed promising results in separation of the isomers as well as lower back pressure. However, due to limitations with the number of MPs on the instrument, this was not pursued further. Building on the ideas in this method, a gradient was developed using an MP already available and utilized for other methods, 0.1% formic acid in water (A1) and 0.01% formic acid in methanol (B2), which resulted in great separation of Δ^9- and Δ^8-THC as well as THC-COOH and partial separation of Δ^9- and Δ^8-THC-OH. While the THC-OH isomers still coeluted, there was sufficient separation to conclusively identify and differentiate the two isomers. These were the selected MPs with which the validation was performed. During the initial stages of method development, the backpressure was on the higher end at around 580 bar, but this was alleviated by decreasing the flow rate from 0.8 to 0.75 mL/min, increasing the column temperature from 30°C to 40°C, and increasing the organic percentage of the starting composition from 68% to 72%. These adjustments reduced the starting pressure to ∼510 bar. To avoid reaching the maximum pressure of 600 bar during equilibration, a "pre-equilibration" method was set up at 100% organic MP and 0.6 mL/min flow rate.

The laboratory's prior method and many published methods used the transition 345.2 → 299.2 for Δ^9-THC-COOH [7, 10, 11, 14]. During the initial matrix studies, drug-free blood exhibited varying-sized peaks for this transition (but not the qualifier ion 345.2 → 193.1) at the RT of Δ^9-THC-COOH. Initially, a large portion of the validation was completed, as the interference appeared to be minor, however, out of concern and unknown identity of the 345.2 → 299.2

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interference, additional blood donor volunteers were sought out. The additional matrix sources evaluated revealed even greater variability of this interference, ultimately affecting the LOD of Δ^9-THC-COOH. Consequently, new MRM transitions were explored through compound optimization, and all affected studies were repeated with the newly selected MRM transitions. All studies and results described in the previous sections were performed using the final method parameters.

Although there are some limitations to the method, it also demonstrated significant potential for expanding the scope of analysis even further. In the interest of implementing the method in a timely manner, Δ^8-THC-OH was only evaluated for qualitative analysis due to coelution with Δ^9-THC-OH, a quantitative analyte. Preliminary studies showed promising results for quantitative analysis. Additionally, in this study, Δ^8-THC-COOH utilized Δ^9-THC-COOH-d_3 for ISTD. Using Δ^8-THC-COOH-d_3 instead would likely produce more consistent results and would be worth evaluating if seeking quantitative results. Furthermore, good separation was demonstrated during the interference study for other isomers and homologs, such as (6aR,9 R)-Δ^{10}-THC and Δ^9-THCP, respectively, and their addition to the method would be worth evaluating.

Based on the previous research [12], the Agilent Poroshell EC-C18 with a longer length and diameter was evaluated. Since the laboratory already had columns of the same chemistry at different lengths, an Agilent Poroshell 120 EC-C18, 2.7 µm, 2.1 × 100 mm column was initially evaluated side by side with the 150-mm length column. Results looked very similar, with the 100 mm column achieving just slightly less separation, so it could potentially be a viable alternative to try out if a laboratory already has that on hand. Also, the choice of the "mismatched" formic acid concentration between aqueous and organic MP was not based on the best performance, but rather that those reagents were already in use on the instrument and provided satisfactory results.

During the compound interference study, CBL, a constitutional isomer of THC, was found to co-elute with Δ^8-THC. CBL is a minor phytocannabinoid, a degradation product of CBC, present in plant material at trace amounts [18]. The transition ion ratios are significantly different, ~10–20%, compared to that of Δ^8-THC. A review of the prior year's casework data, specifically specimens not confirmed for Δ^8-THC, showed little evidence of a significant amount of CBL presence. Preliminary studies also indicated that the presence of a greater amount of CBL in combination with Δ^8-THC would be noticeable on the MRM transition ion ratios; however, further studies would be beneficial to have concrete data on what specific ratios would look like.

On the other hand, exo-THC coelutes with Δ^9-THC, and the MRM ratios closely match that of Δ^9-THC. At a quick glance, the two could easily be mistaken for one another. However, relative to the RT of the ISTD Δ^9-THC-d_3, exo-THC elutes slightly earlier, whereas Δ^9-THC elutes after; therefore, monitoring the RRT of the compound may help in distinguishing the two compounds. Being presented with a mixture of the two compounds would cause more difficulty in recognizing the presence of exo-THC. See Supplemental Fig. S1 for example chromatograms. There is insufficient data in the literature to demonstrate the presence and concentration of exo-THC in body fluid samples. However, as previously stated, numerous byproducts, including exo-THC, may be found in a variety of products on the market. To avoid reporting potentially increased quantitative values, data is being closely reviewed for any inconsistencies that may indicate the presence of interference in Δ^9-THC. This includes a larger full width max height (FWMH) compared to calibrators, which can be an indication of multiple peaks or coelution, as well as any shift in RRT, even if the RT is still within the acceptable range. If there is any evidence another analyte may be contributing to the peak area counts, Δ^9-THC would be reported qualitatively. The application of this can be seen in Fig. 1. Furthermore, the laboratory's new screening method will be able to distinguish between exo-THC and Δ^9-THC, providing an additional tool in monitoring for the presence of exo-THC to ensure proper reporting.

Further studies would provide valuable insights to strengthen this method. Since limited sources of human whole blood did not allow evaluation of 10 whole blood samples during matrix studies, additional matrix sources to evaluate would provide further insightful information. Furthermore, additional evaluation of exo-THC as well as including additional isomers and related compounds for evaluation, such as $\Delta^{6a,10a}$-THC, $\Delta^{6a,10a}$-THC-COOH, Δ^{10}-THC-COOH and cannabicitran, among others, would benefit the study. At this point, it has not been evaluated what ratios of Δ^9-THC-OH and Δ^8-THC-OH allow accurate quantitation of Δ^9-THC-OH. Based on prior casework, it has been observed that a small shoulder remained clearly visible and easily distinguishable, even when peaks were <10% of the area counts of the more abundant isomer present in the sample. However, further evaluation across a range of concentrations of the two isomers is needed to give more insight on the ability to accurately quantitate Δ^9-THC-OH in the presence of Δ^8-THC-OH, and vice versa, if quantitative validation were to be performed for Δ^8-THC-OH.

Conclusion

Successful separation and identification of Δ^8 and Δ^9 isomers of THC, and THC-COOH and partial separation of Δ^8- and Δ^9-THC-OH in blood was achieved using liquid–liquid extraction and liquid chromatography–tandem mass spectrometry. Wide quantification ranges were established for Δ^9-THC, Δ^9-THC-OH, and Δ^8-THC in the 0.5–100 ng/mL range and Δ^9-THC-COOH in the 2.5–250 ng/mL range. Δ^8-THC-OH and Δ^8-THC-COOH achieved LODs at 0.5 and 2.5 ng/mL, respectively, with great potential for implementing quantitative capability for the two compounds with additional studies. The ANSI/ASB Standard 036 guidelines were used as a guidance document for performing this project. The data obtained from the study demonstrated that the method is reliable and can be routinely used for antemortem blood samples.

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Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Disclaimer

The opinions expressed in this paper are those of the author alone and do not represent those of the Ventura County Sheriff's Office Forensic Services Bureau.

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