Document Title: Expanded Quantitative Cannabinoid Testing in Biological Specimens to Combat the Ever Changing Cannabis Landscape

Author(s): Rebecca Wagner, Ph.D.

Document Number: 308568

Date Received: January 2024

Award Number: 2020-DQ-BX-0017

Federal Agency:	U.S. Department of Justice Office of Justice Programs National Institute of Justice
Award Number:	2020-DQ-BX-0017
Project Title:	Expanded Quantitative Cannabinoid Testing in Biological Specimens to Combat the Ever-Changing Cannabis Landscape
Principal Investigator:	Rebecca Wagner, Ph.D., Research Section Supervisor 700 North Fifth Street, Richmond, VA 23219 804-588-4382
Submitting Official:	Alka B. Lohmann, Director of Technical Services 804-588-4092 alka.lohmann@dfs.virginia.gov
Submission Date:	Original: December 21, 2023 Revised: February 7, 2024
UEI:	QNNDFMV8DJU7
EIN:	42-1668853
Recipient:	Virginia Department of Forensic Science 700 North Fifth Street Richmond, VA 23219-1416
Project Period:	January 1, 2021 – December 31, 2023
Reporting Frequency:	Final Summary

Alka B. Lohmann
Signature of Submitting Official

02/07/2024
Date

PROJECT SUMMARY

- **Goals and Objectives**

The goal of this research project is to develop and validate an automated sample preparation technique for the quantitative evaluation of an expanded cannabinoid panel in whole blood and additional biological matrices, using liquid chromatography tandem mass spectrometry (LCMSMS) in accordance with the standard promulgated by the American National Standards Institute/Academy Standards Board (ANSI/ASB) 036, *Standard Practices for Method Validation in Forensic Toxicology*.

The objectives for the project are: 1) expand the traditional scope for cannabinoid testing for forensic toxicology laboratories to include phytocannabinoid constituents from plant material and metabolites that are used in consumer products; 2) investigate commercially available stationary phase substrates and instrumental conditions to increase selectivity and mitigate ionization suppression commonly encountered in the analysis of cannabinoids in biological matrices; and 3) develop and validate an automated sample preparation technique for the quantitative analysis of cannabinoids in biological matrices using LCMSMS.

- **Research Design/Methods**

A method was developed for the evaluation of cannabinoids in biological matrices. The method development was twofold, consisting of the development of a sample preparation procedure and instrumental parameters.

Note: Published internally as an independent document (Figures and Tables are chronological)

The following compounds were evaluated during method development:

Quantitative Targets	Internal Standard
(-)-Δ^9-Tetrahydrocannabinol	Δ^9-Tetrahydrocannabinol-D_3
(-)-Δ^8-Tetrahydrocannabinol	Δ^9-Tetrahydrocannabinol-D_3
(±)-11-Hydroxy- Δ^9-tetrahydrocannabinol	11-Hydroxy- Δ^9-tetrahydrocannabinol-D_3
(±)-11-nor-9-Carboxy- Δ^9-tetrahydrocannabinol	11-nor-9-Carboxy- Δ^9-tetrahydrocannabinol-D_3
Cannabidiol	Cannabidiol-D_3
Qualitative Targets	**Internal Standard**
(±)-11-Hydroxy- Δ^8-tetrahydrocannabinol	11-Hydroxy- Δ^9-tetrahydrocannabinol-D_3
(±)-11-nor-9-Carboxy- Δ^8-tetrahydrocannabinol	11-nor-9-Carboxy- Δ^9-tetrahydrocannabinol-D_3

Instrumental Method Development

Method development was aimed to develop a quantitative method for the analysis of cannabinoids in biological matrices. All target compounds that were not previously developed were optimized on an Agilent Technologies LCMSMS using Agilent Technologies Optimizer software. All compounds were optimized with positive ionization polarity. The two data acquisition methods developed employed dynamic MRM and were designed to separate tetrahydrocannabinol isomers.

The acquisition method was intended to be for the quantitation of (-)-Δ^9-tetrahydrocannabinol (Δ^9-THC), (±)-11-hydroxy-Δ^9-THC (Δ^9-OH-THC), (±)-11-nor-9-carboxy-Δ^9-THC (Δ^9-carboxy-THC), (-)-Δ^8-tetrahydrocannabinol (Δ^8-THC), and cannabidiol. An Agilent Technologies Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm column with a gradient elution was used to separate tetrahydrocannabinol isomers. This chromatographic method separates tetrahydrocannabinol isomers with the exception of Δ^9-THC and exo-THC which are indistinguishable within the method. The column is maintained at 50°C for the entirety of the gradient. Mobile phase A consists of 0.1% formic acid in water while mobile phase B consists of 80:20 methanol:acetonitrile. The optimized instrumental parameters are delineated in Table 1.

Table 1 Optimized instrumental parameters

Parameter	Setting			
Column	Agilent Technologies Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm			
Injection Volume	10 uL			
Needle Wash	5 seconds			
Flow Rate	1.0 mL/min			
Mobile Phase A	0.1% Formic acid in water			
Mobile Phase B	Methanol:acetonitrile (80:20)			
Gradient	Time (min)	% A	% B	Flow Rate (mL/min)
	0.0	40	60	1.0
	1.0	40	60	1.0
	7.0	23	77	1.0
	11.0	5	95	1.0
Post Time	1.5 minutes			
Column Temperature	50°C			

The total run time is 12.5 minutes including the post run. The optimized electrospray ionization source conditions are listed in Table 2.

Table 2 Optimized source conditions

Parameter	Setting
Gas Temperature	350°C
Gas Flow	10 L/min
Nebulizer	40 psi
Capillary	4000 V

As mentioned, the instrument was utilized in positive ionization mode with dynamic MRM analysis. The precursor ions, product ions, and instrumental settings are delineated in Table 3. The compounds are listed in order of retention time.

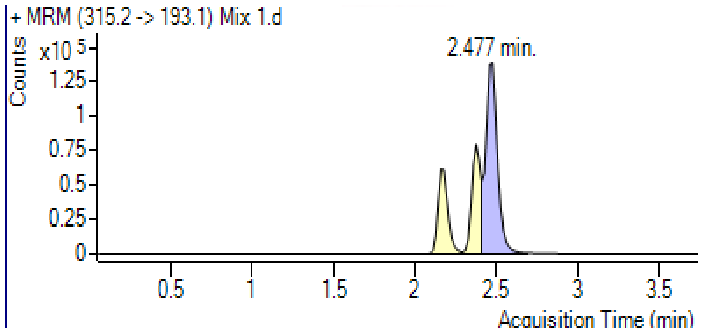
Table 3 Dynamic MRM Settings

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Retention Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator (V)
Δ^9-OH-THC	331.2	**313.2**	3.8	105	8	7
		193.1			20	
Δ^9-OH-THC-D_3	334.2	**316.2**	3.8	120	8	7
		196.3			20	
Δ^9-Carboxy-THC	345.2	**299.1**	4.3	125	16	7
		193.1			24	
Δ^9-Carboxy-THC-D_3	348.2	**330.1**	4.3	125	12	7
		302.1			16	
Cannabidiol	315.2	**193.1**	4.7	110	20	7
		123			32	
Cannabidiol-D_3	318.2	**196.1**	4.7	110	20	7
		123			32	
Δ^9-THC	315.2	**193**	6.8	120	20	7
		122.9			32	
Δ^9-THC-D_3	318.2	**196**	6.8	120	20	7
		123			32	

The product ions that are in bold represent the product ions that were utilized as the quantitation ion transition. Given the structural similarities between isomeric compounds, the Δ^8 isomers (both quantitative and qualitative) will be acquired using the Δ^9-THC parameters.

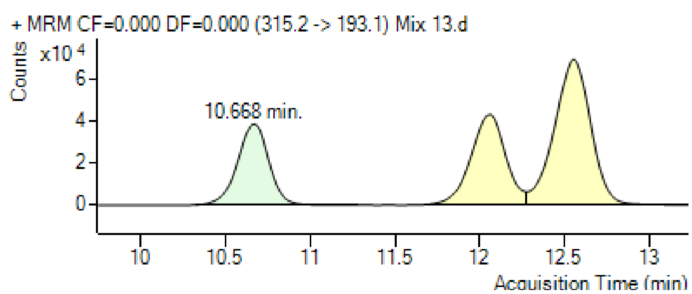
Given the increasing prevalence of tetrahydrocannabinol isomers, a secondary chromatographic technique was developed and evaluated. The acquisition method intended for the enhanced confirmation of Δ^9-THC employs a Restek Raptor fluorophenyl 3.0 x 100 mm, 2.7 µm column. An open access method developed by Restek suggested the separation of exo-THC and Δ^9-THC with an isocratic elution within 4.0 minutes. During development, the suggested method was evaluated. Mobile phase A consisted of 5 mM ammonium formate and 0.1% formic acid in water. Mobile phase B consisted of methanol fortified with 0.1% formic acid. The isocratic method was 75% mobile phase B. During the initial evaluation, the resolution between exo-THC, Δ^8-THC, and Δ^9-THC did not produce baseline resolution. The resolution obtained is shown in Figure 1.

Figure 1 Open access chromatographic method



Although the method fully resolves exo-THC from Δ^9-THC (first peak and third peak), Δ^8-THC and Δ^9-THC are not fully resolved. Therefore, chromatographic optimization was performed to increase the resolution between the tetrahydrocannabinol isomers. The isocratic composition and the flow rate were modified to improve the chromatographic resolution. The optimal composition was 65% mobile phase B with a flow rate of 0.5 mL/min. The resolution is shown in Figure 2.

Figure 2 Optimized chromatographic method

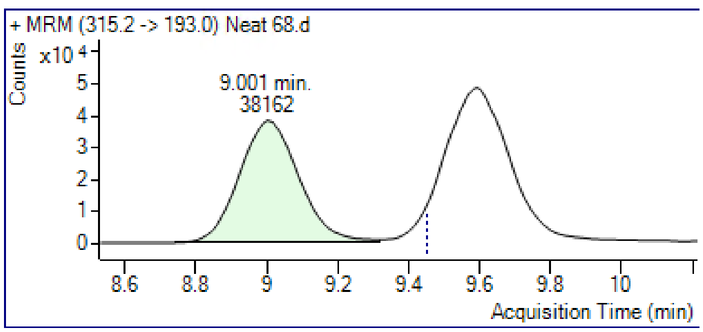


The resolution of the isomers has significantly improved at the expense of the overall runtime of the method. The initial method proposed by Restek indicated resolution of isomers within an instrumental run time of 4.0 minutes. To achieve appropriate separation between isomers, the instrumental run time was extended to 14.0 minutes. The method is isocratic between 0.0 and 13.0 minutes (65% mobile phase B) followed by a gradient 95% mobile phase B by 13.5 minutes to allow for column/instrument flushing. The end time for the run is 14.0 minutes with a 1.5-minute post run. All other instrumental settings were as denoted in the quantitative method.

During method development, inconsistencies in the Restek Raptor column were identified. Over time, the retention times shifted from the originally optimized method by nearly three minutes. The analytical column had minimal injections and the cause of the shift was unable to be identified. Therefore, an Agilent Technologies Poroshell pentafluorophenyl column was evaluated. The column dimensions (3.0 x 100 mm, 2.7 µm) were identical to the Restek Raptor column. Upon analysis, the retention time of Δ^{9}-THC was approximately 13.453 minutes using the previously optimized isocratic conditions (65% Mobile Phase B).

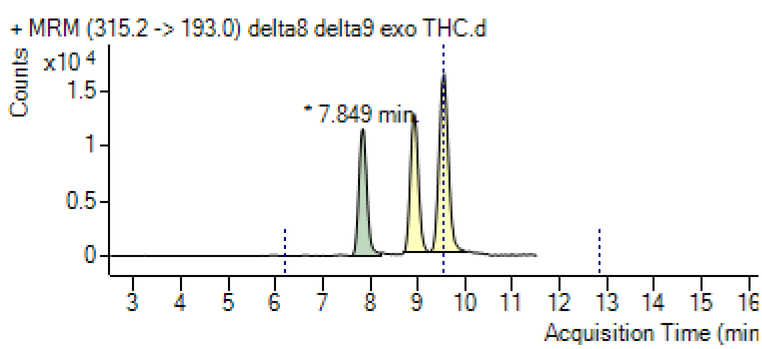
An evaluation into mobile phase composition was performed by assessing 65, 68, 70, and 75% mobile phase B. A composition of 68% mobile phase B enabled baseline resolution between Δ^{9}-THC and Δ^{8}-THC. The resolution is shown in Figure 3.

Figure 3 Optimized chromatographic method



In addition to baseline resolution between Δ^{9}-THC and Δ^{8}-THC, exo-THC elutes at approximately 7.849 minutes.

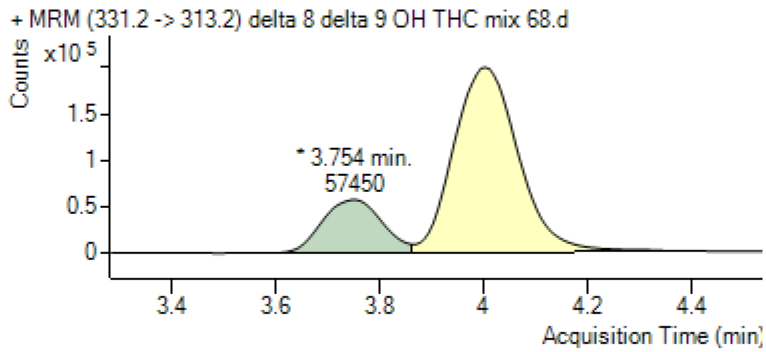
Figure 4 exo-THC, Δ^{9}-THC, and Δ^{8}-THC chromatogram



Exo-THC was the first eluting compound followed by Δ^{8}-THC and Δ^{9}-THC. Furthermore, carboxy-THC and OH-THC isomers were evaluated for chromatographic separation. Δ^{9}-Carboxy-THC and Δ^{8}-carboxy-THC were baseline resolved with retention times of 3.943 minutes and 4.478 minutes, respectively. The Δ^{9}-

OH-THC and Δ^8-OH-THC isomers did not have baseline resolution but had acceptable separation with retention times of 4.004 minutes and 3.754 minutes, respectively. The chromatography is shown in Figure 5.

Figure 5 OH-THC isomer chromatographic evaluation



The first eluting compound in the chromatogram was Δ^8-OH-THC (green) followed by Δ^9-OH-THC (yellow). Given the improved separation between isomeric compounds and the slight decrease in runtime, the Agilent Technologies pentafluorophenyl column was chosen for the secondary chromatographic method.

Extraction Method

Two different sample preparation procedures were developed and evaluated during method development. The first sample preparation procedure was a solid phase extraction using United Chemical Technologies (UCT) Clean Screen THC extraction columns with 200 mg bed mass and 10 mL total volume. In addition to these columns, the UCT Styre Screen THC columns and UCT DAU Clean Screen columns were evaluated. The optimized solid phase extraction procedure requires 1.0 mL of biological specimen with a protein precipitation prior to solid phase extraction. The method was optimized using blank blood, antemortem blood, postmortem blood, and urine. The optimized solid phase extraction procedure is delineated in Table 4.

Table 4 Optimized solid phase extraction procedure

Solid Phase Extraction Procedure
Add 100 µL of 0.1 µg/mL of internal standard to 1.0 mL biological specimen
Add 3.0 mL of cold acetonitrile drop-wise while vortexing
Centrifuge at approximately 2300 rpm for 10 minutes
Transfer supernatant (acetonitrile layer) into a clean test tube
Add 3.0 mL of 0.1 M acetate buffer (pH 3.5)
Add 2.0 mL of water
Vortex
Solid phase extraction • Condition column with 2.0 mL methanol • Condition column with 2.0 mL water • Add 1.0 mL of 0.1 M acetate buffer (pH 3.5) • Load sample • Wash column with 2.0 mL water • Wash column with 2.0 mL (95:5) 0.1 M HCl:acetonitrile • Dry column under full vacuum or pressure for 5 minutes • Elute with 3.0 mL (80:20) n-hexane:ethyl acetate
Transfer topmost layer to clean test tube
Add 40 µL of 0.2% HCl in 2-propanol
Evaporate to dryness at approximately 40°C
Reconstitute with 50 µL of acetonitrile fortified with 0.1% formic acid
Vortex
Add 50 µL of water fortified with 0.1% formic acid
Vortex
Transfer to autosampler vials for analysis

This multistep procedure requires an acetonitrile protein precipitation prior to solid phase extraction. Each aspect of the procedure was individually optimized.

The second sample preparation procedure developed was a supported liquid extraction (SLE). Biotage Isolute SLE 1.0 mL sample columns were employed during method development. Additionally, the 2.0 mL sample volume columns were evaluated. In comparison to the solid phase extraction, the supported liquid extraction procedure utilizes only 0.5 mL of biological specimen and has fewer steps. The optimized supported liquid extraction procedure is delineated in Table 5.

Table 5 Supported liquid extraction procedure

Supported Liquid Extraction
Add 50 µL of 0.1 µg/mL of internal standard to 0.5 mL biological specimen
Add 0.2 mL of 0.1% formic acid in water
Vortex
Decant sample onto column and allow to incubate for 5 minutes
Add 3.0 mL ethyl acetate and allow to incubate for 10 minutes prior to elution
Add 3.0 mL n-hexane and allow to incubate for 15 minutes prior to elution
Evaporate to dryness at approximately 50°C
Reconstitute in 50 µL methanol
Transfer to autosampler vial for analysis

During the development of the two sample preparation methods, an evaluation of the impact of glassware silanization was performed. Initially, all glassware utilized in each extraction was silanized including autosampler vials. To silanize glassware, the glassware was filled with 5% dichlorodimethylsilane in toluene solution. The glassware was allowed to incubate under standard laboratory conditions for at least 20 minutes. The silanizing solution was removed from the glassware and a series of rinses were performed. The first rinse was toluene followed by methanol, then toluene, and finally methanol. The glassware was then dried in an oven at approximately 80°C for at least 20 minutes. The silanization of glassware significantly improved the instrumental response for Δ^9-OH-THC and Δ^9-carboxy-THC. A stepwise removal of silanized glassware was performed to determine the critical steps that are required to be silanized for optimal performance. The first step, in each method, was determined to be a critical step.

The working range evaluated for each method was 0.001/0.005 mg/L (Δ^9-THC, Δ^9-OH-THC/Δ^9-carboxy-THC) to 0.1/0.5 mg/L (Δ^9-THC, Δ^9-OH-THC/Δ^9-carboxy-THC). During the initial assessment of each method, the calibration curve and instrumental responses between the two methods were compared. Each method was capable of achieving the desired working range for both Δ^9-THC and Δ^9-carboxy-THC. The instrumental response for Δ^9-THC was slightly higher for the supported liquid extraction compared to the solid phase extraction. Neither method was able to reach the desired limit of quantitation for OH-THC. The solid phase extraction procedure was able to consistently meet a lower limit of quantitation of 0.004 mg/L while the supported liquid extraction consistently met a lower limit of quantitation of 0.002 mg/L in blank blood, antemortem blood, and postmortem blood.

For quantitative analysis using the solid phase extraction procedure, the working range would be 0.001/0.004/0.005 mg/L (Δ^9-THC/Δ^9-OH-THC, cannabidiol/Δ^9-carboxy-THC) to 0.1/0.4/0.5 mg/L (Δ^9-THC/Δ^9-OH-THC, cannabidiol/Δ^9-Carboxy-THC). The calibrator preparation for the working range of the solid phase extraction procedure is listed in Table 6.

Table 6 Solid phase extraction calibrator preparation

SPE Calibrator Preparation		
Amount of 1/4/5 µg/mL solution (µL)	Amount of 0.1/0.4/0.5 µg/mL solution (µL)	Final concentration of cannabinoids (mg/L)
100		0.1/0.4/0.5
50		0.05/0.20/0.25
25		0.025/0.100/0.125
10		0.01/0.04/0.05
	50	0.005/0.020/0.025
	25	0.0025/0.0100/0.0125
	10	0.001/0.004/0.005

For quantitative analysis using the supported liquid extraction procedure, the working range would be 0.001/0.002/0.005 mg/L (Δ^9-THC/Δ^9-OH-THC, cannabidiol/Δ^9-Carboxy-THC) to 0.1/0.2/0.5 mg/L (Δ^9-THC/Δ^9-OH-THC, cannabidiol/Δ^9-Carboxy-THC). The calibrator preparation for the working range of the supported liquid extraction procedure is listed in Table 7.

Table 7 Supported liquid extraction calibrator preparation

SLE Calibrator Preparation		
Amount of 0.5/1/2.5 µg/mL solution (µL)	Amount of 0.05/0.1/0.25 µg/mL solution (µL)	Final concentration of cannabinoids (mg/L)
100		0.1/0.2/0.5
50		0.05/0.10/0.25
25		0.025/0.05/0.125
	100	0.01/0.02/0.05
	50	0.005/0.010/0.025
	25	0.0025/0.0050/0.0125
	10	0.001/0.002/0.005

As noted previously, the solid phase extraction utilizes 1.0 mL of biological specimen while the supported liquid extraction utilizes 0.5 mL of biological specimen.

Extraction Efficiency

A comparison of the efficiency of the methods was performed by evaluating ionization suppression/enhancement and recovery. Two blank blood sources and three postmortem blood sources were used during the evaluation. For this preliminary analysis, only two replicates of each matrix was analyzed by comparing the instrumental response of the post-extraction fortified sample and the instrumental response of a neat standard. Table 8 describes the ionization suppression/enhancement for each procedure.

Table 8 Ionization suppression/enhancement

Ionization Suppression/Enhancement (%)						
	SPE			SLE		
	Δ^9-OH-THC	Δ^9-Carboxy-THC	Δ^9-THC	Δ^9-OH-THC	Δ^9-Carboxy-THC	Δ^9-THC
Blank Blood 1	100	96	101	85	80	82
Blank Blood 2	126	105	64	104	84	89
Postmortem Blood 1	107	82	93	100	57	90
Postmortem Blood 2	109	83	99	91	53	80
Postmortem Blood 3	83	72	95	102	63	77

Postmortem blood 1 and postmortem blood 2 had significant ionization suppression for Δ^9-carboxy-THC when using the supported liquid extraction procedure. Slight enhancement was observed with the solid phase extraction procedure for Δ^9-OH-THC in blank blood 2. Otherwise, the methods were comparable with similar ionization suppression/enhancement.

The recovery of each sample preparation method was evaluated by comparing duplicate pre-extraction fortified and duplicate post-extraction fortified samples of the five aforementioned blood sources. Table 9 describes the recovery for each procedure in the various matrix sources.

Table 9 Recovery

Recovery (%)						
	SPE			SLE		
	Δ^9-OH-THC	Δ^9-Carboxy-THC	Δ^9-THC	Δ^9-OH-THC	Δ^9-Carboxy-THC	Δ^9-THC
Blank Blood 1	84	76	90	83	75	90
Blank Blood 2	64	51	107	86	82	81
Postmortem Blood 1	61	50	68	58	58	55
Postmortem Blood 2	71	63	86	76	83	74
Postmortem Blood 3	24	35	21	88	84	97

There were significant differences in recovery for the postmortem samples, specifically postmortem blood 3. Postmortem blood 3 had significantly higher recovery with the supported liquid extraction procedures for all analytes compared to the solid phase extraction. The recovery of the compounds was so poor for the solid phase extraction procedure that the chromatographic data did not meet the requirements for appropriate peak shape. Postmortem blood specimens can be complex and highly variable between sources. The supported liquid extraction presented a more consistent recovery amongst postmortem blood and blank blood sources.

Interferences

The method was preliminarily evaluated for interferences associated with tetrahydrocannabinol isomers and other cannabinoids. Additionally, an evaluation of analytes without the presence of internal standard and internal standard without the presence of analytes was performed. Table 10 lists the compounds evaluated for interferences.

Table 10 Interferent analysis

Cannabinoids	
(±) Cannabicyclol (CBL)	Cannabigerovarinic Acid (CBGVA)
(6aR,9R)-Δ^{10}-THC	Cannabinol (CBN)
(6aR,9S)-Δ^{10}-THC	Cannabinolic Acid (CBNA)
±cis-Δ^{9}-THC	Cannabivarin (CBV)
9R-$\Delta^{6a,10a}$-THC	exo-THC
9R-Δ^{7}-THC	Tetrahydrocannabivarinic (THCV)
9S-$\Delta^{6a,10a}$-THC	Tetrahydrocannabivarinic Acid (THCVA)
9S-Δ^{7}-THC	Δ^{8}-Iso-THC
Cannabichromene (CBC)	Δ^{8}-THC Acetate (Δ^{8}-THC-O-Acetate)
Cannabichromenic Acid (CBCA)	Δ^{8}-Tetrahydrocannabiphorol (Δ^{8}-THCP)
Cannabicyclolic Acid (CBLA)	Δ^{9}-Tetrahydrocannabinolic Acid A
Cannabidiolic Acid (CBDA)	Δ^{9}-THC Acetate (Δ^{9}-THC-O-Acetate)
Cannabidivarin (CBDV)	Δ^{9}-Tetrahydrocannabutol (Δ^{9}-THCB)
Cannabidivarinic Acid (CBDVA)	Δ^{9}-Tetrahydrocannabihexol (Δ^{9}-THCH)
Cannabigerol (CBG)	Δ^{9}-Tetrahydrocannabiorcol (Δ^{9}-THCO)
Cannabigerolic Acid (CBGA)	Δ^{9}-Tetrahydrocannabiphorol (Δ^{9}-THCP)

Each compound was prepared as a neat standard at a concentration of 1 µg/mL and evaluated for an instrumental response in the detection windows for each compound within the method. Both optimized analytical methods were evaluated for interferences.

<u>Agilent Technologies Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm Column</u>

When evaluating for an instrumental response in the Δ^{9}-OH-THC detection window, the following compounds listed in Table 11 provided an instrumental response.

Table 11 Δ^{9}-OH-THC detection window

Δ^{9}-OH-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^{9}-OH-THC	3.824	696645	16.3
Δ^{8}-OH-THC	3.890	323954	38.5
(6aR,9R)-Δ^{10}-THC	3.840	7621*	No Quantifier Peak
(6aR,9S)-Δ^{10}-THC	3.840	13680*	No Quantifier Peak
Cannabidivarinic Acid (CBDVA)	3.316	2387242	No Qualifier Peak
Cannabigerovarinic Acid (CBGVA)	4.147	2484	No Qualifier Peak

*The instrumental responses are of the qualifier peak as no quantifier peak was present. The Δ^{9}-OH-THC qualifier peak was 113436 area counts.

The retention times for CBDVA and CBGVA were outside of the ±3% acceptance criteria. Although the retention times for (6aR,9R)-Δ^{10}-THC and (6aR,9S)-Δ^{10}-THC were within retention time acceptance criterion, no quantifier peak was present, and the qualifier peak was significantly lower than the Δ^{9}-OH-THC qualifier peak. Δ^{8}-OH-THC was evaluated and determined to have a similar retention time as Δ^{9}-OH-THC but the qualifier ratios were significantly different.

A neat standard containing both isomers was evaluated. Figure 6 shows the separation obtained between the two isomers.

Figure 6 Δ^{9}-OH-THC and Δ^{8}-OH-THC

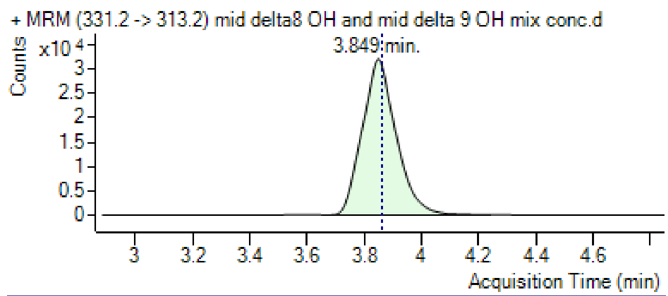


Figure 6 contains equal concentrations of Δ^{9}-OH-THC and Δ^{8}-OH-THC. When evaluated individually, Δ^{9}-OH-THC has a retention time of 3.824 minutes while Δ^{8}-OH-THC has a retention time of 3.890 minutes. The qualifier ratio for Δ^{9}-OH-THC was 16.7 while the qualifier ratio for Δ^{8}-OH-THC was 39.4. Given the similarities in retention time but differences in qualifier ratios, samples at different ratios of the two targets were evaluated. A high concentration of Δ^{8}-OH-THC (high calibrator) was evaluated with a low (low calibrator), two mid concentrations, and a high concentration of Δ^{9}-OH-THC was evaluated. The reverse was also evaluated with the Δ^{9}-OH-THC concentration being the highest calibrator concentration and different Δ^{8}-OH-THC concentrations evaluated. Furthermore, equal portions of Δ^{9}-OH-THC and Δ^{8}-OH-THC was evaluated at a mid-calibrator concentration.

When a high concentration of Δ^{8}-OH-THC and a low concentration of Δ^{9}-OH-THC was evaluated, the qualifier ratio was outside of ±20% acceptance (38.5). When a high concentration of Δ^{8}-OH-THC and a mid-concentration of Δ^{9}-OH-THC was evaluated, the qualifier ratio was outside of ±20% acceptance (24.1, 32.9). When a low concentration of Δ^{8}-OH-THC and a high concentration of Δ^{9}-OH-THC was evaluated, the qualifier ratio was within ±20% acceptance (16.7). Additionally, when a mid-concentration of Δ^8-OH-THC and a high concentration of Δ^9-OH-THC was evaluated, the qualifier ratio was within ±20% acceptance (19.4, 16.9). Finally, when equal concentrations of Δ^8-OH-THC and Δ^9-OH-THC were evaluated at mid-concentrations, the qualifier ratios were outside of ±20% acceptance (21.2, 23.1). It is challenging to visualize any chromatographic differences when evaluating the various ratios of compounds.

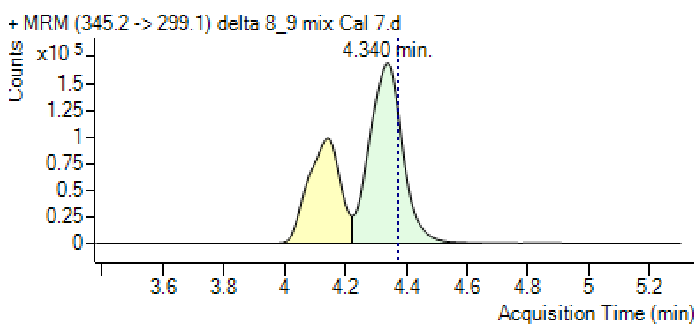
When evaluating for an instrumental response in the Δ^9-carboxy-THC detection window, the following compounds listed in Table 12 provided an instrumental response.

Table 12 Δ^9-Carboxy-THC detection window

Δ^9-Carboxy-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^9-Carboxy-THC	4.335	165445	53.1
Δ^8-Carboxy-THC	4.094	228632	92.6

The retention time of Δ^8-carboxy-THC was outside of ±3% minutes. Additionally, the qualifier ratio for Δ^8-carboxy-THC was outside of ±20%. To evaluate the separation of the carboxy-THC isomers, a sample fortified with Δ^9-carboxy-THC and Δ^8-carboxy-THC was extracted using the supported liquid extraction procedure and analyzed. The two compounds are not fully resolved as shown in Figure 7.

Figure 7 Δ^9-Carboxy-THC and Δ^8-carboxy-THC



The first peak (yellow) was Δ^8-carboxy-THC while the second peak (green) was Δ^9-carboxy-THC.

When evaluating for an instrumental response in the cannabidiol detection window, the following compounds listed in Table 13 provided an instrumental response.

Table 13 Cannabidiol detection window

Cannabidiol Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Cannabidiol	4.680	312799	69.8
Cannabidiolic Acid (CBDA)	4.639	1071	50.2
Cannabigerol (CBG)	4.829	2440	22.0
Cannabigerovarinic Acid (CBGVA)	4.190	19127	22.1

Both CBDA and CBG have small peaks that do not meet peak shape acceptance criterion. Additionally, CBDA and CBG have qualifier ratios outside of ±20%. CBGVA does not meet the retention time acceptance criteria and has a qualifier ratio outside of ±20%.

When evaluating for an instrumental response in the Δ^9-THC detection window, the following compounds listed in Table 14 provided an instrumental response.

Table 14 Δ^9-THC detection window

Δ^9-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^9-THC	6.773	297489	73.6
Δ^8-THC	7.040	253019	79.8
(±) Cannabicyclol (CBL)	7.065	55705	389.9
(6aR,9R)-Δ^{10}-THC	7.439	313607	62.4
(6aR,9S)-Δ^{10}-THC	7.282	300797	60.6
±cis-Δ^9-THC	6.424	645247	73.1
9R-$\Delta^{6a,10a}$-THC	7.281	549950	54.4
9R-Δ^7-THC	6.981	317137	75.9
9S-$\Delta^{6a,10a}$-THC	7.289	433919	54.9
9S-Δ^7-THC	7.106	275130	72.2
Cannabinol (CBN)	7.006	2384	75.6
exo-THC	6.715	355796	76.1
Tetrahydrocannabivarinic Acid (THCVA)	6.690	1199	No Qualifier Peak
Δ^8-Iso-THC	7.023	354016	69.8
Δ^8-THC Acetate (Δ^8-THC-O-Acetate)	7.006	2684	81.5
Δ^9-Tetrahydrocannabinolic Acid A	6.740	1930	82.3
Δ^9-THC Acetate (Δ^9-THC-O-Acetate)	6.732	2219	73.9

All compounds were outside of a ±3% minute retention time window when compared to the retention time of Δ^9-THC with the exception of exo-THC, THCVA, Δ^9-tetrahydrocannabinolic acid A, and Δ^9-THC acetate (Δ^9-THC-O-acetate). In addition to the retention time acceptance criterion not being met, CBL, 9R-$^{\Delta 6a,10a}$-THC, and 9S-$^{\Delta 6a,10a}$-THC were also outside of the ±20% qualifier ratio acceptance criterion. THCVA had an instrumental response of 1199 area counts with no qualifier transition noted. Compared to the instrumental response of Δ^9-THC (297489 area counts) the peak area for THCVA was determined not to be an interferent. Similarly, Δ^9-tetrahydrocannabinolic acid A and Δ^9-THC acetate (Δ^9-THC-O-acetate) had peak areas of 1930 and 2219, respectively and were determined to not be an interferent with Δ^9-THC. Exo-THC was the only compound that was determined to be a potential interferent with Δ^9-THC.

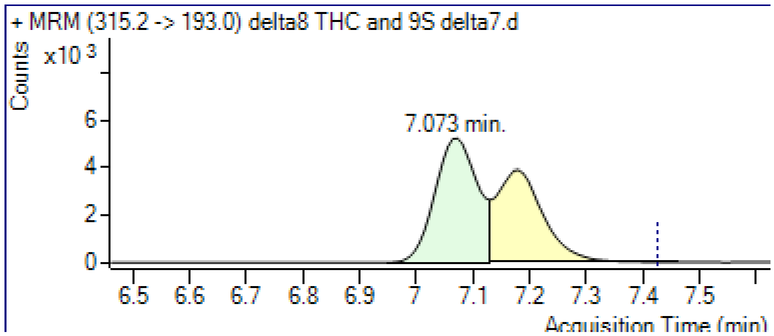
When evaluating for an instrumental response in the Δ^8-THC detection window, the following compounds listed in Table 15 provided an instrumental response.

Table 15 Δ^8-THC detection window

Δ^8-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^8-THC	7.040	253019	79.8
Δ^9-THC	6.773	297489	73.6
(±) Cannabicyclol (CBL)	7.065	55705	389.9
(6aR,9R)-Δ^{10}-THC	7.439	313607	62.4
(6aR,9S)-Δ^{10}-THC	7.282	300797	60.6
±cis-Δ^9-THC	6.424	645247	73.1
9R-Δ^{6a,10a}-THC	7.281	549950	54.4
9R-Δ^7-THC	6.981	317137	75.9
9S-Δ^{6a,10a}-THC	7.289	433919	54.9
9S-Δ^7-THC	7.106	275130	72.2
Cannabinol (CBN)	7.006	2384	75.6
exo-THC	6.715	355796	76.1
Tetrahydrocannabivarinic Acid (THCVA)	6.690	1199	No Qualifier Peak
Δ^8-Iso-THC	7.023	354016	69.8
Δ^8-THC Acetate (Δ^8-THC-O-Acetate)	7.006	2684	81.5
Δ^9-Tetrahydrocannabinolic Acid A	6.740	1930	82.3
Δ^9-THC Acetate (Δ^9-THC-O-Acetate)	6.732	2219	73.9

All compounds were outside of a ±3% minute retention time window when compared to the retention time of Δ^8-THC with the exception of CBL, 9R-Δ^7-THC, 9S-Δ^7-THC, CBN, Δ^8-iso-THC, and Δ^8-THC acetate (Δ^8-THC-O-acetate). The qualifier ratio for CBL was outside of the ±20% acceptance criteria. If present, the extreme ratio would skew the Δ^8-THC qualifier ratio results. 9R-Δ^7-THC, 9S-Δ^7-THC, CBN, Δ^8-iso-THC, and Δ^8-THC acetate (Δ^8-THC-O-acetate) met the predetermined acceptance criteria for both retention time and qualifier ratios. The instrumental response for CBN and Δ^8-THC acetate (Δ^8-THC-O-acetate) were significantly lower than the 253019 peak area response of Δ^8-THC and therefore not considered to be an interferent. 9R-Δ^7-THC, 9S-Δ^7-THC, and Δ^8-iso-THC are indistinguishable with the current acceptance criteria for retention time and qualifier ratios. 9S-Δ^7-THC has a retention time of nearly 0.1 minute later than Δ^8-THC. The chromatographic separation is shown in Figure 8.

Figure 8 9S-Δ^7-THC and Δ^8-THC



An evaluation into the source of the chromatographic response in the Δ^9-THC/Δ^8-THC detection window from Δ^9-THC acetate (Δ^9-THC-O-acetate), Δ^8-THC acetate (Δ^8-THC-O-acetate), and Δ^8-iso-THC was performed; the mass spectrometer was optimized for each compound. The retention times were noted to be 8.805, 8.788, 7.047 minutes, respectively. Additionally, cannabicyclol, cannabinol, and Δ^9-tetrahydrocannabinolic acid A were optimized to determine their retention times. The retention times were noted to be 7.105, 6.127, and 8.664 minutes, respectively. Further evaluation into the presence of Δ^9-THC and Δ^8-THC when analyzing their respective THC-O-acetates was performed and is later described.

Agilent Technologies Poroshell Pentafluorophenyl 3.0 x 100 mm, 2.7 µm Column

When evaluating for an instrumental response in the Δ^9-OH-THC detection window, the following compounds listed in Table 16 provided an instrumental response.

Table 16 Δ^9-OH-THC detection window

Δ^9-OH-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^9-OH-THC	3.970	710029	15.6
Δ^8-OH-THC	3.705	196679	36.4
Cannabidivarinic Acid (CBDVA)	3.929	782742	No Qualifier Peak

The retention time for CBDVA was within ±3% minutes of Δ^9-OH-THC. The qualifier ratio was outside of acceptance criterion for CBDVA when evaluating the detection window for Δ^9-OH-THC. The retention time for Δ^8-OH-THC was outside of acceptance criteria for Δ^9-OH-THC in addition to the qualifier ratio being out side of ±20%. The resolution of Δ^8-OH-THC and Δ^9-OH-THC is shown in Figure 9.

Figure 9 Δ^{9}-OH-THC and Δ^{8}-OH-THC

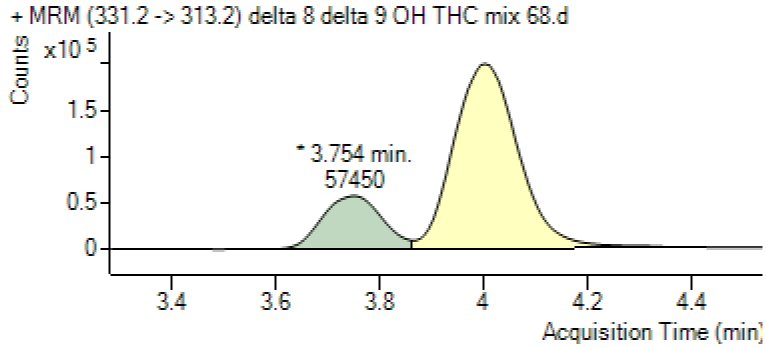


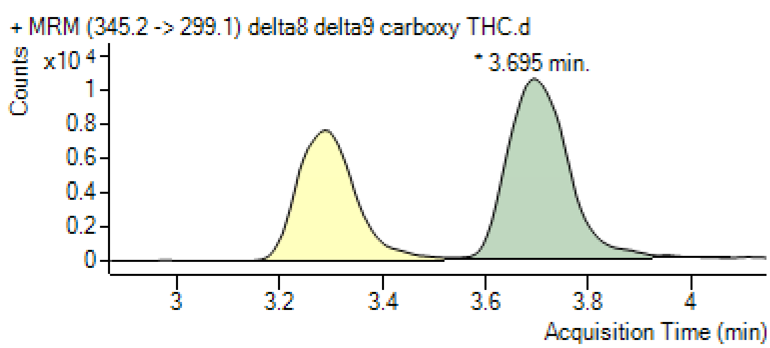
Figure 9 contains equal concentration of Δ^{8}-OH-THC and Δ^{9}-OH-THC. With this column, Δ^{8}-OH-THC elutes first at 3.754 minutes and Δ^{9}-OH-THC elutes second at 3.970 minutes. When evaluating for an instrumental response in the Δ^{9}-carboxy-THC detection window, the following compounds listed in Table 17 provided an instrumental response.

Table 17 Δ^{9}-Carboxy-THC detection window

Δ^{9}-Carboxy-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^{9}-Carboxy-THC	4.482	95056	49.9
Δ^{8}-Carboxy-THC	3.943	68118	91.5

The retention time of Δ^{8}-carboxy-THC was outside of ±3% minutes. Additionally, the qualifier ratio for Δ^{8}-carboxy-THC was outside of ±20%. To evaluate the resolution of the two isomers, a mixed standard was prepared and analyzed with equal concentrations of the isomers. The chromatographic separation is shown in Figure10.

Figure 10 Δ^{8}-carboxy-THC and Δ^{9}-carboxy-THC



The first peak in the chromatographic window was Δ^8-carboxy-THC while the second peak was Δ^9-carboxy-THC. When evaluating for an instrumental response in the cannabidiol detection window, the following compounds listed in Table 18 provided an instrumental response.

Table 18 Cannabidiol detection window

Cannabidiol Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Cannabidiol	4.553	482242	75.0
Cannabidiolic Acid (CBDA)	4.594	372	68.3
Cannabidivarinic Acid (CBDVA)	3.948	1911	3.5
Cannabigerol (CBG)	4.644	7985	21.1
Cannabigerovarinic Acid (CBGVA)	6.449	9200	4.9

All peaks noted including CBDA, CBDVA, CBG, and CBGVA were small peaks that did not meet peak shape acceptance criterion. Additionally, CBDVA and CBGVA were outside of retention time acceptance criterion of ±3% and qualifier ratio acceptance of ±20%. CBDA and CBG were within the retention time acceptance criterion and CBDA was also within the qualifier ratio acceptance criterion.

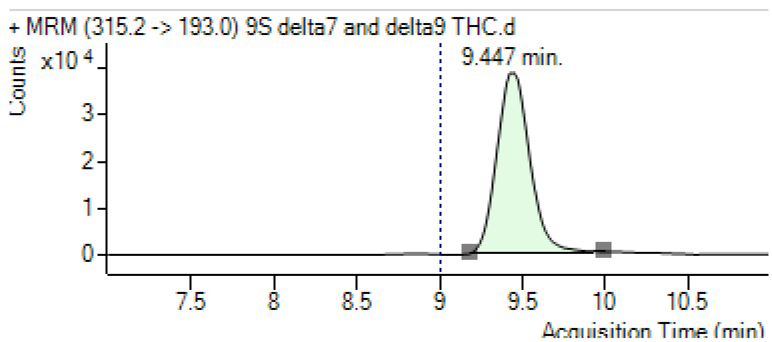
When evaluating for an instrumental response in the Δ^9-THC detection window, the following compounds listed in Table 19 provided an instrumental response.

Table 19 Δ^9-THC detection window

Δ^9-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^9-THC	9.521	944870	38.3
Δ^8-THC	8.909	681434	40.1
(±) Cannabicyclol (CBL)	9.596	115815	186.7
±cis-Δ^9-THC	6.623	1109876	38.0
9R-Δ^7-THC	8.925	272471	40.6
9S-Δ^7-THC	9.497	296313	37.1
Cannabinol (CBN)	8.892	4027	36.9
exo-THC	7.815	484647	38.8
Δ^8-Iso-THC	8.544	405636	38.5
Δ^8-THC Acetate (Δ^8-THC-O-Acetate)	8.925	18458	37.1
Δ^9-Tetrahydrocannabinolic Acid A	9.530	3217	35.9
Δ^9-THC Acetate (Δ^9-THC-O-Acetate)	9.513	23737	40.1

All compounds were outside of a ±3% minute retention time window when compared to the retention time of Δ^9-THC with the exception of CBL, 9S-Δ^7-THC, Δ^9-tetrahydrocannabinolic acid A, and Δ^9-THC-O-acetate. Although the retention time acceptance criterion was met for CBL, the qualifier ratio was outside of ±20%. Δ^9-Tetrahydrocannabinolic acid A had an instrumental response of 3217 and the peak shape was not acceptable. 9S-Δ^7-THC and Δ^9-THC-O-acetate were the only two compounds identified as potential interferents with Δ^{9}-THC. Figure 11 is a chromatogram of 9S-Δ^{7}-THC and Δ^{9}-THC at equal concentrations in a neat sample.

Figure 11 9S-Δ^{7}-THC and Δ^{9}-THC



The Δ^{9}-THC-O-acetate was investigated to identify the source of the interferent. A neat standard of Δ^{9}-THC-O-acetate was prepared and analyzed alongside an extracted sample fortified with Δ^{9}-THC-O-acetate. The instrumental response for Δ^{9}-THC was monitored with each sample. The retention time of the THC-O-acetate elutes much later than Δ^{9}-THC indicating that the presence of Δ^{9}-THC is not from degradation of Δ^{9}-THC-O-acetate into Δ^{9}-THC in the ionization source. The acidic mobile phase is imperative to the ionization of Δ^{9}-THC. Therefore, the analysis of Δ^{9}-THC-O-acetate without an acidic mobile phase produced no instrumental response for Δ^{9}-THC.

To determine the contribution of each step, the instrumental response of Δ^{9}-THC was compared between the neat standard and extracted sample. No instrumental response for Δ^{9}-THC was noted in either sample. For this investigation, a new stock solution of Δ^{9}-THC-O-acetate was prepared. Given the differences in analytical results, the stability of the Δ^{9}-THC-O-acetate stock standard was evaluated. The previously prepared interferent stock solution was reanalyzed alongside the freshly prepared stock solution to confirm the presence of Δ^{9}-THC. Δ^{9}-THC was observed in the old stock solution and not the freshly prepared solution.

To further investigate the degradation of Δ^{9}-THC-O-acetate to Δ^{9}-THC, the autosampler vial containing neat standard of the freshly prepared stock was injected 24-hours after the initial injection. The vial was then injected again at a time point of 72-hours after initial injection. The samples remained on the instrument in autosampler vials under standard laboratory conditions. The initial response of Δ^{9}-THC was approximately 450 area counts. After 24-hours the instrumental response of Δ^{9}-THC increased to approximately 3700 area counts. Finally, after 72-hours, the instrumental response of Δ^9-THC increased to approximately 8600 area counts. This indicates that Δ^9-THC-O-acetate degrades to Δ^9-THC in solution and that the presence of an instrumental peak for Δ^9-THC during the initial interferent study was from sample degradation and not a production of Δ^9-THC during extraction or analysis.

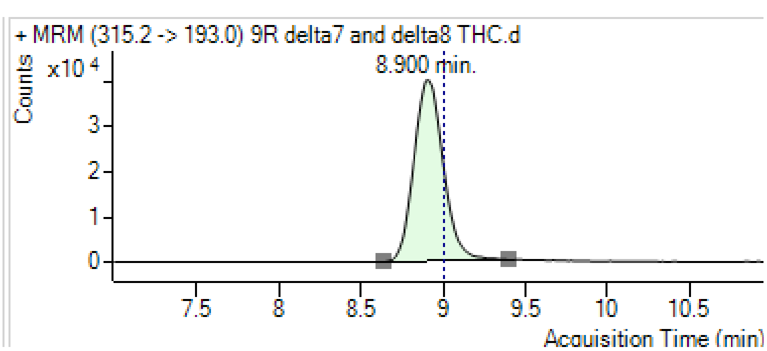
When evaluating for an instrumental response in the Δ^8-THC detection window, the following compounds listed in Table 20 provided an instrumental response.

Table 20 Δ^8-THC detection window

Δ^8-THC Detection Window			
Compound	Retention Time (minutes)	Peak Area	Qualifier Ratio
Δ^8-THC	8.909	681434	40.1
Δ^9-THC	9.521	944870	38.3
(±) Cannabicyclol (CBL)	9.596	115815	186.7
±cis-Δ^9-THC	6.623	1109876	38.0
9R-Δ^7-THC	8.925	272471	40.6
9S-Δ^7-THC	9.497	296313	37.1
Cannabinol (CBN)	8.892	4027	36.9
exo-THC	7.815	484647	38.8
Δ^8-Iso-THC	8.544	405636	38.5
Δ^8-THC Acetate (Δ^8-THC-O-Acetate)	8.925	18458	37.1
Δ^9-Tetrahydrocannabinolic Acid A	9.530	3217	35.9
Δ^9-THC Acetate (Δ^9-THC-O-Acetate)	9.513	23737	40.1

All compounds were outside of a ±3% minute retention time window when compared to the retention time of Δ^8-THC with the exception of 9R-Δ^7-THC, CBN, and Δ^8-THC-O-acetate. The aforementioned compounds were also within the qualifier ratio acceptance criterion of ±20%. CBN did not have acceptable peak shape with a peak area of 4027 counts. The Δ^8-THC-O-acetate was investigated to identify the source of the interferent. 9R-Δ^7-THC is indistinguishable with the current acceptance criteria for retention time and qualifier ratios. Figure 12 is a chromatogram of 9R-Δ^7-THC and Δ^8-THC at equal concentrations in a neat standard.

Figure 12 9R-Δ^7-THC and Δ^8-THC



The Δ^8-THC-O-acetate was investigated to identify the source of the interferent. A neat standard of Δ^8-THC-O-acetate was prepared and analyzed alongside an extracted sample fortified with Δ^8-THC-O-acetate. The instrumental response for Δ^8-THC was monitored with each sample. The retention time of the THC-O-acetate elutes much later than Δ^8-THC indicating that the presence of Δ^8-THC is not from degradation of Δ^8-THC-O-acetate into Δ^8-THC in the ionization source. The acidic mobile phase is imperative to the ionization of Δ^8-THC. Therefore, the analysis of Δ^8-THC-O-acetate without an acidic mobile phase produced no instrumental response for Δ^8-THC.

To determine the contribution of each step, the instrumental response of Δ^8-THC was compared between the neat standard and extracted sample. No instrumental response for Δ^8-THC was noted in either sample. For this investigation, a new stock solution of Δ^8-THC-O-acetate was prepared. Given the differences in analytical results, the stability of the Δ^8-THC-O-acetate stock standard was evaluated. The previously prepared interferent stock solution was reanalyzed alongside the freshly prepared stock solution to confirm the presence of Δ^8-THC. Δ^8-THC was observed in the old stock solution and not the freshly prepared solution.

To further investigate the degradation of Δ^8-THC-O-acetate to Δ^8-THC, the autosampler vial containing neat standard of the freshly prepared stock was injected 24-hours after the initial injection. The vial was then injected again at a time point of 72-hours after initial injection. The samples remained on the instrument in autosampler vials under standard laboratory conditions. The initial response of Δ^8-THC was approximately 300 area counts. After 24-hours the instrumental response of Δ^8-THC increased to approximately 2700 area counts. Finally, after 72-hours, the instrumental response of Δ^8-THC increased to approximately 6800 area counts. This indicates that Δ^8-THC-O-acetate degrades to Δ^8-THC in solution and that the presence of an instrumental peak for Δ^8-THC during the initial interferent study was from sample degradation and not a production of Δ^8-THC during extraction or analysis.

In summary, Table 21 lists the compounds that are unable to be distinguished from the target compound using retention time and qualifier ratios for each column.

Table 21 Interfering compound summary

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^9-OH-THC		Δ^8-OH-THC
Δ^8-OH-THC		Δ^9-OH-THC
Δ^9-Carboxy-THC		Δ^8-Carboxy-THC
Δ^8-Carboxy-THC		Δ^9-Carboxy-THC
Cannabidiol		
Δ^9-THC	9S-Δ^7-THC	exo-THC
Δ^8-THC	9R-Δ^7-THC	Δ^8-Iso-THC, 9R-Δ^7-THC, 9S-Δ^7-THC

Table 22 describes the compounds that produced an instrumental response within the retention time acceptance criterion for the target compound. Low instrumental response with poor peak shape was not included in the table. Table 22 includes interferences on either the quantifier transition or the qualifier transition.

Table 22 Interfering instrumental response

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^9-OH-THC	CBDVA	Δ^8-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^8-OH-THC		Δ^9-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^9-Carboxy-THC		Δ^8-Carboxy-THC*
Δ^8-Carboxy-THC		Δ^9-Carboxy-THC*
Cannabidiol	CBG	
Δ^9-THC	9S-Δ^7-THC, CBL	exo-THC
Δ^8-THC	9R-Δ^7-THC	Δ^8-Iso-THC, 9R-Δ^7-THC, 9S-Δ^7-THC, CBL

Process Comparison

During method development an investigation into various instrumental techniques for the most efficient analysis of cannabinoids using two analytical columns was performed. The reconstitution volume and solvent for the supported liquid extraction is 50 µL of methanol. Taking into consideration the potential for solvent evaporation, two potential processes were identified. The first process (Instrumental Process 1) involves the injection of samples in series injecting all samples on the first column followed by injection of samples on the second column. The second process (Instrumental Process 2) involves the injection of a sample on the first column and immediately following injection on the second column. An example of Instrumental Process 1 is shown in Table 23.

Table 23 Instrumental Process 1

Sample Number	Sample Name	Method
1	Sample 1	Column 1
2	Sample 2	Column 1
3	Sample 3	Column 1
4	Sample 4	Column 1
5	Sample 1	Column 2
6	Sample 2	Column 2
7	Sample 3	Column 2
8	Sample 4	Column 2

Assuming 48 samples are extracted in a single batch, the total runtime for column 1 would be approximately 11 hours prior to beginning the injections on column 2. Column 2 would also have a runtime of approximately 11 hours for a batch of 48 samples. Given the reconstitution volume and solvent, evaporation of samples shall be considered. To evaluate this possibility, neat samples were prepared and injected at time point 0. The samples were subsequently re-injected after approximately 10 hours. This was performed 3 times to account for variability in laboratory conditions and vial/vial caps. Although enough sample remained for a second injection, this does not eliminate the potential for this to occur in all circumstances.

Instrumental Process 2 was developed to limit the time between the two injections of a single sample. The process injects a single sample on column 1 with the injection on column 2 immediately following. Instrumental Process 2 is shown in Table 24.

Table 24 Instrumental Process 2

Sample Number	Sample Name	Method
1	Sample 1	Column 1
2	Sample 1	Column 2
3	Sample 2	Column 1
4	Sample 2	Column 2
5	Sample 3	Column 1
6	Sample 3	Column 2
7	Sample 4	Column 1
8	Sample 4	Column 2

When changing between analytical methods, an equilibration time is required between injections of samples. Although the columns maintain their respective mobile phase compositions, the binary pumps and plumbing to the columns must be equilibrated with the appropriate mobile phase. This equilibration takes approximately four minutes causing an increase in runtime for a batch of 48 samples from approximately 11 hours to approximately 13 hours. Therefore, the total runtime for a batch of 48 samples analyzed on column 1 and column 2 would be approximately 26 hours.

An alternative option to the abovementioned options is to equip the multisampler of the instrument with a thermostat control. The addition of the thermostat would allow for the multisampler to be cooled to approximately 4°C preventing sample evaporation. This would allow for a variation in the sample injection sequence.

Conclusions

The solid phase extraction and supported liquid extraction procedures were capable of achieving similar lower limits of detection and quantitation for Δ^9-THC and Δ^9-carboxy-THC. The lower limit of detection and quantitation of Δ^9-OH-THC was estimated to be 0.4 mg/L for the solid phase extraction and 0.2 mg/L for the supported liquid extraction.

When evaluating the ionization suppression/enhancement and recovery for each method, slight differences in the ionization suppression were noted. There was significant difference in the recovery of postmortem specimens between the two methods. The supported liquid extraction was capable of achieving consistent recovery across matrix types and sources whereas the solid phase extraction noted more significant variability in recovery based on matrix.

Additionally, the solid phase extraction method is a more laborious time consuming process that requires 1.0 mL of biological specimen. The supported liquid extraction only requires 0.5 mL of biological specimen for analysis. Furthermore, the supported liquid extraction has significantly fewer steps in the extraction process and does not include the requirement for a protein precipitation prior to extraction. Therefore, the supported liquid extraction procedure will be validated for the quantitative analysis of cannabinoids in biological specimens using LCMSMS. The dual column process will include quantitative analysis on the Poroshell 120 EC-C18 with enhanced confirmation on the Poroshell pentafluorophenyl column.

References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 24, **2022.**

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 23, **2022**.

York, J., "Analyzing THC Concentrates? Look for Isomers!" ChromaBLOGraphy Restek Corporation, https://www.restek.com/en/chromablography/chromablography/analyzing-thc-concentrates-look-for-isomers/. Access **2022**.

United Chemical Technologies, "Analysis of Natural Cannabinoids and Metabolites from Blood Using Clean Screen THC and Selectra Core C18 Column on LC-MS/MS." Application note. May **2022**.

United Chemical Technologies, "Analysis of Natural Cannabinoids and Metabolites from Urine Using Styre Screen HBL and Selectra Core C18 Column on LC-MS/MS." Application note. March **2022**.

Appendix A: Cannabinoid Sources

The table describes the sources of various cannabinoids. The identification of a phytocannabinoid does not exclude it from being synthetically prepared. Additionally, some synthetic preparations are fully synthetic while others are derived from phytocannabinoids. For example, Δ^9-Tetrahydrocannabiphorol (Δ^9-THCP) can be prepared in a fully synthetic manner or can be synthesized from Δ^9-THC that was extracted from plant material.

Cannabinoids	Source	Pharmacological Activity
Δ^9-THC	Phytocannabinoid	Active
Δ^8-THC	Phytocannabinoid	Active
Δ^9-OH-THC	Metabolite	
Δ^8-OH-THC	Metabolite	
Δ^9-Carboxy-THC	Metabolite	
Δ^8-Carboxy-THC	Metabolite	
Cannabidiol (CBD)	Phytocannabinoid	Active
(±) Cannabicyclol (CBL)	Phytocannabinoid	
(6aR,9R)-Δ^{10}-THC	Trace Phytocannabinoid/impurity in Δ^8-THC synthesis from CBD	
(6aR,9S)-Δ^{10}-THC	Trace Phytocannabinoid/impurity in Δ^8-THC synthesis from CBD	
±cis-Δ^9-THC	Phytocannabinoid found in high CBD plant material	
9R-$\Delta^{6a,10a}$-THC	Trace Phytocannabinoid	Active
9R-Δ^7-THC	Synthetic	Inactive
9S-$\Delta^{6a,10a}$-THC	Trace Phytocannabinoid	Active
9S-Δ^7-THC	Synthetic	Inactive
Cannabichromene (CBC)	Phytocannabinoid	
Cannabichromenic Acid (CBCA)	Phytocannabinoid	
Cannabicyclolic Acid (CBLA)	Phytocannabinoid	
Cannabidiolic Acid (CBDA)	Phytocannabinoid	
Cannabidivarin (CBDV)	Phytocannabinoid	
Cannabidivarinic Acid (CBDVA)	Phytocannabinoid	
Cannabigerol (CBG)	Phytocannabinoid	
Cannabigerolic Acid (CBGA)	Phytocannabinoid	
Cannabigerovarinic Acid (CBGVA)	Phytocannabinoid	
Cannabinol (CBN)	Phytocannabinoid/Degradation product of THC	
Cannabinolic Acid (CBNA)	Phytocannabinoid	
Cannabivarin (CBV)	Phytocannabinoid	
exo-THC	Impurity in THC synthesis	
Tetrahydrocannabivarinic (THCV)	Phytocannabinoid	
Tetrahydrocannabivarinic Acid (THCVA)	Phytocannabinoid	
Δ^8-Iso-THC	Potential impurity in the synthesis of Δ^9-THC and Δ^8-THC	
Δ^8-THC Acetate (Δ^8-THC-O-Acetate)	Synthetic	
Δ^8-Tetrahydrocannabiphorol (Δ^8-THCP)	Trace Phytocannabinoid (isolated in 2019)	
Δ^9-Tetrahydrocannabinolic Acid A	Phytocannabinoid	
Δ^9-THC Acetate (Δ^9-THC-O-Acetate)	Synthetic	
Δ^9-Tetrahydrocannabutol (Δ^9-THCB)	Trace Phytocannabinoid (isolated in 2019)	
Δ^9-Tetrahydrocannabihexol (Δ^9-THCH)	Phytocannabinoid (isolated in 2020)	
Δ^9-Tetrahydrocannabiorcol (Δ^9-THCO)	Phytocannabinoid	
Δ^9-Tetrahydrocannabiphorol (Δ^9-THCP)	Trace Phytocannabinoid (isolated in 2019)	

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Participants

Name:	Rebecca Wagner, Ph.D.
Project Role:	Principal Investigator
Funding Support:	Virginia Department of Forensic Science
Foreign Country Collaboration:	No

Name:	Denise Herr, Ph.D.
Project Role:	Forensic Laboratory Specialist VI
Funding Support:	Virginia Department of Forensic Science
Foreign Country Collaboration:	No

Name:	Ali Siddiqi
Project Role:	Forensic Laboratory Specialist VI
Funding Support:	Virginia Department of Forensic Science
Foreign Country Collaboration:	No

OUTCOMES

1. Validation of the Quantitative Analysis of Cannabinoids using LCMSMS

Method Validation Summary:

Note: Published internally as an independent document (Figures and Tables are chronological)

Method Validation Summary for the Quantitative Analysis of Cannabinoids in Biological Matrices using LCMSMS

1. Bias and Precision
    a. Bias
    b. Within-run Precision
    c. Intermediate Precision
2. Sensitivity
    a. Estimated Limit of Detection (LOD)
    b. Lower Limit of Quantitation (LLOQ)
3. Linearity and Calibration Model
4. Ionization Suppression/Enhancement
5. Carryover
6. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
7. Dilution Integrity
8. Stability
9. Robustness
10. Summary
11. References

An Agilent Technologies 1260 binary pump liquid chromatograph coupled independently to both an Agilent Technologies 6460 and 6470 tandem mass spectrometer was used during validation. Validation experiments were performed in accordance with the approved validation plan. The biological matrices evaluated during the validation included blank blood, antemortem blood, and postmortem blood for quantitative analysis. Urine was only evaluated during lower limit of quantitation, ionization suppression/enhancement, carryover, interferences, dilution integrity, and stability experiments.

1. Bias and Precision
    a. Bias

Bias was assessed by analyzing pooled blank blood, antemortem blood, and postmortem blood fortified with the target compounds at three different concentrations (low, medium, and high) over a total of five batch analyses. Each concentration, for each matrix, was evaluated in triplicate. The calibration range of the method was established to be 1/2/5 mg/mL to 100/200/500 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC). The three concentrations evaluated for bias included 3/6/15 ng/mL, 10/20/50 ng/mL, and 75/150/375 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC).

The pooled fortified samples were prepared by spiking a large volume of matrix (blank blood, antemortem blood, postmortem blood) with the respective concentrations of cannabinoids. Aliquots of 0.5 mL were subsequently removed from the pooled samples and extracted prior to quantitative analysis using LCMSMS. Bias was assessed using Equation 1

Equation 1

$$\text{Bias (\%) Concentration}_x = \left(\frac{\text{Mean of Calculated Concentration}_x - \text{Expected Concentration}_x}{\text{Expected Concentration}_x}\right) \times 100$$

The acceptance criterion for pooled bias was ±20% for all three concentration levels. All back calculated concentrations were utilized in determining the overall bias of the method. The back calculated concentrations were established using the calibration curve prepared in blank blood matrix. The pooled bias for each matrix using the C18 analytical column is represented in Table 1.

Table 1 Cannabinoids bias C18 analytical column

Pooled Bias C18 Analytical Column % Bias; n=15			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	-4.33	-1.10	-1.14
Carboxy-THC	-8.40	-5.64	-3.72
Cannabidiol	-0.11	-0.43	5.02
Δ^9-THC	-6.67	-5.07	-0.96
Δ^8-THC	-2.22	0.87	4.26
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	8.67	11.50	8.46
Carboxy-THC	-1.11	3.29	2.63
Cannabidiol	15.89	14.10	16.24
Δ^9-THC	7.33	7.93	8.27
Δ^8-THC	9.11	9.33	9.36
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	8.33	8.07	2.89
Carboxy-THC	3.02	3.53	-0.40
Cannabidiol	9.89	5.17	4.47
Δ^9-THC	2.44	-2.13	-2.12
Δ^8-THC	3.33	3.73	1.41

All matrix types had bias values within the predetermined acceptance criterion of ±20% of the target compound. No significant impact on bias was noted for antemortem blood or postmortem blood when evaluating against a blank blood calibration curve. To investigate the impact of the non-matched matrix calibration curve, calibration curves were prepared in each matrix type and compared. All matrices were evaluated for their relationship with the blank blood calibration curve. All matrices were consistent when compared to the blank blood matrix calibration curve.

Bias was also evaluated for the PFP analytical column. The pooled bias for each matrix using the PFP analytical column is represented in Table 2.

Table 2 Cannabinoids bias PFP analytical column

Pooled Bias PFP Analytical Column % Bias; n=15			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	-7.22	-2.13	-1.02
Carboxy-THC	-7.87	-3.35	-1.79
Cannabidiol	-0.56	0.80	4.35
Δ^9-THC	-5.78	-5.27	-0.82
Δ^8-THC	-2.22	1.00	-2.29
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	2.00	7.27	5.68
Carboxy-THC	0.71	4.39	2.93
Cannabidiol	15.22	13.60	15.40
Δ^9-THC	7.11	5.67	8.40
Δ^8-THC	6.22	5.40	1.80
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	2.11	3.73	0.45
Carboxy-THC	4.58	4.13	-1.14
Cannabidiol	10.11	6.00	4.24
Δ^9-THC	3.11	-2.07	-1.98
Δ^8-THC	-5.11	-3.87	-9.97

All matrix types had bias values within the predetermined acceptance criterion of ±20% of the target compound. No significant impact on bias was noted for antemortem blood or postmortem blood when evaluating against a blank blood calibration curve. To investigate the impact of the non-matched matrix calibration curve, calibration curves were prepared in each matrix type and compared. All matrices were evaluated for their relationship with the blank blood calibration curve. All matrices were consistent when compared to the blank blood matrix calibration curve.

b. Within-run Precision

The within-run precision was assessed using pooled blank blood, antemortem blood, and postmortem blood fortified with the target compounds at three different concentrations (low, medium, high) for a total of five batch analyses. Each concentration, for each matrix, was evaluated in triplicate. The three concentrations evaluated for bias included 3/6/15 ng/mL, 10/20/50 ng/mL, and 75/150/375 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC).

The pooled fortified samples were prepared by spiking a large volume of matrix (blank blood, antemortem blood, postmortem blood) with the respective concentrations of the target analyte. Aliquots (0.5 mL) were subsequently removed from the pooled samples and extracted prior to quantitative analysis using LCMSMS. Within-run precision was calculated using the Equation 2.

Equation 2

$$\text{Within-run Precision (\%CV)} = \left(\frac{\text{Standard Deviation of Batch Mean}}{\text{Calculated Mean of Batch}}\right) \times 100\%$$

The acceptance criterion for within-run precision was ≤20% for the coefficient of variation (%CV) at each concentration level. Table 3 represents the within-run precision data for the fortified pooled samples at three concentrations for each matrix type using the C18 analytical column.

Table 3 Cannabinoids within-run precision C18 analytical column

Pooled Within-run Precision C18 Analytical Column Mean±SD(%CV); n=3			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.00±0.17(3)	20.20±0.44(2)	150.7±2.2(2)
Carboxy-THC	13.87±0.59(4)	42.87±0.75(2)	360.9±6.8(2)
Cannabidiol	6.23±0.47(8)	19.90±0.52(3)	156.8±4.6(3)
Δ^9-THC	2.80±0.10(4)	9.93±0.29(3)	76.23±2.20(3)
Δ^8-THC	2.90±0.17(6)	10.23±0.32(3)	82.70±2.91(4)
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.33±0.21(3)	22.23±0.47(2)	1456.4±5.8(4)
Carboxy-THC	14.47±0.91(6)	52.63±1.52(3)	372.4±15.6(4)
Cannabidiol	7.57±0.55(7)	22.20±0.95(4)	176.9±9.7(6)
Δ^9-THC	3.20±0.17(5)	11.00±0.60(5)	78.67±2.50(3)
Δ^8-THC	3.17±0.15(5)	11.13±0.60(5)	74.93±4.38(6)
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.40±0.30(5)	22.17±0.93(4)	157.3±5.5(4)
Carboxy-THC	14.50±0.66(5)	53.87±2.28(4)	383.7±12.4(3)
Cannabidiol	6.50±0.66(10)	21.43±1.35(6)	164.3±11.7(7)
Δ^9-THC	3.17±0.21(7)	9.70±0.60(6)	74.07±4.22(6)
Δ^8-THC	3.03±0.25(8)	10.03±0.96(10)	79.27±5.07(6)

As shown in Table 3, the coefficient of variation was within the predetermined acceptance criterion of ≤20% for within-run precision for all matrices evaluated. The largest percent coefficient of variation was observed to be 10% for the 6 ng/mL cannabidiol and 10 ng/mL Δ^8-THC in postmortem blood. The within-run precision was also evaluated when using the PFP analytical column. The within-run precision is shown in Table 4.

Table 4 Cannabinoids within-run precision PFP analytical column

Pooled Within-run Precision CPFP Analytical Column Mean±SD(%CV); n=3			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	5.00±0.17(3)	19.67±0.47(2)	149.8±7.7(5)
Carboxy-THC	14.47±0.51(4)	45.63±1.12(2)	367.9±15.2(4)
Cannabidiol	5.87±0.25(4)	19.90±0.53(3)	155.1±3.9(3)
Δ^9-THC	2.87±0.15(5)	9.40±0.35(4)	71.93±2.99(4)
Δ^8-THC	3.03±0.15(5)	10.17±0.32(3)	65.80±10.19(15)
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.53±0.35(5)	21.70±0.44(2)	167.7±6.0(4)
Carboxy-THC	16.13±1.00(6)	51.60±1.92(4)	374.0±14.7(4)
Cannabidiol	7.43±0.42(6)	22.83±1.17(5)	164.9±8.7(5)
Δ^9-THC	3.37±0.12(3)	10.53±0.38(4)	77.23±2.87(4)
Δ^8-THC	3.17±0.21(7)	10.03±0.75(7)	73.03±4.5(6)
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	5.60±0.40(7)	20.93±0.76(4)	153.4±7.3(5)
Carboxy-THC	14.77±0.50(3)	54.13±2.67(5)	378.3±18.1(5)
Cannabidiol	7.03±0.47(7)	22.10±0.96(4)	164.4±14.5(9)
Δ^9-THC	3.30±0.20(6)	9.80±0.26(3)	75.77±3.67(5)
Δ^8-THC	3.10±0.17(6)	10.03±0.47(5)	62.80±4.19(7)

The percent coefficient of variation was within the predetermined acceptance criterion of ≤20% for the within-run precision using the PFP analytical column. The largest precision was observed to be 15% for the 75 ng/mL Δ^8-THC.

c. Intermediate Precision

The intermediate precision was evaluated using the C18 and PFP analytical columns. The same fortified pooled blank blood, antemortem blood, and postmortem blood used in the bias evaluation was used in the intermediate precision. The intermediate precision was calculated using Equation 3.

Equation 3

$$\text{Intermediate Precision (\%CV)} = \left(\frac{\text{Standard deviation of combined means}}{\text{Calculated grand mean}}\right) \times 100\%$$

The acceptance criterion for intermediate precision was within ≤20% for the %CV at each concentration level. Table 5 represents the intermediate precision for the fortified pooled samples evaluated for each matrix type.

Table 5 Cannabinoids intermediate precision C18 analytical column

Pooled Intermediate Precision C18 Analytical Column Mean±SD(%CV); n=15			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	5.74±0.19(3)	19.78±0.65(3)	148.3±2.9(2)
Carboxy-THC	13.74±0.54(4)	47.18±2.33(5)	361.0±7.2(2)
Cannabidiol	5.99±0.29(5)	19.91±0.42(2)	157.5±3.2(2)
Δ^9-THC	2.80±0.08(3)	9.49±0.32(3)	74.28±2.38(3)
Δ^8-THC	2.93±0.15(5)	10.09±0.20(2)	78.19±2.94(4)
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.52±0.24(4)	22.30±0.73(3)	162.7±6.5(4)
Carboxy-THC	14.83±1.05(7)	51.65±2.18(4)	384.9±17.2(4)
Cannabidiol	6.95±0.48(7)	22.82±0.70(3)	174.4±7.75(4)
Δ^9-THC	3.22±0.17(5)	10.79±0.47(4)	81.20±3.10(4)
Δ^8-THC	3.27±0.18(5)	10.93±0.39(4)	82.02±5.6(7)
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.50±0.33(5)	21.61±0.71(3)	154.3±6.8(4)
Carboxy-THC	15.45±0.81(5)	51.77±2.95(6)	373.5±19.3(5)
Cannabidiol	6.59±0.44(7)	21.03±0.87(4)	156.7±8.56(5)
Δ^9-THC	3.07±0.17(6)	9.79±0.39(4)	73.41±3.95(5)
Δ^8-THC	3.10±0.18(6)	10.37±0.59(6)	76.06±6.23(8)

All compounds evaluated were within the predetermined acceptance criterion for intermediate precision when using the C18 analytical column. The intermediate precision ranged from 2% to 8% for all matrix types. The intermediate precision was also determined for the PFP analytical column. Table 6 shows the data obtained from the intermediate precision evaluation.

Table 6 Cannabinoids intermediate precision PFP analytical column

Pooled Intermediate Precision PFP Analytical Column Mean±SD(%CV); n=15			
Blank Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	5.57±0.35(6)	19.57±0.54(3)	148.5±5.4(4)
Carboxy-THC	13.82±0.46(4)	48.33±1.65(3)	368.3±10.7(3)
Cannabidiol	5.97±0.26(4)	20.16±0.42(2)	156.5±2.4(2)
Δ^9-THC	2.83±0.07(2)	9.47±0.28(3)	74.39±2.35(3)
Δ^8-THC	2.93±0.14(5)	10.10±0.34(3)	73.28±6.49(9)
Antemortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.12±0.47(8)	21.45±0.69(3)	158.5±8.0(5)
Carboxy-THC	15.11±0.84(6)	52.19±2.02(4)	386.0±16.3(4)
Cannabidiol	6.91±0.46(7)	22.72±0.68(3)	173.1±8.0(5)
Δ^9-THC	3.21±0.11(3)	10.57±0.31(3)	81.30±3.84(5)
Δ^8-THC	3.19±0.21(7)	10.54±0.68(6)	76.35±6.76(9)
Postmortem Blood	3/6/15 ng/mL	10/20/50ng/mL	75/150/375 ng/mL
OH-THC	6.13±0.37(6)	20.75±0.69(3)	150.7±9.7(6)
Carboxy-THC	15.69±0.75(5)	52.07±2.31(4)	370.7±20.6(6)
Cannabidiol	6.61±0.35(5)	21.20±0.82(4)	156.4±11.0(7)
Δ^9-THC	3.09±0.15(5)	9.79±0.29(3)	73.51±4.39(6)
Δ^8-THC	2.85±0.28(10)	9.61±0.62(6)	67.52±6.71(10)

The intermediate precision for all compounds evaluated was between 2% and 10% for all matrix types. All compounds at all concentrations met the predetermined acceptance criterion for intermediate precision.

2. Sensitivity

a. Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation was defined as an administratively defined decision point (threshold concentration). The limit of detection was evaluated on all instrumentation models and is understood to be an estimate based on the condition of the instruments at the time of the evaluation. The lowest calibrator concentration within the method was 1/2/5 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC). Therefore, concentrations of 0.75/1.5/3.75 ng/mL and 0.5/1/2.5 ng/mL were evaluated for blank blood over three batch analyses. Nine blank blood matrix sources were utilized in the determination of the estimated limit of detection. The peak shape, retention time, qualifier ratio, and signal to noise ratio were evaluated for each compound at each concentration. The predetermined identification criteria included a retention time within ±3%, a qualifier ratio within ±20%, and a signal to noise ratio ≥3.3.

The estimated limit of detection for all target compounds, with the exception of carboxy-THC, was determined to be at the method's lower limit of quantitation. Carboxy-THC was determined to have an estimated limit of detection of 2.5 ng/mL. Given the limitation in blank blood of reaching a limit of detection lower than the lower limit of quantitation, the other matrix types evaluated within the validation (antemortem blood, postmortem blood, and urine) were only assessed at the lower limit of quantitation.

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation was established by evaluating the lowest non-zero calibrator for the method. For each matrix type (blank blood, antemortem blood, postmortem blood, and urine), nine different blank matrix sources were fortified at the lowest calibrator concentration (1/2/5 ng/mL [Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC]) and analyzed, in triplicate, over three analyses. The replicates were utilized to demonstrate that all detection, identification, bias, and precision criteria were met even in the presence of ionization suppression. For postmortem matrices, concentrations of 2/4/5 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC) were evaluated for the lower limit of quantitation.

Predetermined acceptance criteria:

Retention Time: ±3%

Qualifier Ratio: ±20%

Signal-to-Noise: ≥10

Back Calculated Concentration: ±20%

In addition to the predetermined acceptance criteria, chromatographic peak shape was also monitored. Several replicates for blank blood, antemortem blood, postmortem blood, and urine were outside of the accuracy predetermined acceptance criteria of ±20% for both analytical columns. Section 2.4.3.5 of the Toxicology Procedures Manual (Qualtrax Revision 26) states that values of ±30% from the target calibrator concentration are acceptable for the lowest calibrator. Therefore, all targets were evaluated against the ±30% bias acceptance criteria. All replicates for OH-THC, carboxy-THC, and Δ^9-THC for all matrices were within ±30% for the C18 and PFP analytical columns. Additionally, cannabidiol met the acceptance criteria of ±30% when using the PFP analytical column. When evaluating the C18 analytical column for cannabidiol one (1) blank blood replicate out of 81 replicates was outside of ±30%. Further, three (3) urine replicates out of 81 replicates were outside of ±30%. Five (5) antemortem replicates for Δ^8-THC using the C18 analytical column were outside of ±30% out of 81 total replicates.

When evaluating Δ^8-THC using the PFP analytical column, 5 blank blood replicates, 7 postmortem blood replicates and 6 antemortem replicates out of 81 replicates for each matrix type were outside of ±30% acceptance criterion. In addition to bias, the qualifier ratio of the replicates was evaluated. A total of 81 replicates including nine matrix sources for each matrix type were evaluated for their qualifier ratio acceptance.

When evaluating the qualifier ratio for OH-THC using the C18 analytical column, 2 qualifier ratios were outside of ±20% when evaluating antemortem blood. On the PFP analytical column, there were a total of 11 out of 81 replicates outside of the ±20% acceptance criteria. Given the low qualifier ratio (approximately 10%), the acceptance criterion was adjusted in accordance with ANSI/ASB 098 Standard, Standard for Mass Spectral Analysis in Forensic Toxicology to ±30% (10 to 20% relative intensity). After reassessment of the data with a ±30% qualifier ratio acceptance criterion, all replicates, with the exception of 1 postmortem replicate evaluated for OH-THC, on both analytical columns, were within acceptance.

When evaluating the qualifier ratio for carboxy-THC using the C18 and PFP analytical column, all qualifier ratios for all matrices were within the predetermined acceptance criterion of ±20%. When evaluating the qualifier ratio for Δ^9-THC using the C18 analytical column, one qualifier ratio was observed to be outside of the ±20% acceptance criterion for antemortem blood. When evaluating the PFP analytical column, all qualifier ratios were within the predetermined acceptance criterion for Δ^9-THC.

Both cannabidiol and Δ^8-THC had a significant number of qualifier ratio failures (qualifier ratio outside of ±20%) for all matrices using both the C18 and PFP analytical columns. Cannabidiol on the PFP analytical column had one antemortem specimen with poor peak shape and one blank blood qualifier ratio failure. When evaluating urine, several qualifier ratio failures were noted along with accuracy failures for cannabidiol using the C18 analytical column. When evaluating cannabidiol on the PFP analytical column, qualifier ratio failures were only noted for cannabidiol. All other target compounds met the predetermined acceptance criteria.

3. Linearity and Calibration Model

The best fit calibration model was determined using multiple statistical analysis techniques as well as the analysis of residual plots. A total of 31 batch analyses, using blank blood, were analyzed to determine the best fit calibration model for each target. Three different calibration ranges were used within the validation depending on the target compound. Table 7 delineates the non-zero calibrators that were evaluated to determine the best fit calibration model.

Table 7 Target compound calibration range and calibrators

Calibration Range	
Target Compound	Calibrator Concentration (ng/mL)
OH-THC, Cannabidiol	2 5 10 20 50 100 200
Carboxy-THC	5 12.5 25 50 125 250 500
Δ^9-THC, Δ^8-THC	1 2.5 5 10 25 50 100

To determine the linear/quadratic nature of the model, ANOVA was used to compare the standard deviation of the residuals from all batches evaluated within the calibration range. The t-test and f-test were utilized from the ANOVA. The t-test determined if there was a statistically significant difference between linear and quadratic models.

If p-value < 0.05 (level of significance), the null hypothesis was rejected,

If p-value > 0.05 (level of significance), the null hypothesis was not rejected,

The null-hypothesis states that there was no statistically significant difference between groups.

The f-test was utilized to determine if there was a statistically significant difference in the variance between the two groups.

If $f > F_{crit}$, the null hypothesis was rejected,

If $f < F_{crit}$, the null hypothesis was not rejected,

The null hypothesis states that the variances between the two groups were equal.

A comparison of linear weighted (1/x) and quadratic weighted (1/x) models was also performed to demonstrate consistent results. If the two groups were determined not to be statistically different, a linear calibration model was applied to the target. If the two groups were determined to be statistically significantly different, the quadratic calibration model was applied to the target.

To determine the weighting of the calibration model (non-weighted or 1/x weighting), a t-test was used to assess if there was a significant difference between the two groups. The t-test was completed after the linear/quadratic nature of the model was established. The weighted and non-weighted sum of the relative error for the residual was compared using the t-test.

If p-value < 0.05 (level of significance), the null hypothesis was rejected,

If p-value > 0.05 (level of significance), the null hypothesis was not rejected,

The null hypothesis states that there was no statistically significant difference between groups.

The weighting of the calibration model was also determined by applying the weighting that minimizes the sum of relative error for the residuals. The sum of relative error was averaged for an overall relative sum over the batches analyzed for the working range. The relative residual error was calculated using Equation 4 for each concentration in the calibration curve.

Equation 4

$$\text{Relative Residual Error} = \frac{|\text{Residual Error}|}{\text{Theoretical Concentration}}$$

After calculating the relative residual errors, the values were summed. The sums of the relative errors for the batches evaluated for the working range were then averaged and the lowest average between the weighted and non-weighted groups was determined to be the best fit weighting model for the curve.

In addition to statistical analyses, residual plots were constructed to help visually assist in the evaluation of the best fit calibration model. Additional calibration model evaluations were completed including one antemortem blood, one postmortem blood, and one urine matrix source with the 31 previously evaluated blank blood analyses.

With the addition of the other matrices, no change was observed in the best fit calibration model indicating the appropriateness of using blank blood for establishing the calibration curve. This is further shown in Section 1 with the evaluation of bias and precision for each matrix type using a blank blood matrix for the establishment of the calibration curve. Appendix A details the best fit calibration model determination for each target compound within the analytical method.

4. Ionization Suppression/Enhancement

Ionization suppression and enhancement was evaluated by assessing the instrumental response of post-extraction fortified samples and neat standards. Post-extraction fortified samples were prepared from blank matrix that was subject to the supported liquid extraction protocol. After extraction, the blank samples were fortified with both target and internal standard. The neat samples were prepared by spiking an appropriate volume of the target analyte, internal standard, in methanol directly into the autosampler vial. Neat samples were not dried down during preparation.

Equation 5 was used to calculate the ionization suppression/enhancement for the target compounds and the internal standards. The ionization suppression/enhancement was assessed at two different concentrations: 5/10/25 ng/mL and 50/100/250 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC).

Equation 5

$$\text{Ion Suppression/Enhancement} = \left(\frac{\text{Average Post-Extraction Fortified Sample}}{\text{Average Neat Sample}}\right) \times 100$$

To fully evaluate the impact of ionization suppression/enhancement, duplicate determinations of each concentration for each matrix source were evaluated. A total of ten different matrix sources per matrix type was used in the evaluation. The post-extraction fortified samples were compared to six replicate injections of neat standards. The overall ionization suppression or enhancement was calculated for both the C18 analytical column and the PFP analytical column. Table 8 shows the data associated with the C18 analytical column whereas Table 9 shows the data associated with the PFP analytical column.

Table 8 Ionization suppression and enhancement C18 analytical column

Ionization Suppression and Enhancement % Suppression/Enhancement (Standard Deviation)				
Target Compound	Blank Blood (n=36)	Antemortem Blood (n=36)	Postmortem Blood (n=36)	Urine (n=36)
OH-THC	105.8±16.3	69.5±5.7	77.2±10.9	48.9±14.9
Carboxy-THC	96.3±19.9	53.4±7.0	59.4±11.7	49.3±17.6
Cannabidiol	93.8±9.1	65.3±6.9	63.6±10.2	38.9±9.8
Δ^9-THC	107.6±4.8	87.6±4.9	88.4±8.4	50.3±8.1
Δ^8-THC	110.2±4.3	92.3±5.9	92.7±8.4	51.3±7.9
OH-THC-D_3	90.3±14.4	68.5±6.3	75.0±11.7	45.2±12.6
Carboxy-THC-D_3	90.2±21.3	51.4±7.0	58.0±11.7	44.4±15.7
Cannabidiol-D_3	88.9±10.3	63.9±8.2	60.4±11.4	37.2±9.0
Δ^9-THC-D_3	104.5±3.8	88.8±6.0	87.2±8.1	47.2±7.2

Table 9 Ionization suppression and enhancement PFP analytical column

Ionization Suppression and Enhancement % Suppression/Enhancement (Standard Deviation)				
Target Compound	Blank Blood (n=36)	Antemortem Blood (n=36)	Postmortem Blood (n=36)	Urine (n=36)
OH-THC	99.3±25.9	115.7±23.0	74.8±22.1	101.1±22.1
Carboxy-THC	113.0±30.5	123.6±19.8	99.0±25.0	115.1±22.6
Cannabidiol	76.9±19.1	93.0±16.5	61.0±16.2	87.5±18.0
Δ^9-THC	123.6±20.1	123.0±21.5	104.2±15.7	98.9±16.2
Δ^8-THC	102.8±17.0	108.2±15.3	99.9±15.5	94.6±16.9
OH-THC-D_3	93.1±25.8	118.7±24.8	73.8±21.7	102.5±21.5
Carboxy-THC-D_3	106.8±26.2	125.0±21.5	96.7±24.2	117.7±22.5
Cannabidiol-D_3	73.5±19.6	95.1±17.1	59.4±15.3	90.8±18.7
Δ^9-THC-D_3	98.3±15.5	107.6±14.9	96.8±12.2	95.1±15.3

The values of 100% are indicative of no ionization suppression or enhancement in the samples. Values greater than 100% indicate ionization enhancement and values less than 100% indicate ionization suppression. Values greater than ±25% are indicative of significant ionization suppression or enhancement. The ionization enhancement did not exceed 25% for either analytical column. Ionization suppression was noted in several instances. The C18 analytical column demonstrated the most ionization suppression between the two column types evaluated. Antemortem blood, postmortem blood, and urine all had indications of significant ionization suppression when using the C18 column and less notably with the PFP analytical column. No ionization suppression was noted with the PFP column and urine.

In addition to the average ionization suppression or enhancement, the variability between the matrices was also evaluated by assessing the %CV. The %CV was calculated for each matrix type and should not exceed ±20%. The %CV exceeded 20% for carboxy-THC and associated internal standard in blank blood for the C18 analytical column. Additionally, the %CV exceeded 20% for the majority of compounds evaluated in urine. The PFP analytical column provided more variability than the C18 analytical column. Values greater than 20%CV were noted with blank blood, antemortem blood, postmortem blood, and urine.

Given the significant ionization suppression noted with the C18 and PFP analytical columns, and the variability between matrices exceeding a %CV of 20%, additional matrices were evaluated for the estimated limit of detection and lower limit of quantitation.

5. Carryover

Carryover was evaluated by analyzing blank matrix samples immediately following progressively higher concentrations of fortified matrix within the injection sequence. Three concentrations, 1/2/5 mg/L, 2/4/10 mg/L, and 4/8/20 mg/L (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC), were evaluated in three sources each of blank blood, antemortem blood, postmortem blood, and urine. The blank sample immediately following the fortified matrix sample was evaluated for an instrumental response greater than 10% of the LLOQ (0.001/0.002/0.005 mg/L). No blank matrix samples immediately following any fortified matrix sample had indications of carryover.

6. Interferences

To assess for interference, the qualifier and quantifier ions for the target compounds and internal standards were monitored. If an instrumental response was noted and was less than 10% of the LLOQ response for the qualifier and quantifier ions, the impact of the instrumental response was deemed insignificant.

a. Endogenous Compounds

To evaluate samples for endogenous interferences, a total of ten matrix sources per matrix type (blank blood, antemortem blood, postmortem blood, and urine) were extracted and evaluated without the addition of internal standard. The samples were evaluated for the presence of instrumental response for the analyte and internal standard. No endogenous interferences were identified.

b. Internal Standard

To evaluate potential interferences of internal standard by a high concentration of analyte, samples were fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single matrix sample, per matrix type was evaluated. No interferences from a high concentration of analyte were detected.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, a single matrix sample, per matrix type was fortified with an appropriate concentration of internal standard (10/20/50 ng/mL) without the analyte of interest and analyzed for the absence of response for the analyte. No interferences from internal standard were detected.

c. Commonly Encountered Analytes

Interferences from commonly encountered compounds were evaluated by analyzing three sources of blank matrix fortified with high concentrations of commonly encountered drugs and metabolites. Table 10 depicts the compounds that were assessed for interferences.

Table 10 Commonly encountered analytes

Drug Class	Drug	Concentration
Opioids and Cocaine	Oxymorphone, Hydromorphone, 6-Monoacetylmorphine, Acetylfentanyl, Fentanyl, Benzoylecgonine, Meperidine, Tramadol, Methadone, Morphine, Codeine, Oxycodone, Hydrocodone, Cocaethylene, Cocaine	0.2/2.0/1.0 mg/L
Anti-Epileptic Drugs	Gabapentin, Levetiracetam, Lamotrigine, Zonisamide, 10,11-dihydro-10-hydroxycarbamazepine, Oxcarbazepine, Topiramate, Carbamazepine, Phenytoin, Pregabalin, Lacosamide	0.01 mg/mL
Benzodiazepines	Alprazolam, Clonazepam, Lorazepam, Diazepam, Nordiazepam, Oxazepam, Temazepam, Zolpidem	0.002 mg/mL
NPS	Dibutylone, N-ethyl Pentylone, Tenocyclidine, Clonazolam, 4-Chloro-alpha-PVP, PV8, 6-MAPB, SDB-006, 3-Fluoro AMB, 4-Fluoro AMB, MMB-FUBINACA, MMB-CHMICA, 5F-AB-PINACA, MAB-CHMINICA, ADB-FUBICA, 4F-ADB, 4-APDB, 5-APDB, 6-APDB, MDMB-FUBINACA, 25I-NBOMe, 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOH, 25I-NBF, 25I-NBMD, Pentylone, 3-Methoxy-PCP, Methoxphenidine, Mitragynine, Methiopropamine, 5-DBFPV, 5F-PB-22, AB-FUBINACA, AB-PINACA, 3-Fluoro-phenmetrazine, PB-22	1.0 mg/L
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	0.1 mg/mL
Fentanyls	3-Fluorofentanyl, 4-Methoxybutyrylfentanyl, Acetylfentanyl, Acrylfentanyl, alpha-Methylacetylfentanyl, alpha-Methylfentanyl, Benzodioxolefentanyl, beta-Hydroxythiofentanyl, Butyrylfentanyl, Carfentanil, cis-3-Methylfentanyl, Cyclopropylfentanyl, Despropionylfentanyl, Fentanyl, Furanylfentanyl, Methoxyacetylfentanyl, Ocfentanil, ortho-Fluoroacrylfentanyl, ortho-Fluorobutyrylfentanyl, ortho-Fluorofentanyl, ortho-Fluoroisobutyrylfentanyl, para-Fluoroacrylfentanyl, para-Fluorobutyrylfentanyl, para-Fluorofentanyl, para-Fluoroisobutyrylfentanyl, Phenylfentanyl, Tetrahydrofuranfentanyl, trans-3-Methylfentanyl, U-47700, U-49900, Valerylfentanyl	0.05/0.1 mg/L
Acid/Neutral Drugs	Acetaminophen, Carbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, Glutethimide, Ibuprofen, Levetiracetam, Oxcarbazepine, Phenytoin, Salicylic Acid	0.006 mg/mL
Base Drugs	Amitriptyline, Citalopram, Cyclobenzaprine, Diphenhydramine, Nortriptyline, PCP, Trazodone, Dextromethorphan	0.006 mg/mL
Amphetamines	Amphetamine, Methamphetamine, MDA, MDMA, Bupropion, Phentermine	0.002 mg/mL
Barbiturates	Butalbital, Phenobarbital, Butabarbital Pentobatbital, Secobarbital	0.04 mg/mL

Three sources of blank blood, antemortem blood, postmortem blood, and urine were evaluated for interferences. No interferences from commonly encountered compounds were noted.

Individual cannabinoids were extracted and evaluated for an instrumental response for the target compounds and internal standards within the analytical methods. Table 11 lists the cannabinoid interferences evaluated during the validation.

Table 11 Cannabinoid interferent analysis

Cannabinoids	
(±) Cannabicyclol (CBL)	Cannabigerovarinic Acid (CBGVA)
(6aR,9R)-Δ^{10}-THC	Cannabinol (CBN)
(6aR,9S)-Δ^{10}-THC	Cannabinolic Acid (CBNA)
±cis-Δ^{9}-THC	Cannabivarin (CBV)
9R-$\Delta^{6a,10a}$-THC	exo-THC
9R-Δ^{7}-THC	Tetrahydrocannabivarin (THCV)
9S-$\Delta^{6a,10a}$-THC	Tetrahydrocannabivarinic Acid (THCVA)
9S-Δ^{7}-THC	Δ^{8}-Iso-THC
Cannabichromene (CBC)	Δ^{8}-THC Acetate (Δ^{8}-THC-O-Acetate)
Cannabichromenic Acid (CBCA)	Δ^{8}-Tetrahydrocannabiphorol (Δ^{8}-THCP)
Cannabicyclolic Acid (CBLA)	Δ^{9}-Tetrahydrocannabinolic Acid A
Cannabidiolic Acid (CBDA)	Δ^{9}-THC Acetate (Δ^{9}-THC-O-Acetate)
Cannabidivarin (CBDV)	Δ^{9}-Tetrahydrocannabutol (Δ^{9}-THCB)
Cannabidivarinic Acid (CBDVA)	Δ^{9}-Tetrahydrocannabihexol (Δ^{9}-THCH)
Cannabigerol (CBG)	Δ^{9}-Tetrahydrocannabiorcol (Δ^{9}-THCO)
Cannabigerolic Acid (CBGA)	Δ^{9}-Tetrahydrocannabiphorol (Δ^{9}-THCP)

Cannabinoid interferences were identified with each individual column. Tables 12 and 13 describe the potentially interfering compounds based on qualifier ratio acceptance and instrumental response.

Table 12 Interfering compound summary based on qualifier ratio and retention time

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^{9}-OH-THC		Δ^{8}-OH-THC
Δ^{8}-OH-THC		Δ^{9}-OH-THC
Δ^{9}-Carboxy-THC		Δ^{8}-Carboxy-THC
Δ^{8}-Carboxy-THC		Δ^{9}-Carboxy-THC
Cannabidiol		
Δ^{9}-THC	9S-Δ^{7}-THC	exo-THC
Δ^{8}-THC	9R-Δ^{7}-THC	Δ^{8}-Iso-THC, 9R-Δ^{7}-THC, 9S-Δ^{7}-THC

Table 13 describes the compounds that produced an instrumental response within the retention time acceptance criterion for the target compound. Low instrumental response with poor peak shape was not included in the table. Table 13 includes interferences on either the quantifier transition or the qualifier transition.

Table 13 Interfering instrumental response

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^9-OH-THC	CBDVA	Δ^8-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^8-OH-THC		Δ^9-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^9-Carboxy-THC		Δ^8-Carboxy-THC
Δ^8-Carboxy-THC		Δ^9-Carboxy-THC
Cannabidiol	CBG	
Δ^9-THC	9S-Δ^7-THC, CBL	exo-THC
Δ^8-THC	9R-Δ^7-THC	Δ^8-Iso-THC, 9R-Δ^7-THC, 9S-Δ^7-THC, CBL

In addition to the aforementioned cannabinoids, the following hexahydrocannabinol isomers were evaluated for interferences with the target compounds and internal standards within the analytical method: 8(S)-hydroxy-9(S)-hexahydrocannabinol, 8(R)-hydroxy-9(R)-hexahydrocannabinol, (±)9β-hydroxy hexahydrocannabinol, (±)9α-hydroxy hexahydrocannabinol, (±)9-nor-9α-hydroxyhexahydrocannabinol, and (±)-9-nor-9β-hydroxyhexahydrocannabinol. When evaluating 8(S)-hydroxy-9(S)-hexahydrocannabinol (3.053 min), an instrumental response within the OH-THC-D_3 (3.623 min) internal standard window was observed. The instrumental response was outside of the retention time acceptance ±3% criterion window. For (±)-9-nor-9β-hydroxyhexahydrocannabinol, an instrumental response appears at the same retention time as OH-THC-D_3. The instrumental response was only on the quantifier ion transition and there was no peak present with the qualifier ion transition. This appears only on the C18 analytical method and not the PFP analytical method. Lastly, (±)-9-nor-9α-hydroxyhexahydrocannabinol produced an instrumental response at a retention time within approximately 4.5% of cannabidiol. The qualifier ratios also pass qualifier ion ratio acceptance criterion. This interferent only appears on the C18 analytical method and not the PFP analytical method.

7. Dilution Integrity

The effect of sample dilution on the bias and precision of samples was evaluated using a large volume dilution. When assessing large volume dilution, a pooled blood sample fortified at the highest calibrator concentration (0.1/0.2/0.5 mg/L [Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC]) was prepared. A 500 µL aliquot of matrix was then diluted with blank matrix per sample. Dilution ratios of 1/2 and 1/10 were evaluated for bias and precision per matrix type. The concentration was adjusted depending upon the dilution factor and the adjusted concentration must be within the ±20% of the undiluted target concentration for both bias and precision.

Dilution integrity studies were performed with one source of blank blood, antemortem blood, postmortem blood, and urine. Each sample was injected on the C18 and PFP analytical columns. The average bias associated with OH-THC in each matrix type is shown in Table 14.

Table 14 Dilution integrity bias OH-THC

Dilution Bias %Bias; n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)
Blank Blood	-0.17	5.07	0.00	-2.28	2.30	0.67
Antemortem Blood	10.28	19.37	-10.83	9.53	18.15*	-6.50
Postmortem Blood	2.67	13.03	9.50	-0.85	11.60	12.67
Urine	0.68	4.23	-11	-2.77	1.57	-11.83

*n=2

All dilutions were within the predetermined acceptance criterion for bias. The largest bias was observed with a 1/2 dilution of antemortem blood when analyzed on the C18 column. The observed bias was 19.37%. The respective sample on the PFP column produced a bias of 18.15%. It was noted that only two replicates were evaluated on the PFP column due to inadequate sample volume in one of the replicate samples. The average bias associated with carboxy-THC in each matrix type is shown in Table 15.

Table 15 Dilution integrity bias carboxy-THC

Dilution Bias %Bias; n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.5 mg/L)	1/2 (0.25 mg/L)	1/10 (0.05 mg/L)	Undiluted (0.5 mg/L)	1/2 (0.25 mg/L)	1/10 (0.05 mg/L)
Blank Blood	-0.79	-0.52	-8.20	-3.74	1.93	-1.73
Antemortem Blood	0.85	8.17	-12.20	1.49	8.74*	-7.60
Postmortem Blood	-5.09	7.56	5.13	-4.44	9.81	10.67
Urine	-2.43	3.85	-9.80	-0.73	4.33	-7.33

*n=2

All dilutions were within the predetermined acceptance criterion for bias. The largest bias observed was -12.20% with the 1/10 dilution of antemortem blood when analyzed on the C18 analytical column. The average bias associated with cannabidiol in each matrix type is shown in Table 16.

Table 16 Dilution integrity bias cannabidiol

Dilution Bias %Bias; n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)
Blank Blood	0.73	6.17	-1.17	-1.67	4.13	-2.67
Antemortem Blood	5.03	24.60	-13.50	9.85	25.35*	-12.67
Postmortem Blood	-0.27	12.33	6.67	-0.03	12.87	6.50
Urine	-9.33	-0.23	-16.50	-6.47	-0.10	-13.83

*n=2

All dilutions with the exception of the 1/2 dilution of antemortem blood was within the predetermined acceptance criterion for bias. Both analytical columns had an observed bias greater than ±20%. Therefore, antemortem blood shall not be diluted for the quantitative analysis of cannabidiol. The average bias associated with Δ^9-THC in each matrix type is shown in Table 17.

Table 17 Dilution integrity bias Δ^9-THC

Dilution Bias %Bias; n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)
Blank Blood	-1.30	-1.33	-5.00	-3.07	2.13	-1.33
Antemortem Blood	0.87	16.07	-10.33	-0.13	14.80*	-9.00
Postmortem Blood	-3.33	10.53	6.00	-6.83	9.40	4.33
Urine	-17.23	-10.40	-21.00	-12.87	-7.93	-24.00

*n=2

All dilutions with the exception of the 1/10 dilution of urine was within the predetermined acceptance criterion for bias. Both analytical columns had an observed bias greater than ±20%. Therefore, urine shall not be diluted greater than 1/2 for quantitative analysis of Δ^9-THC. The average bias associated with Δ^8-THC in each matrix type is shown in Table 18.

Table 18 Dilution integrity bias Δ^8-THC

Dilution Bias %Bias; n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)
Blank Blood	4.27	1.13	-6.00	-7.70	10.80	7.33
Antemortem Blood	0.27	20.40	-9.00	19.00	37.90*	2.00
Postmortem Blood	1.03	10.67	3.33	-2.23	23.53	28.00
Urine	-14.73	-9.93	-23.67	-16.63	-11.47	-22.67

*n=2

When evaluating Δ^8-THC for bias during dilution, the PFP analytical column presented several instances where the bias exceeded the ±20% acceptance criterion. Blank blood was the only matrix within the acceptance criteria for all dilution ratios. Both antemortem blood and postmortem blood exceeded ±20% for the 1/2 dilution. Postmortem blood also exceeded the ±20% bias acceptance criterion for a dilution ratio of 1/10. Urine exceeded the acceptable tolerance for bias at a 1/10 dilution ratio with a bias of -22.67%. Therefore, antemortem blood and postmortem blood shall not be diluted for the quantitative analysis of Δ^8-THC on the PFP analytical column. Additionally, urine shall be diluted with no more than a 1/2 dilution for the quantitative analysis of Δ^8-THC on the PFP analytical column.

When evaluating the C18 analytical column, urine at a 1/10 dilution also exceeded the predetermined acceptance criterion for bias with a bias of -23.67%. All other matrices were within the predetermined acceptance criterion for bias for all dilution ratios.

In addition to an evaluation of bias with common dilution ratios, the precision of the replicate analyses was also evaluated. The data used for bias was also utilized in the evaluation of precision. The precision was calculated for each matrix type undiluted, with a 1/2 dilution, and with a 1/10 dilution. The precision associated with OH-THC in each matrix type is shown in Table 19.

Table 19 Dilution integrity precision OH-THC

Dilution Precision Mean±SD(%CV); n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)
Blank Blood	0.200±0.002(1)	0.105±0.002(2)	0.020±0.002(9)	0.195±0.004(2)	0.102±0.002(1)	0.020±0.001(6)
Antemortem Blood	0.221±0.005(2)	0.119±0.002(2)	0.018±0.000(2)	0.219±0.003(2)	0.118±0.005(4)	0.019±0.000(1)
Postmortem Blood	0.205±0.016(8)	0.113±0.006(5)	0.022±0.000(1)	0.198±0.014(7)	0.112±0.005(4)	0.023±0.000(1)
Urine	0.201±0.006(3)	0.104±0.001(1)	0.018±0.000(2)	0.194±0.002(1)	0.102±0.000(1)	0.018±0.000(1)

All dilutions were less than the predetermined acceptance criterion for precision for both analytical columns. The largest precision observed was 9% which was for the 1/10 dilution of blank blood when using the C18 analytical column. The precision associated with carboxy-THC in each matrix type is shown in Table 20.

Table 20 Dilution integrity precision carboxy-THC

Dilution Precision Mean±SD(%CV); n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.5 mg/L)	1/2 (0.25 mg/L)	1/10 (0.05 mg/L)	Undiluted (0.5 mg/L)	1/2 (0.25 mg/L)	1/10 (0.05 mg/L)
Blank Blood	0.496±0.015(3)	0.249±0.010(4)	0.046±0.003(7)	0.481±0.007(1)	0.255±0.006(2)	0.049±0.005(11)
Antemortem Blood	0.504±0.003(1)	0.270±0.001(1)	0.044±0.001(1)	0.507±0.004(1)	0.272±0.010(4)	0.046±0.001(2)
Postmortem Blood	0.475±0.035(7)	0.269±0.012(5)	0.053±0.001(2)	0.478±0.030(6)	0.275±0.011(4)	0.055±0.001(1)
Urine	0.488±0.009(2)	0.260±0.002(1)	0.045±0.000(1)	0.496±0.007(1)	0.261±0.001(1)	0.046±0.001(2)

All dilutions were less than the predetermined acceptance criterion for precision for both analytical columns. The largest precision observed was 11% which was for the 1/10 dilution of blank blood when using the PFP analytical column. The precision associated with cannabidiol in each matrix type is shown in Table 21.

Table 21 Dilution integrity precision cannabidiol

Dilution Precision Mean±SD(%CV); n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)	Undiluted (0.2 mg/L)	1/2 (0.1 mg/L)	1/10 (0.02 mg/L)
Blank Blood	0.201±0.004(2)	0.106±0.006(5)	0.020±0.002(8)	0.197±0.006(3)	0.104±0.002(2)	0.019±0.002(10)
Antemortem Blood	0.210±0.005(3)	0.125±0.000(1)	0.017±0.001(5)	0.220±0.007(3)	0.125±0.002(1)	0.017±0.001(4)
Postmortem Blood	0.199±0.016(8)	0.112±0.006(5)	0.021±0.000(1)	0.200±0.013(7)	0.113±0.005(4)	0.021±0.001(3)
Urine	0.181±0.003(2)	0.100±0.003(3)	0.017±0.000(1)	0.187±0.001(1)	0.100±0.002(2)	0.017±0.000(3)

All dilutions for each matrix type were within the predetermined acceptance criterion for precision. The greatest precision observed was the 1/10 dilution of blank blood. The observed precision was 10%. The precision associated with Δ^9-THC in each matrix type is shown in Table 22.

Table 22 Dilution integrity precision Δ^9-THC

Dilution Precision Mean±SD(%CV); n=3						
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)
Blank Blood	0.099±0.002(2)	0.049±0.001(1)	0.010±0.001(7)	0.097±0.001(1)	0.051±0.001(2)	0.010±0.001(7)
Antemortem Blood	0.101±0.003(3)	0.058±0.000(1)	0.009±0.000(4)	0.100±0.003(3)	0.057±0.001(1)	0.009±0.000(1)
Postmortem Blood	0.097±0.006(6)	0.055±0.003(6)	0.011±0.000(2)	0.093±0.007(7)	0.055±0.003(5)	0.010±0.000(1)
Urine	0.083±0.002(2)	0.045±0.001(2)	0.008±0.000(3)	0.087±0.001(1)	0.046±0.000(1)	0.008±0.000(5)

The largest precision observed was 7%. This was observed with the 1/10 dilution of blank blood on both the C18 and PFP analytical columns. All dilutions for all matrix types were within the predetermined acceptance criterion for precision. The precision associated with Δ^8-THC in each matrix type is shown in Table 23.

Table 23 Dilution integrity precision Δ^8-THC

	Dilution Precision Mean±SD(%CV); n=3					
	C18 Column			PFP Column		
Matrix Type	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)	Undiluted (0.1 mg/L)	1/2 (0.05 mg/L)	1/10 (0.01 mg/L)
Blank Blood	0.104±0.004(4)	0.051±0.001(3)	0.009±0.001(6)	0.092±0.001(1)	0.055±0.009(16)	0.011±0.001(6)
Antemortem Blood	0.100±0.003(3)	0.060±0.001(2)	0.009±0.001(6)	0.119±0.006(5)	0.069±0.000(1)	0.010±0.000(3)
Postmortem Blood	0.101±0.007(7)	0.055±0.005(9)	0.010±0.001(5)	0.098±0.007(7)	0.062±0.001(2)	0.013±0.000(2)
Urine	0.085±0.002(2)	0.045±0.001(2)	0.008±0.000(3)	0.083±0.003(3)	0.044±0.001(3)	0.008±0.001(7)

The largest observed precision was 16% which was associated with the 1/2 dilution of blank blood when using the PFP analytical column. All dilutions for all matrices met the predetermined acceptance criterion of 20%.

8. Stability

The stability of extracted samples that were not analyzed immediately was evaluated at two concentrations (5/10/25 ng/mL [Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC] and 50/100/250 mg/L) for each matrix type (blank blood, antemortem blood, postmortem blood, and urine). The samples were extracted and injected immediately in triplicate to establish the Day 1 instrumental response. Both concentration levels were subsequently injected in triplicate every twenty-four hours over a six-day period. It was intended to evaluate the stability for a seven-day period but due to sample evaporation the stability study ended after six days. Further, the stability for the PFP analytical column was only evaluated for five days due to sample evaporation. The stability study was performed in a cooled autosampler that was maintained at approximately 4°C to minimize evaporation.

The instrumental response was compared for each time point. If the average instrumental response decreased below 80% or increased above 120% of the average Day 1 response, then the target was considered unstable after that time. Table 24 shows the stability for both analytical columns at low and high concentrations for OH-THC in each matrix type.

Table 24 OH-THC stability study

Stability Study OH-THC Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	119	115	115	130	109	100	81	62	63	72	-
Antemortem Blood	100	102	100	117	82	132	100	92	91	90	110	-
Postmortem Blood	100	84	90	88	93	104	100	85	95	82	101	-
Urine	100	115	112	116	125	120	100	97	85	118	108	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	94	96	99	104	100	100	91	53	79	79	-
Antemortem Blood	100	105	106	113	117	127	100	93	125	118	154	-
Postmortem Blood	100	99	101	121	108	147	100	79	81	80	92	-
Urine	100	106	104	105	114	108	100	115	118	134	134	-

When evaluating the stability of the C18 column, OH-THC was stable for three days in postmortem blood, four days for blank blood and urine, and five days for antemortem blood. The stability when using the PFP column was slightly different than the C18 column. At the low concentration, antemortem blood, postmortem blood, and urine were all stable for five days. Blank blood was stable for two days. At the high concentration, blank blood and antemortem blood were stable for two days while postmortem blood was only stable for one day. Urine was stable for three days. The stability of carboxy-THC is shown in Table 25.

Table 25 Carboxy-THC stability study

Stability Study Carboxy-THC Deviation(%); n=3												
25 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	132	148	146	161	135	100	96	68	72	80	-
Antemortem Blood	100	87	89	119	63	122	100	94	97	98	120	-
Postmortem Blood	100	83	83	82	88	102	100	92	109	92	116	-
Urine	100	107	107	111	121	116	100	99	83	115	106	-
250 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	93	99	100	106	109	100	96	53	87	80	-
Antemortem Blood	100	90	92	107	106	100	100	99	138	126	168	-
Postmortem Blood	100	91	92	114	101	131	100	77	80	78	91	-
Urine	100	106	106	103	115	106	100	128	128	144	147	-

The stability of carboxy-THC showed similar variability as the OH-THC stability. The instrumental response was variable causing observed fluctuations in stability. The PFP analytical column demonstrated less stability than the C18 column in most matrices. Table 23 shows the stability of cannabidiol with both analytical columns at low and high concentrations.

Table 26 Cannabidiol stability study

Stability Study Cannabidiol Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	107	106	109	118	96	100	72	55	54	61	-
Antemortem Blood	100	100	98	111	76	124	100	87	86	90	100	-
Postmortem Blood	100	96	94	93	100	107	100	74	83	70	89	-
Urine	100	113	110	105	114	115	100	88	73	96	87	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	99	99	100	106	99	100	73	46	68	65	-
Antemortem Blood	100	106	106	108	112	123	100	88	113	109	136	-
Postmortem Blood	100	106	107	116	111	137	100	71	70	69	78	-
Urine	100	100	96	96	102	98	100	113	114	124	123	-

Cannabidiol appears to be more stable with the C18 column compared to the PFP column. This is a presumed stability based on the observation of instrumental response of the target compounds. The same sample was injected on both columns during the stability study. Therefore, this presumed instability is truly variability in the instrumental response of the instrument and does not indicate that the sample/compound is deteriorating. The stability of Δ^9-THC is shown in Table 27.

Table 27 Δ^9-THC stability study

Stability Study Δ^9-THC Deviation(%); n=3												
5 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	105	112	111	119	100	100	74	73	74	83	-
Antemortem Blood	100	101	96	105	66	112	100	88	90	95	104	-
Postmortem Blood	100	104	103	100	101	110	100	77	74	71	82	-
Urine	100	110	108	106	110	107	100	92	88	99	104	-
50 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	104	107	105	109	104	100	84	78	81	89	-
Antemortem Blood	100	106	106	104	109	123	100	95	96	98	107	-
Postmortem Blood	100	107	105	108	107	123	100	84	80	83	89	-
Urine	100	101	97	95	98	96	100	96	92	102	105	-

Both Δ^9-THC and Δ^8-THC were the most stable of the compounds when evaluated on the C18 analytical column. Although presumed stable on the C18 analytical column, the PFP analytical column often only had a stability of one to two days. The stability data using the instrumental response for Δ^8-THC is shown in Table 28.

Table 28 Δ^8-THC stability study

Stability Study Δ^8-THC Deviation(%); n=3												
5 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	106	105	114	94	100	72	76	73	86	-
Antemortem Blood	100	99	102	102	68	115	100	91	91	98	121	-
Postmortem Blood	100	102	102	98	104	107	100	83	83	75	84	-
Urine	100	114	113	106	113	105	100	96	94	104	112	-
50 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	102	102	108	101	100	81	74	79	87	-
Antemortem Blood	100	104	103	100	105	116	100	93	96	99	110	-
Postmortem Blood	100	107	104	106	106	121	100	89	87	89	94	-
Urine	100	101	100	94	98	95	100	98	96	105	110	-

The stability of the internal standards within the method were also evaluated. This evaluation was performed using the instrumental response of the internal standard in each sample. Tables 29, 30, 31, 32 show the stability of each internal standard.

Table 29 OH-THC-D_3 stability study

Stability Study OH-THC-D_3 Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	116	115	117	132	110	100	79	58	60	69	-
Antemortem Blood	100	104	102	122	78	132	100	89	93	92	111	-
Postmortem Blood	100	83	86	87	88	107	100	84	95	80	100	-
Urine	100	112	115	115	126	125	100	94	80	107	103	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	95	96	99	105	101	100	91	51	78	79	-
Antemortem Blood	100	105	106	115	119	130	100	93	127	117	156	-
Postmortem Blood	100	100	101	121	107	153	100	79	81	79	90	-
Urine	100	112	122	120	131	127	100	117	120	133	133	-

Table 30 Carboxy-THC-D_3 stability study

Stability Study Carboxy-THC-D_3 Deviation(%); n=3												
25 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	130	143	142	157	135	100	95	66	66	78	-
Antemortem Blood	100	92	91	122	68	129	100	92	98	97	120	-
Postmortem Blood	100	79	80	83	85	99	100	85	100	83	105	-
Urine	100	110	109	114	122	122	100	98	80	112	105	-
250 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	88	89	90	97	97	100	99	51	86	81	-
Antemortem Blood	100	93	94	109	111	100	100	97	136	126	168	-
Postmortem Blood	100	92	95	118	102	131	100	77	79	78	94	-
Urine	100	121	122	120	134	121	100	128	131	144	149	-

Table 31 Cannabidiol-D_3 stability study

Stability Study Cannabidiol-D_3 Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	109	108	108	121	98	100	69	52	51	58	-
Antemortem Blood	100	105	105	120	77	132	100	83	86	84	98	-
Postmortem Blood	100	96	95	98	99	112	100	75	90	74	88	-
Urine	100	109	104	106	110	108	100	91	72	101	89	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	99	99	100	105	99	100	73	44	66	64	-
Antemortem Blood	100	104	104	104	111	121	100	91	114	109	137	-
Postmortem Blood	100	107	106	119	111	137	100	70	71	72	80	-
Urine	100	116	116	114	120	113	100	117	116	129	127	-

Table 32 Δ^9-THC-D_3 stability study

Stability Study Δ^9-THC-D_3 Deviation(%); n=3												
5 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	108	112	112	123	103	100	75	74	72	83	-
Antemortem Blood	100	100	103	107	66	117	100	86	87	87	99	-
Postmortem Blood	100	101	102	97	103	109	100	84	85	81	88	-
Urine	100	113	109	103	112	107	100	91	87	99	104	-
50 ng/mL												

	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	103	104	104	110	104	100	82	73	79	85	-
Antemortem Blood	100	106	112	106	110	124	100	88	90	91	99	-
Postmortem Blood	100	104	104	106	105	121	100	84	81	82	89	-
Urine	100	119	114	107	110	112	100	96	94	102	109	-

Given the variability in instrumental response during the stability study, the data was normalized to the internal standard response to provide specific detail regarding the impacts of stability. The instrumental response (peak area) of the compound of interest was ratioed with the respective internal standard instrumental response. Table 33 shows the deviation of the ratioed data for each day within the stability study.

Table 33 OH-THC stability study

Stability Study OH-THC Ratio Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	103	100	98	98	100	100	103	107	104	104	-
Antemortem Blood	100	98	97	96	104	100	100	103	98	98	99	-
Postmortem Blood	100	101	105	101	106	98	100	101	100	103	101	-
Urine	100	103	98	101	100	96	100	103	106	105	105	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	100	100	100	99	99	100	100	105	101	101	-
Antemortem Blood	100	100	100	98	98	98	100	100	98	101	99	-
Postmortem Blood	100	99	100	100	101	96	100	100	101	101	102	-
Urine	100	86	86	88	87	85	100	98	98	101	100	-

When evaluating the ratio of analyte to internal standard, OH-THC was determined to be stable for six days using the C18 analytical column and five days for the PFP analytical column. There were no indications that the samples would be unstable on day six of the stability study for the PFP column. The sample was evaporated and unable to inject. Tables 34, 35, 36, and 37 describe the ratioed stability for the remaining compounds.

Table 34 Carboxy-THC stability study

Stability Study Carboxy-THC Ratio Deviation(%); n=3												
25 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	102	103	103	102	100	100	101	102	109	103	-
Antemortem Blood	100	95	98	97	92	95	100	102	99	101	100	-
Postmortem Blood	100	105	104	98	104	104	100	109	108	111	110	-
Urine	100	97	98	97	99	95	100	101	104	102	101	-
250 ng/mL												

	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	106	111	111	109	113	100	96	104	101	100	-
Antemortem Blood	100	97	98	98	96	100	100	102	101	100	100	-
Postmortem Blood	100	98	97	96	99	100	100	100	101	99	97	-
Urine	100	88	87	86	85	87	100	100	97	99	99	-

Table 35 Cannabidiol stability study

Stability Study Cannabidiol Ratio Deviation(%); n=3												
10 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	100	98	98	100	105	107	106	105	-
Antemortem Blood	100	95	93	92	99	94	100	105	99	107	102	-
Postmortem Blood	100	100	100	95	101	96	100	98	92	94	101	-
Urine	100	104	105	99	103	106	100	98	102	95	98	-
100 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	100	100	100	100	99	100	100	104	102	101	-
Antemortem Blood	100	102	102	103	101	102	100	97	99	99	99	-
Postmortem Blood	100	99	101	98	101	100	100	102	98	96	97	-
Urine	100	86	83	84	85	87	100	97	98	96	97	-

Table 36 Δ^9-THC stability study

Stability Study Δ^9-THC Ratio Deviation(%); n=3												
5 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	99	99	97	97	100	99	99	103	100	-
Antemortem Blood	100	101	93	98	100	96	100	103	104	108	105	-
Postmortem Blood	100	103	101	102	99	101	100	93	88	87	93	-
Urine	100	98	99	103	98	100	100	101	101	100	100	-
50 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	101	102	101	100	101	100	103	107	103	105	-
Antemortem Blood	100	100	95	98	99	100	100	109	106	108	109	-
Postmortem Blood	100	103	102	101	102	102	100	100	99	101	100	-
Urine	100	85	85	89	88	86	100	100	98	100	97	-

Table 37 Δ^8-THC stability study

Stability Study Δ^8-THC Ratio Deviation(%); n=3												
5 ng/mL												
	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	94	94	94	93	92	100	95	103	101	104	-
Antemortem Blood	100	99	99	95	102	98	100	106	105	112	122	-
Postmortem Blood	100	101	100	101	102	98	100	99	97	93	96	-
Urine	100	101	103	103	101	98	100	105	109	106	108	-
50 ng/mL												

	C18 Analytical Column						PFP Analytical Column					
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Blank Blood	100	98	98	98	98	98	100	99	101	101	102	-
Antemortem Blood	100	98	92	94	96	94	100	106	106	109	112	-
Postmortem Blood	100	103	100	100	101	100	100	106	107	109	106	-
Urine	100	85	87	88	89	85	100	102	103	103	102	-

All compounds were stabile for six days using the C18 analytical column. Urine demonstrated the largest drift in ratioed response producing deviations nearing 80% for the high concentration sample for all analytes. All compounds were also determined to be stable for five days using the PFP analytical column except for Δ^8-THC in antemortem blood. The ratioed response increased above 120% for antemortem blood on day five. Therefore, the Δ^8-THC when in antemortem blood was determined to be stable for 4 days.

9. Robustness

Analysis for the validation was completed on two instrument models to capture the variability between instrumentation. Agilent Technologies 6460 and 6470 LCMSMS instruments were utilized during validation. Further, care was taken to ensure that critical experiments such as calibration model and limit of detection samples were evaluated using all instrument models.

10. Summary

The cannabinoids evaluated within the comprehensive quantitative validation included OH-THC, carboxy-THC, cannabidiol, Δ^9-THC, and Δ^8-THC. The matrices evaluated included blank blood, antemortem blood, postmortem blood, and urine. Within the validation, urine was assessed qualitatively and was not evaluated during bias and precision experiments. Although quantitation is not intended to be performed on both analytical columns, all experiments within the quantitative validation were performed on both analytical columns including the C18 and PFP columns. Further, during validation, multiple lot numbers of SLE cartridges were used. An interferent with the qualifier transition for Δ^8-THC from the SLE cartridges was identified. This interferent was not always observed on both analytical columns and did not have an impact on the quantifier transition for Δ^8-THC. The signal response associated with the interferent was variable from cartridge to cartridge and not always observed when using the PFP analytical column.

All blood matrix sources evaluated (blank blood, antemortem blood, postmortem blood) passed the predetermined acceptance criterion for bias and precision. The estimated limit of detection was established by being evaluated at two concentrations that were lower than the lower limit of detection. The concentrations within this evaluation included 0.75/1.5/3.75 ng/mL and 0.5/1/2.5 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC). The only compound that passed the predetermined identification was carboxy-THC at a concentration of 2.5 ng/mL. All other compounds did not meet the predetermined identification criteria for blank blood and therefore the estimated limit of detection was adjusted to be equal to the lower limit of quantitation.

Nine matrix sources per matrix type were fortified at the lower limit of quantitation in triplicate. The evaluation was performed over three analyses. The lowest calibrator concentration was 1/2/5 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC) for all matrices with the exception of postmortem blood. Postmortem blood was fortified at 2/4/5 ng/mL (Δ^9-THC, Δ^8-THC/OH-THC, cannabidiol/carboxy-THC). OH-THC passed bias acceptance criteria for all replicates in all matrices. The qualifier ion ratio acceptance criterion was extended to ±30% for OH-THC. With this change, only one qualifier ion ratio failure was observed. Carboxy-THC passed all lower limit of quantitation acceptance criteria for all matrix types. Δ^9-THC passed bias acceptance criteria and only one qualifier ion ratio failure was observed for all replicates in all matrices. Δ^8-THC and cannabidiol had several bias and qualifier ion ratio failures during the evaluation of the lower limit of quantitation.

The best fit calibration model for all target compounds with the method was determined to be quadratic weighted 1/x. In addition to the determination of the best fit calibration model a comparison of the different matrices evaluated in the method were assessed. Calibration curves from antemortem blood, postmortem blood, and urine were compared to blank blood. No changes were observed in the best fit calibration model indicating the appropriateness of using blank blood for establishing the calibration within an analytical run.

Ionization suppression and enhancement was evaluated, and significant ionization suppression was noted on both analytical columns for multiple matrix types and analytes. This identification of ionization suppression prompted additional matrix sources to be evaluated for the estimated limit of detection and lower limit of quantitation. The stability of the analytes post extraction was evaluated using both the C18 and PFP analytical columns. Given the inherent observed evaporation of sample, raw instrumental response was not an appropriate assessment tool for stability. Rather, the ratioed instrumental response of target compound and internal standard was used to assess stability. All compounds were determined to be stable for six days using the C18 analytical column. All compounds were stable for five days using the PFP analytical column except for Δ^8-THC in antemortem blood which was determined to be stable for four days.

The validation criteria for the quantitation for OH-THC, carboxy-THC, and Δ^9-THC have been met in blank blood, antemortem blood, and postmortem blood. The presence of a Δ^8-THC qualifier ion transition interferent significantly impacted the lower limit of quantitation causing ion ratio failures. The interferent was identified to be a component of the SLE column. Once mitigated, verification experiments shall be performed. Based on the validation data, Δ^8-THC should be qualitative only. The evaluation of cannabinoids in urine should be qualitative only. The evaluation of cannabidiol should be qualitative only. Liver was not assessed within this validation.

All data from the validation has been stored on the DTSResearch Shared Drive.

11. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 26, **2023**.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 26, **2023**.

Herr, D., Siddiqi, A., Wagner, R. Cannabinoids quantitation and confirmation by LCMSMS method development. Virginia Department of Forensic Science. **2022**.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. **2019**.

ANSI/ASB 098 Standard, Standard for Mass Spectral Analysis in Forensic Toxicology. 1st Edition. **2023**.

Appendix A: Calibration Curve in Blank Blood Regression Analysis

Calibration Curve in Blank Blood Regression Analysis

OH-THC: quadratic-weighted (1/x) calibration model using C18 analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $8.6995 \times 10^{-6} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 23.6509 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for OH-THC on the C18 analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $2.9267 \times 10^{-7} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for OH-THC using the C18 analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2338 and non-weighted 0.3939).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for OH-THC. Charts 1-4 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for OH-THC respectively.

Chart 1

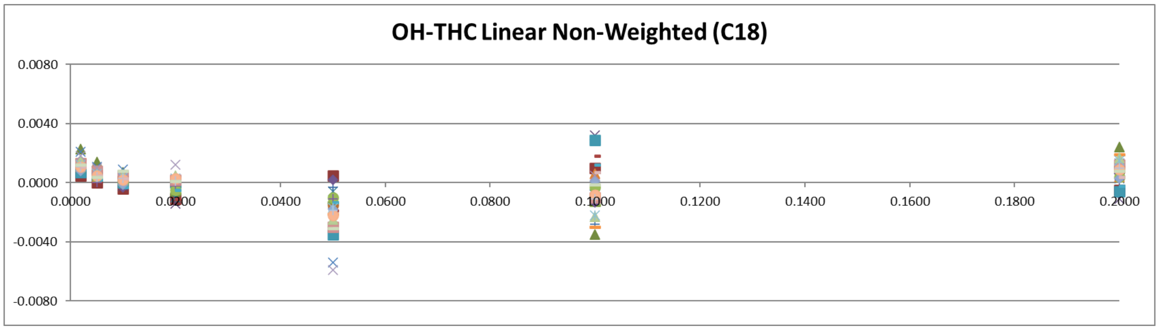


Chart 2

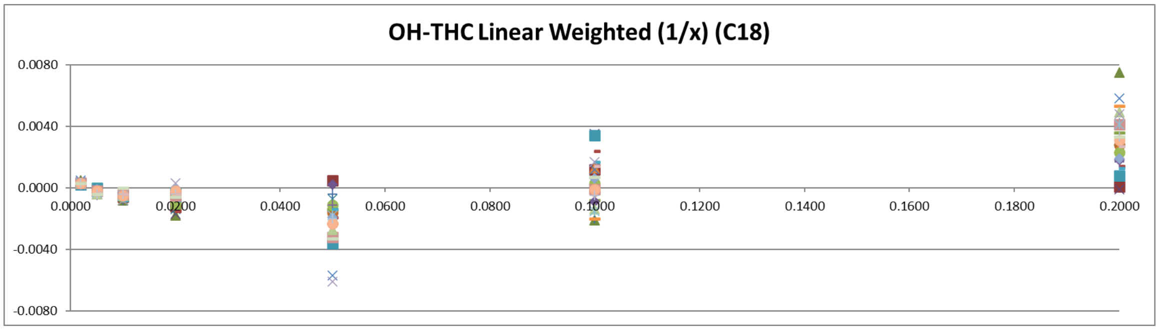


Chart 3

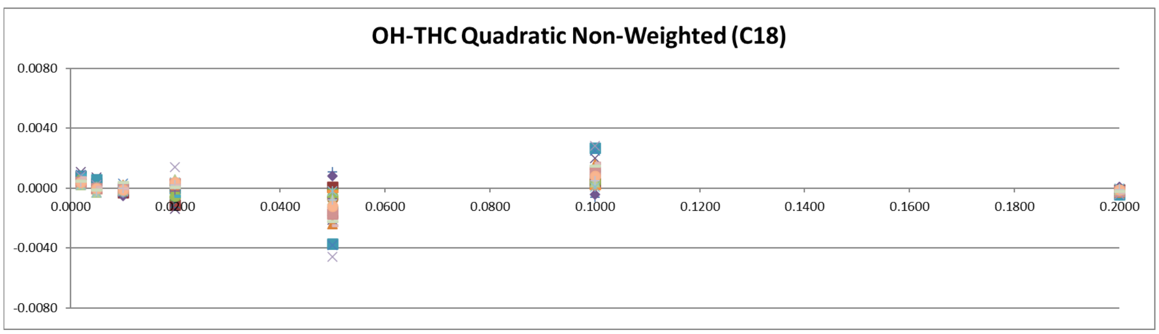


Chart 4

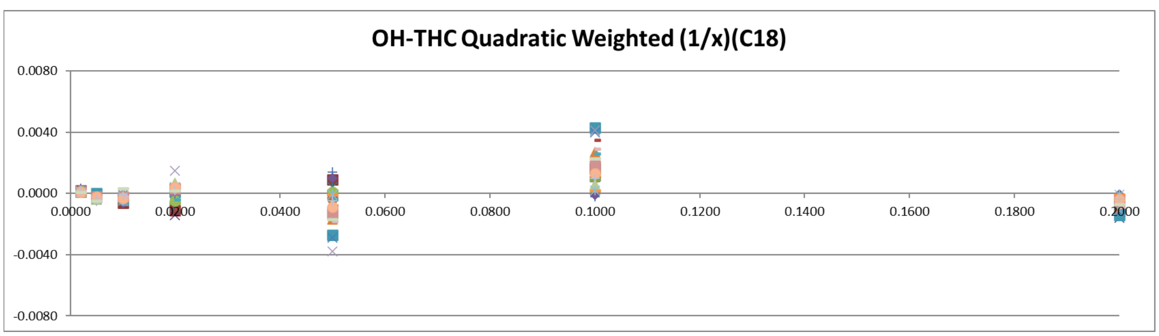


Figure 1 represents a quadratic weighted (1/x) calibration curve for OH-THC on the C18 analytical column with a dynamic range of 2 ng/mL to 200 ng/mL.

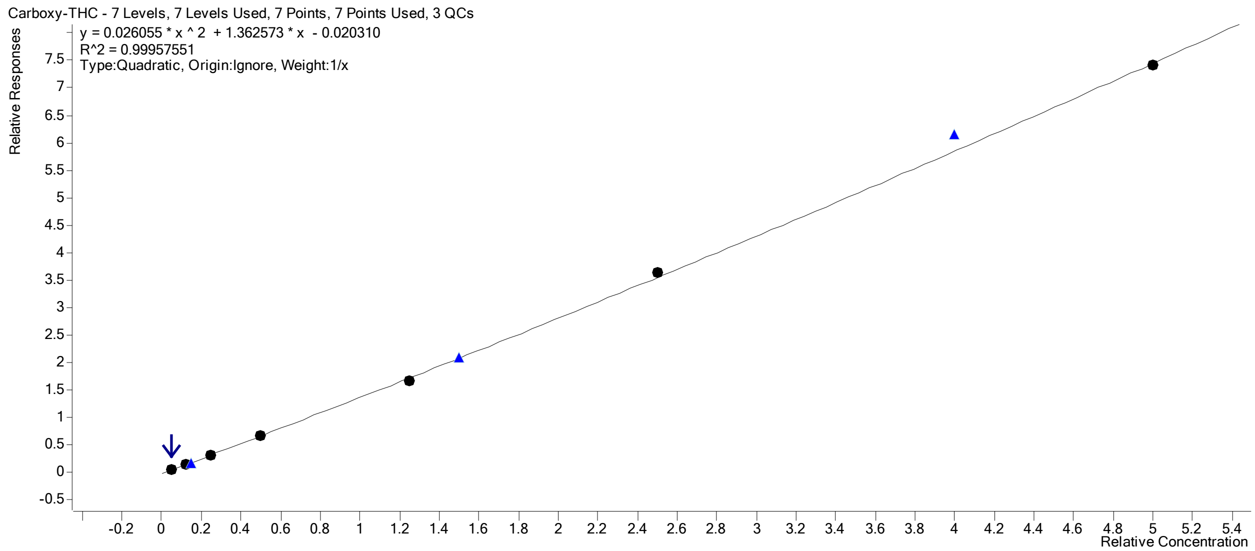


Figure 1 OH-THC calibration curve with C18 analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99927470 and the origin was ignored.

OH-THC: quadratic-weighted (1/x) calibration model using PFP analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $5.7234 \times 10^{-6} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

$F = 24.7840 > 4.0012$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for OH-THC on the PFP analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $2.3603 \times 10^{-6} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for OH-THC using the PFP analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2206 and non-weighted 0.3665).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for OH-THC. Charts 5-8 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for OH-THC respectively.

Chart 5

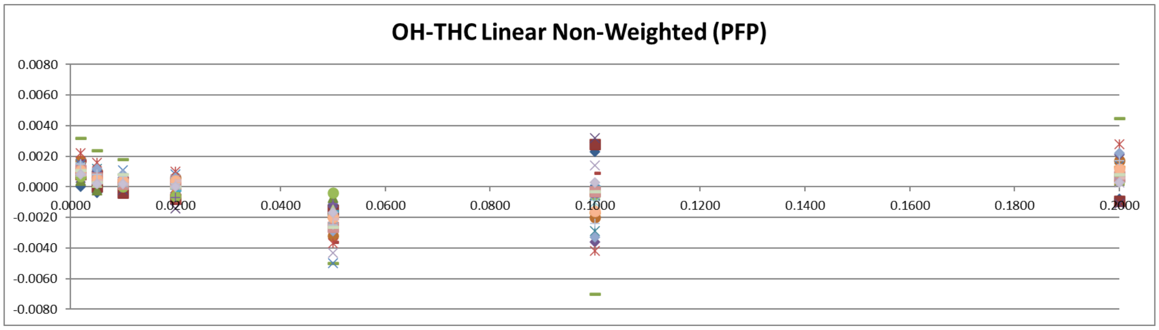


Chart 6

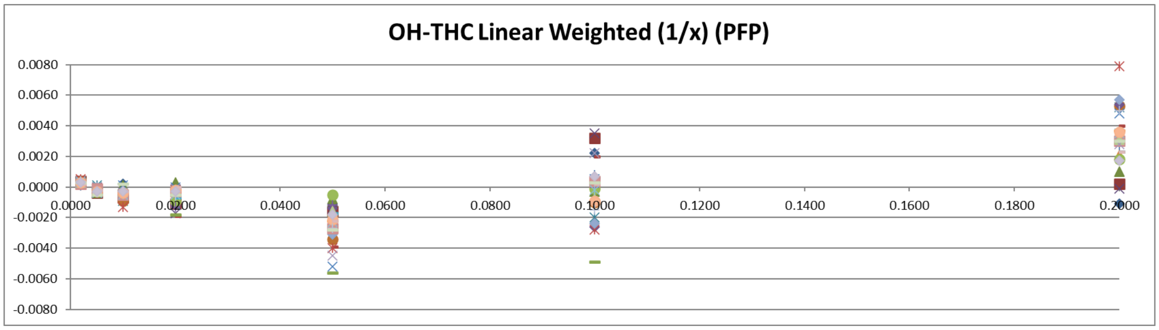


Chart 7

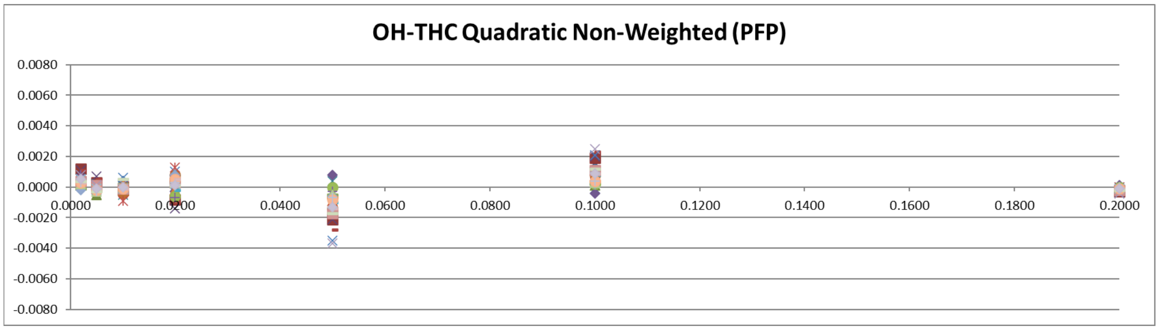


Chart 8

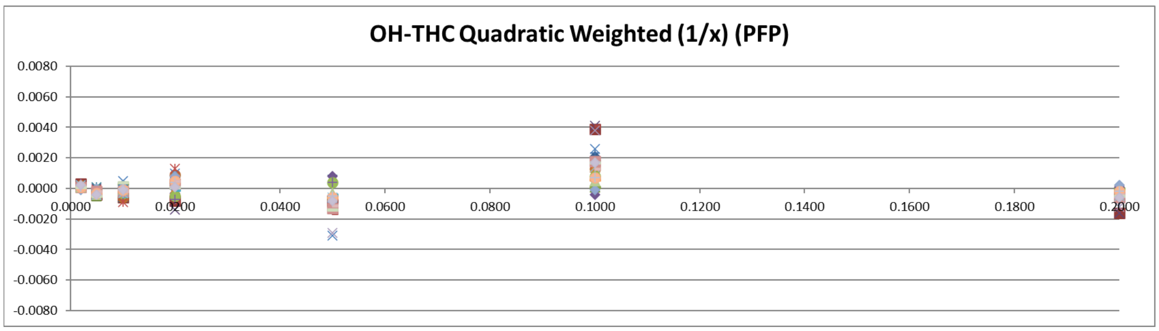


Figure 2 represents a quadratic weighted (1/x) calibration curve for OH-THC on the PFP analytical column with a dynamic range of 2 ng/mL to 200 ng/mL.

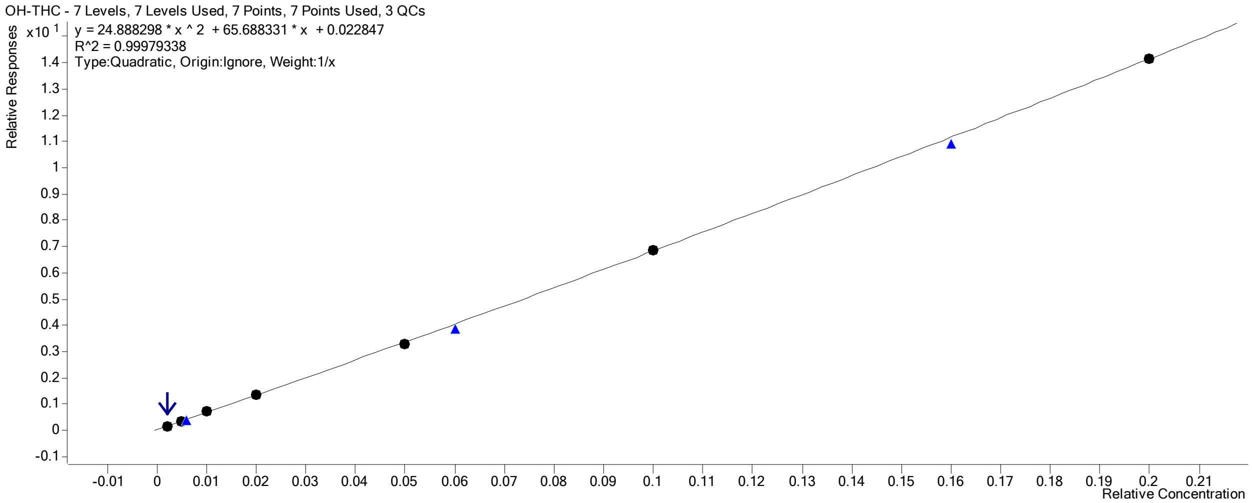


Figure 2 OH-THC calibration curve with PFP analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99979338 and the origin was ignored.

Carboxy-THC: quadratic-weighted (1/x) calibration model using C18 analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $4.3827 \times 10^{-8} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 39.2741 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for carboxy-THC on the C18 analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $6.2030 \times 10^{-10} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for carboxy-THC using the C18 analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2516 and non-weighted 0.4765).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for carboxy-THC. Charts 9-12 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for carboxy-THC respectively.

Chart 9

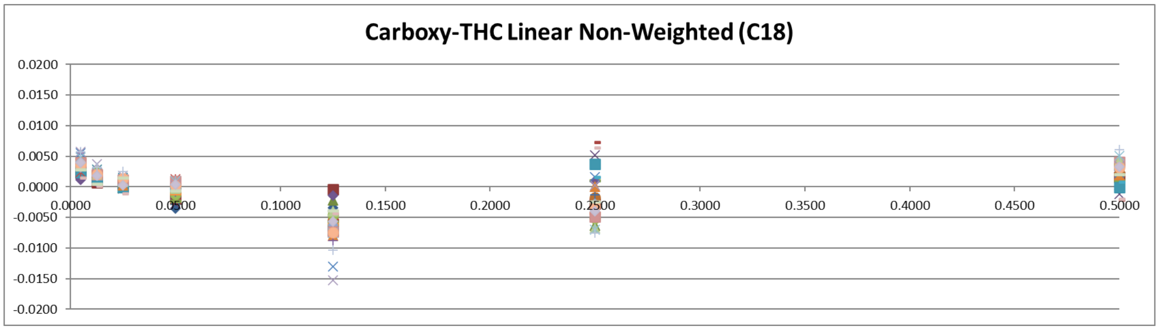


Chart 10

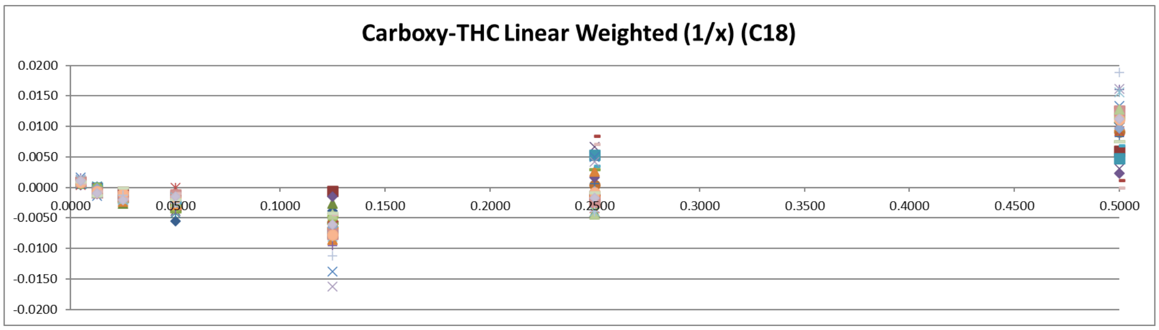


Chart 11

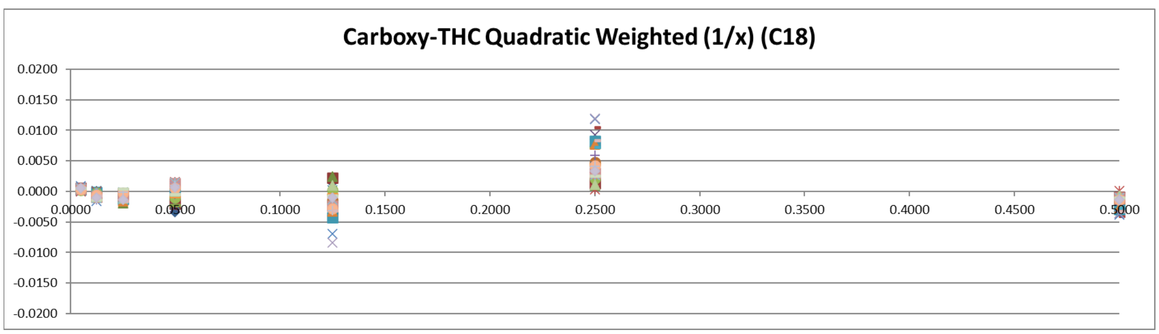


Chart 12

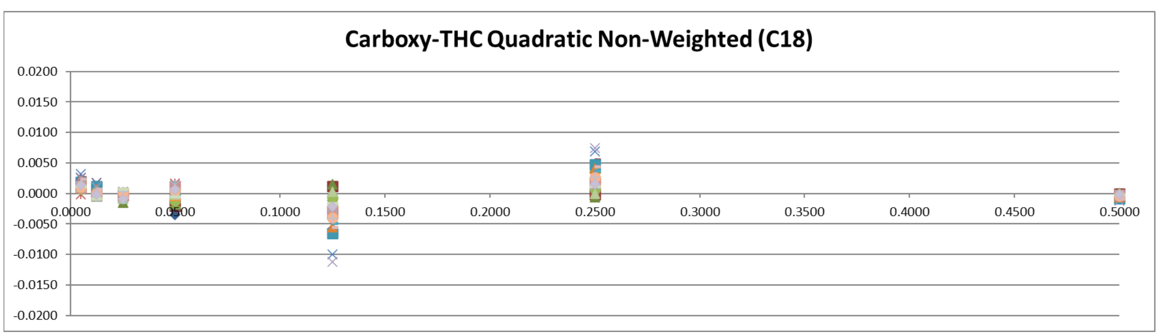


Figure 3 represents a quadratic weighted (1/x) calibration curve for carboxy-THC on the C18 analytical column with a dynamic range of 5 ng/mL to 500 ng/mL.

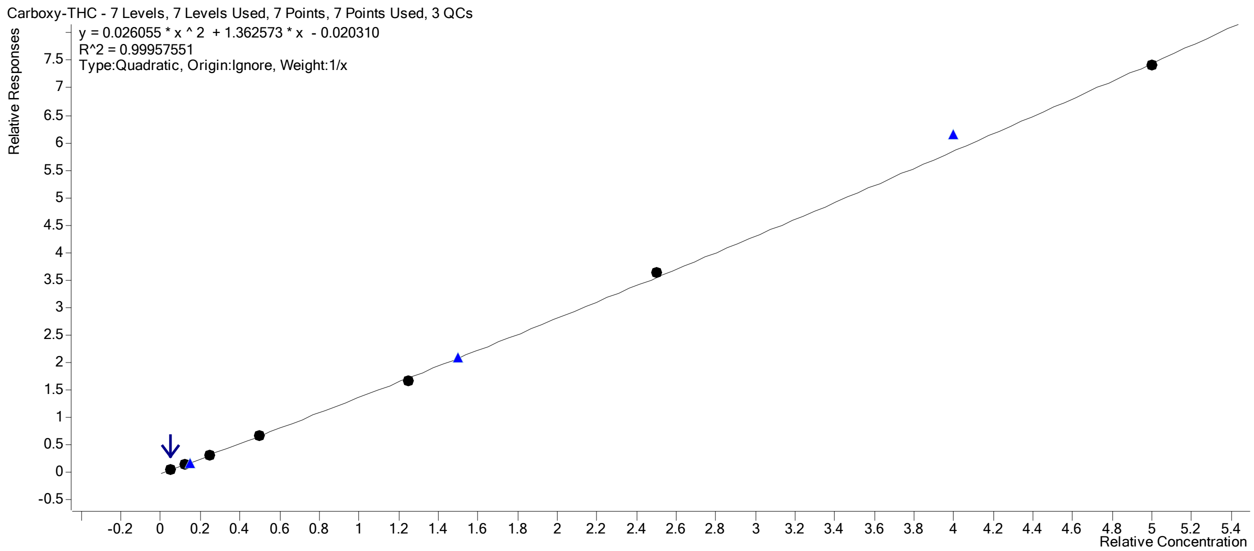


Figure 3 Carboxy-THC calibration curve with C18 analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99871553 and the origin was ignored.

Carboxy-THC: quadratic-weighted (1/x) calibration model using PFP analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $7.9721 \times 10^{-11} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 62.0338 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for carboxy-THC on the PFP analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $7.8815 \times 10^{-8} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for carboxy-THC using the PFP analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2747 and non-weighted 0.5082).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for carboxy-THC. Charts 13-16 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for carboxy-THC respectively.

Chart 13

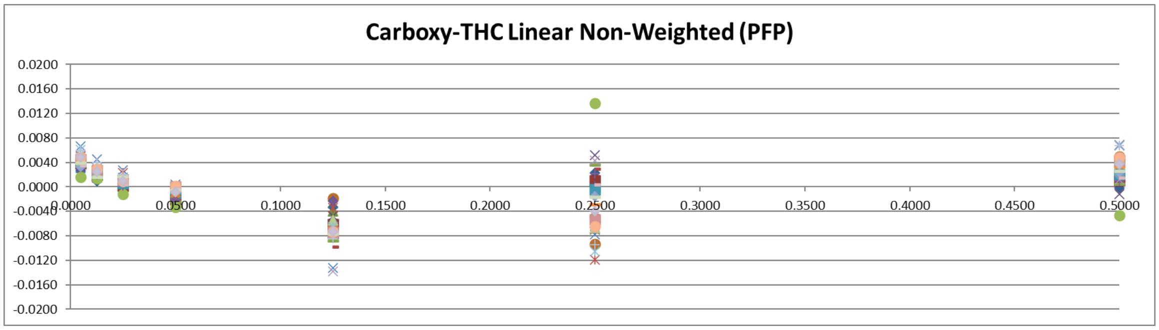


Chart 14

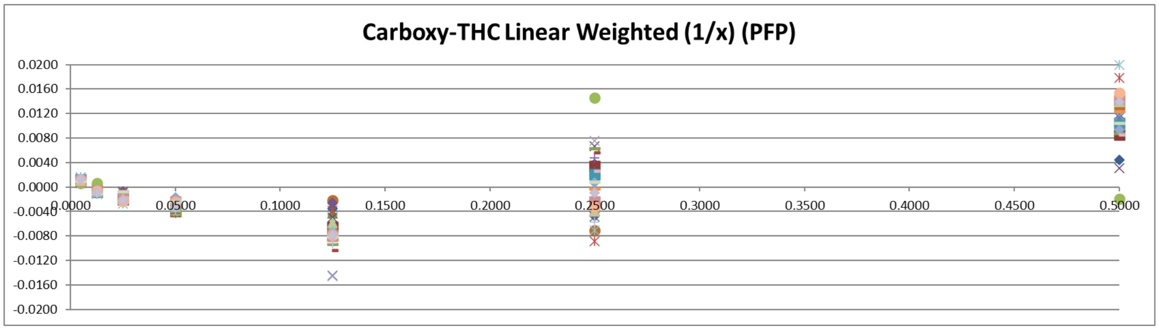


Chart 15

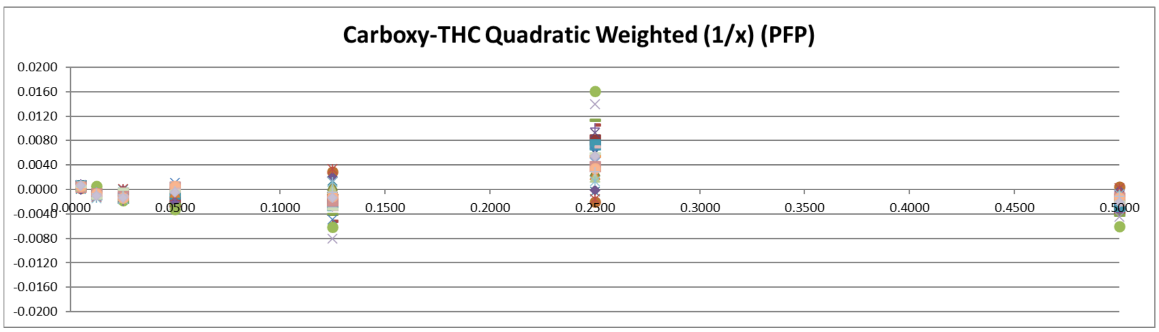


Chart 16

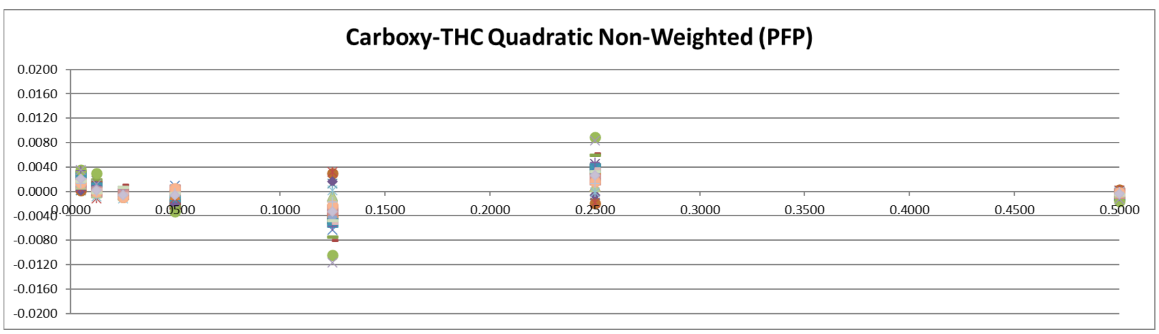


Figure 4 represents a quadratic weighted (1/x) calibration curve for carboxy-THC on the PFP analytical column with a dynamic range of 5 ng/mL to 500 ng/mL.

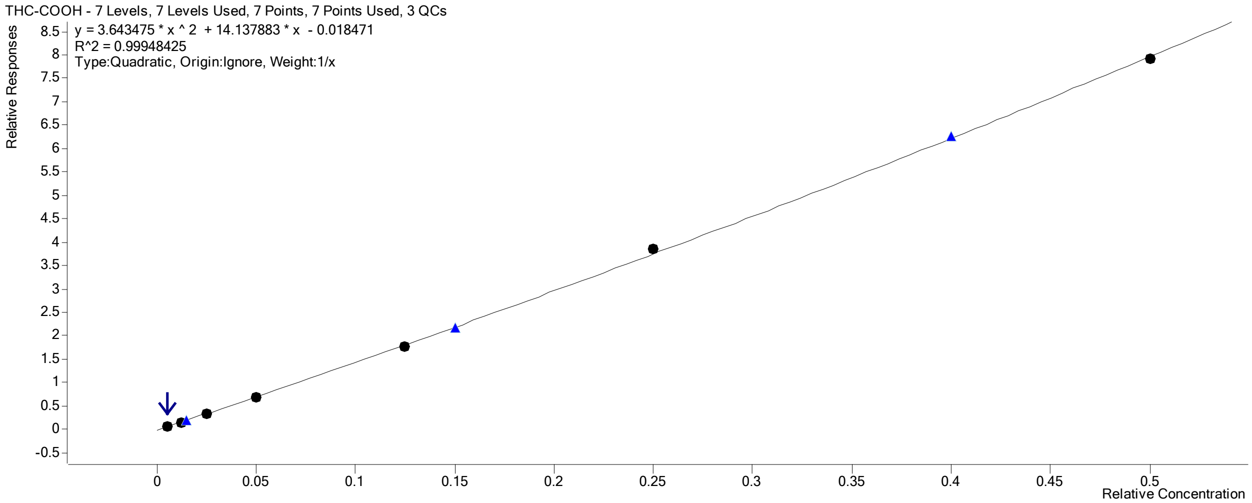


Figure 4 Carboxy-THC calibration curve with PFP analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99948425 and the origin was ignored.

Cannabidiol: quadratic-weighted (1/x) calibration model using C18 analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $2.3949 \times 10^{-19} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

$F = 173.3184 > 4.0012$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for cannabidiol on the C18 analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $3.4686 \times 10^{-12} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for cannabidiol using the C18 analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2996 and non-weighted 0.6234).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for cannabidiol. Charts 17-20 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for cannabidiol respectively.

Chart 17

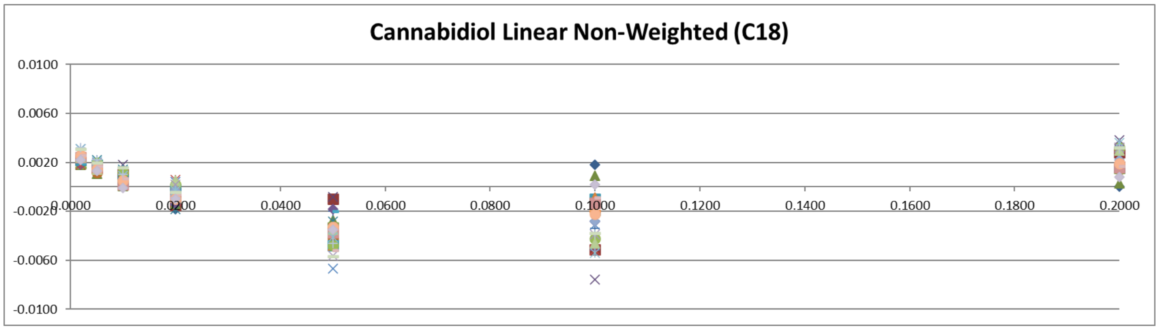


Chart 18

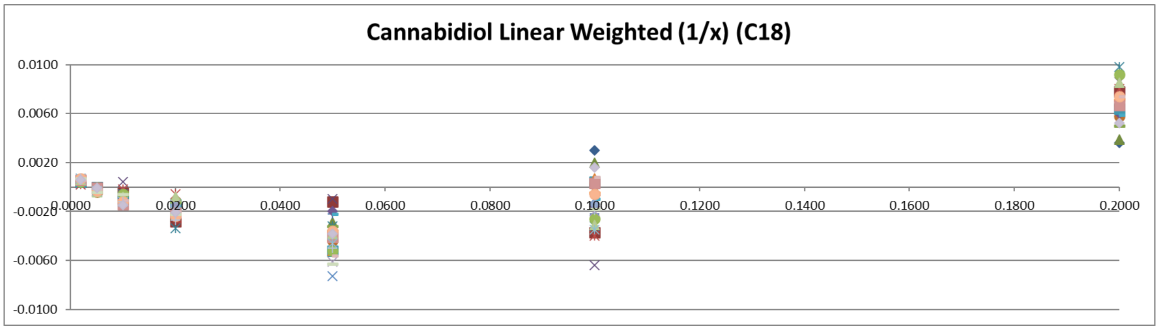


Chart 19

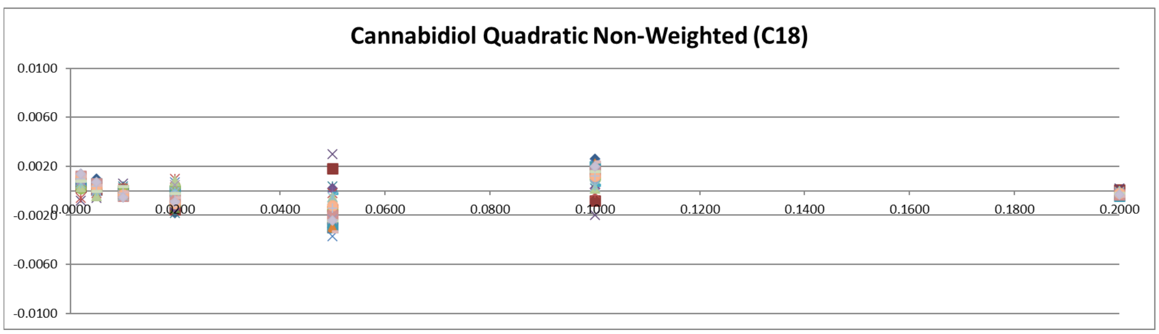


Chart 20

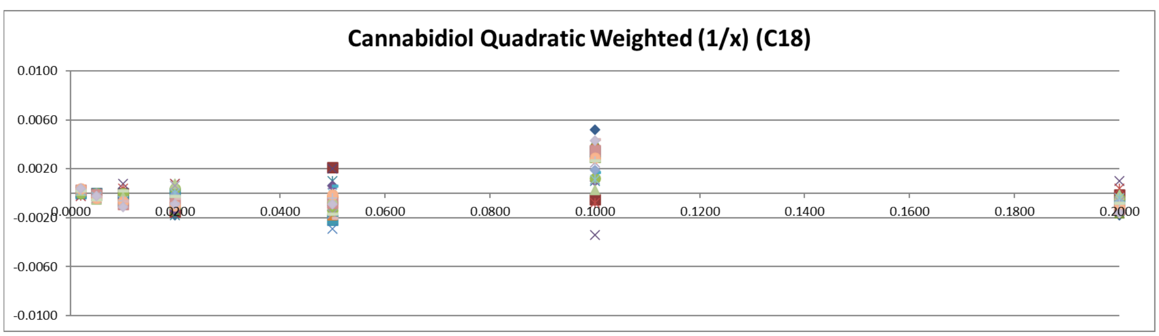


Figure 5 represents a quadratic weighted (1/x) calibration curve for cannabidiol on the C18 analytical column with a dynamic range of 2 ng/mL to 200 ng/mL.

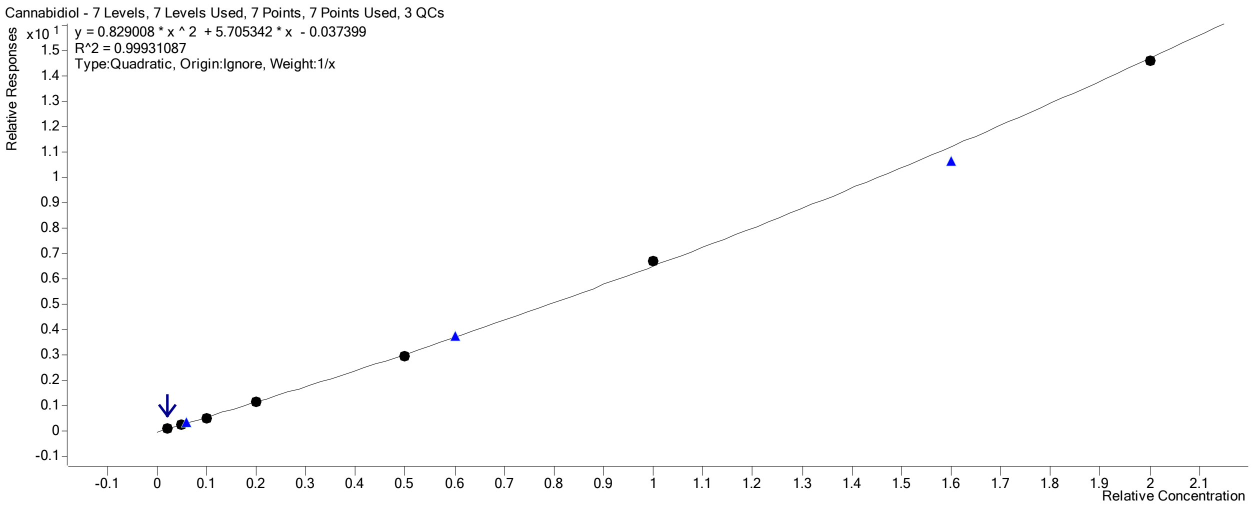


Figure 5 Cannabidiol calibration curve with C18 analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99931087 and the origin was ignored.

Cannabidiol: quadratic-weighted (1/x) calibration model using PFP analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $7.0207 \times 10^{-14} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 93.8412 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for cannabidiol on the PFP analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $3.0768 \times 10^{-12} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for cannabidiol using the PFP analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.3230 and non-weighted 0.6762).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for cannabidiol. Charts 21-24 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for cannabidiol respectively.

Chart 21

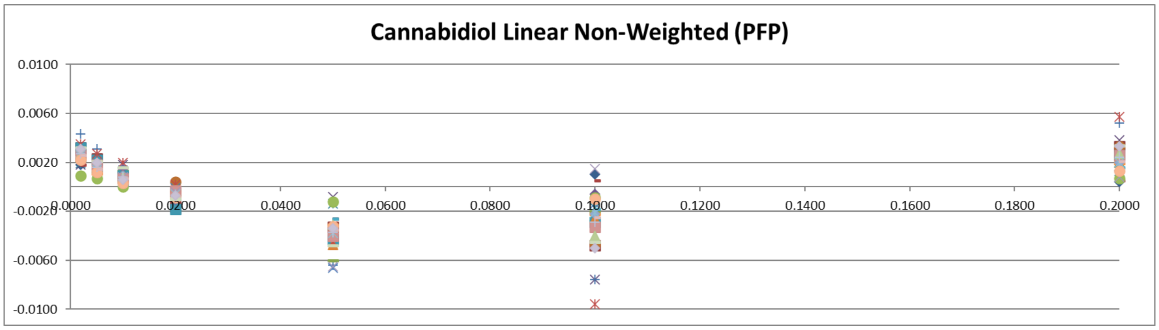


Chart 22

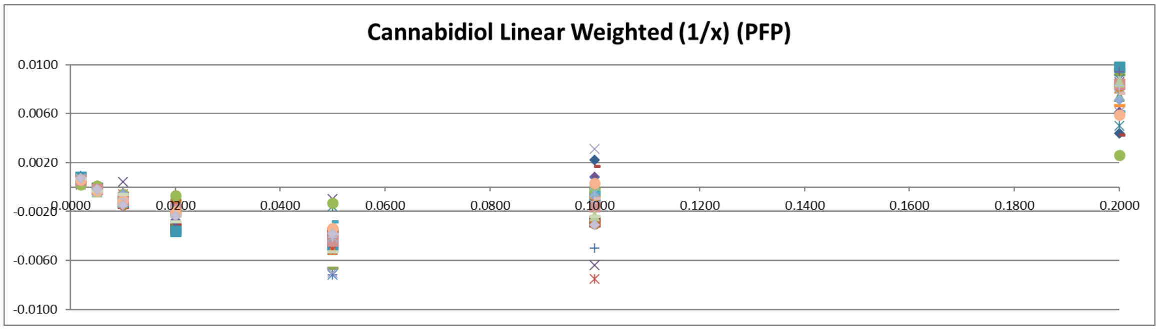


Chart 23

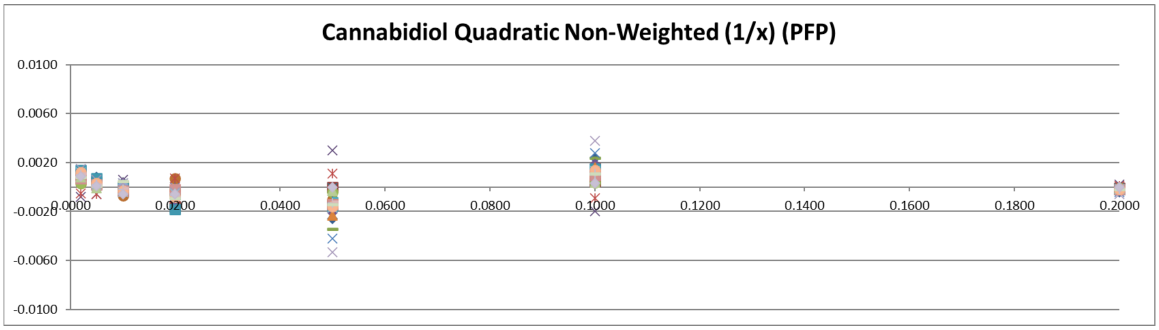


Chart 24

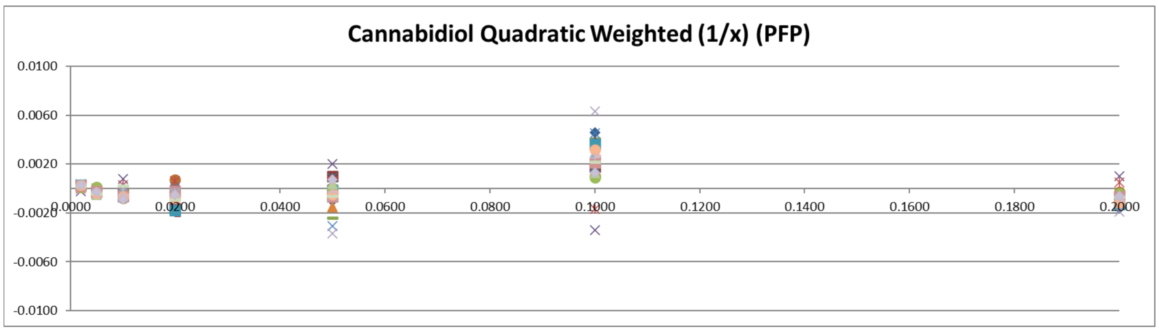


Figure 6 represents a quadratic weighted (1/x) calibration curve for cannabidiol on the PFP analytical column with a dynamic range of 2 ng/mL to 200 ng/mL.

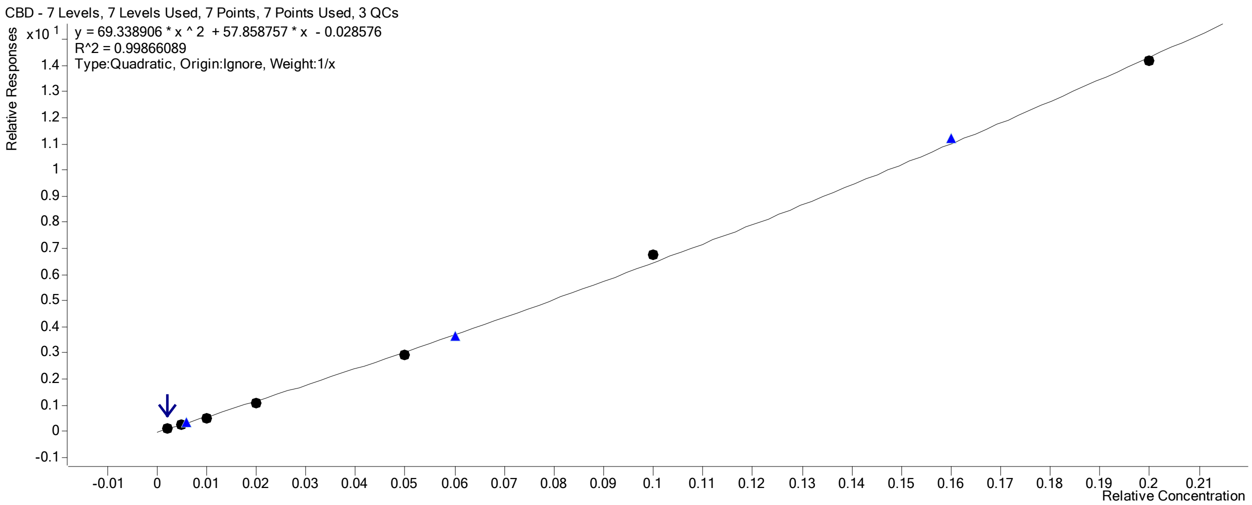


Figure 6 Cannabidiol calibration curve with PFP analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99866089 and the origin was ignored.

Δ⁹-THC: quadratic-weighted (1/x) calibration model using C18 analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $3.7638 \times 10^{-12} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

$F = 74.9197 > 4.0012$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for Δ^9-THC on the C18 analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $9.5420 \times 10^{-9} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for Δ^9-THC using the C18 analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2411 and non-weighted 0.5771).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for Δ^9-THC. Charts 25-28 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for Δ^9-THC respectively.

Chart 25

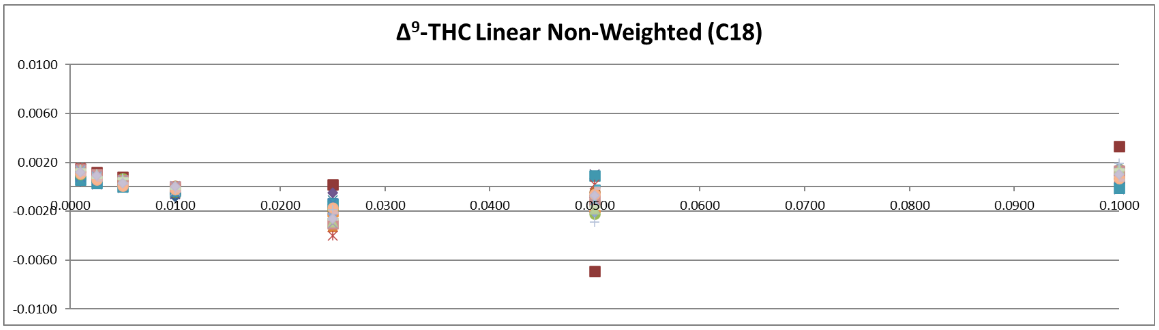


Chart 26

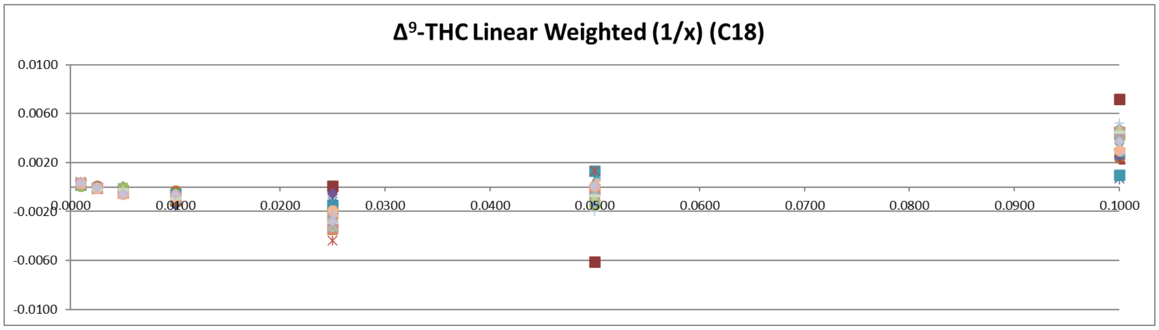


Chart 27

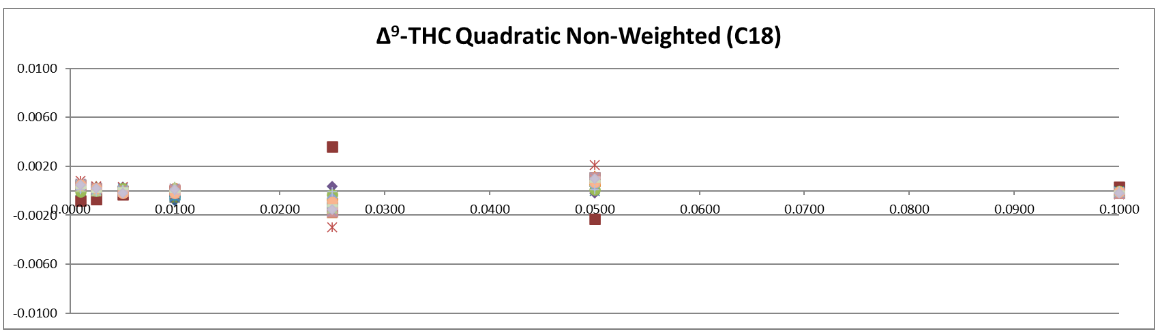


Chart 28

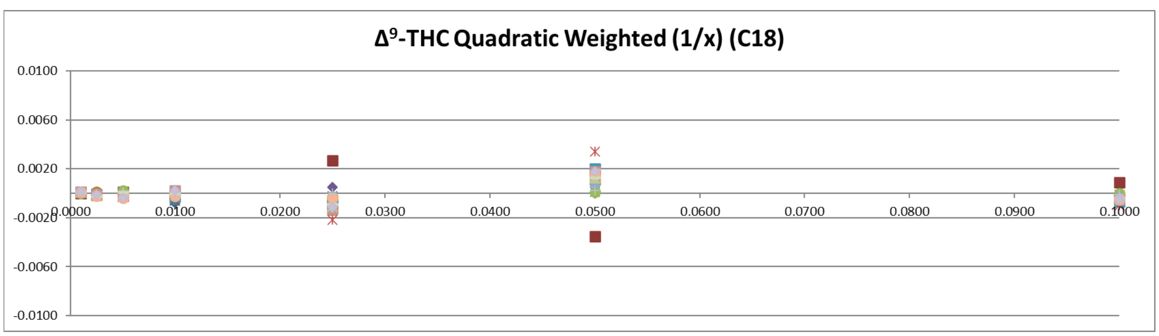


Figure 7 represents a quadratic weighted (1/x) calibration curve for Δ^9-THC on the C18 analytical column with a dynamic range of 1 ng/mL to 100 ng/mL.

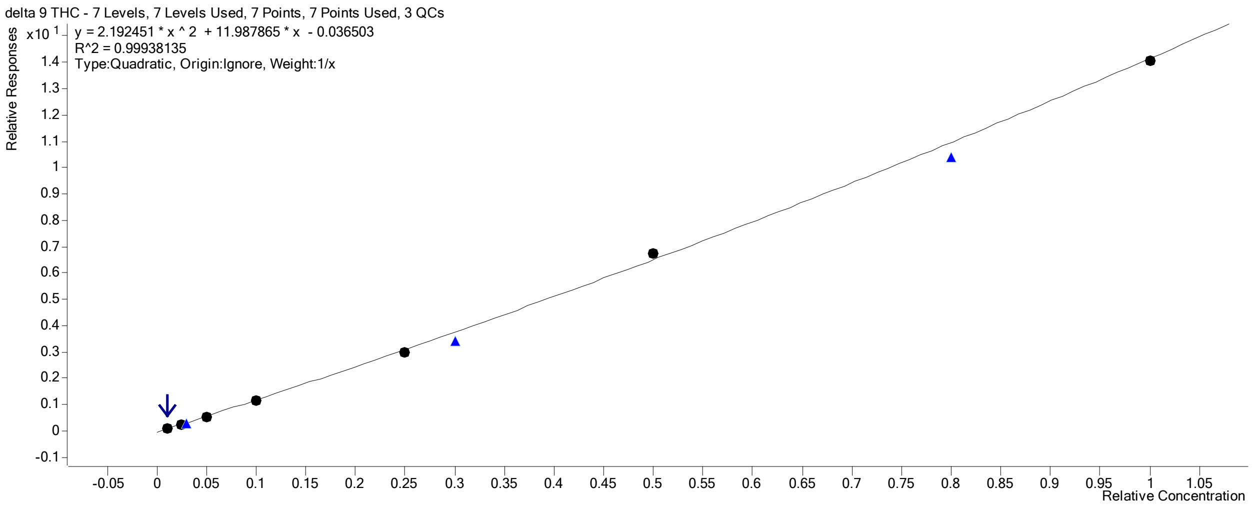


Figure 7 Δ^9-THC calibration curve with C18 analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99938135 and the origin was ignored.

Δ^9-THC: quadratic-weighted (1/x) calibration model using PFP analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $1.1318 \times 10^{-19} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

$F = 179.1891 > 4.0012$ (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for Δ^9-THC on the PFP analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $1.6895 \times 10^{-9} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for Δ^9-THC using the PFP analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2897 and non-weighted 0.6214).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for Δ^9-THC. Charts 29-32 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for Δ^9-THC respectively.

Chart 29

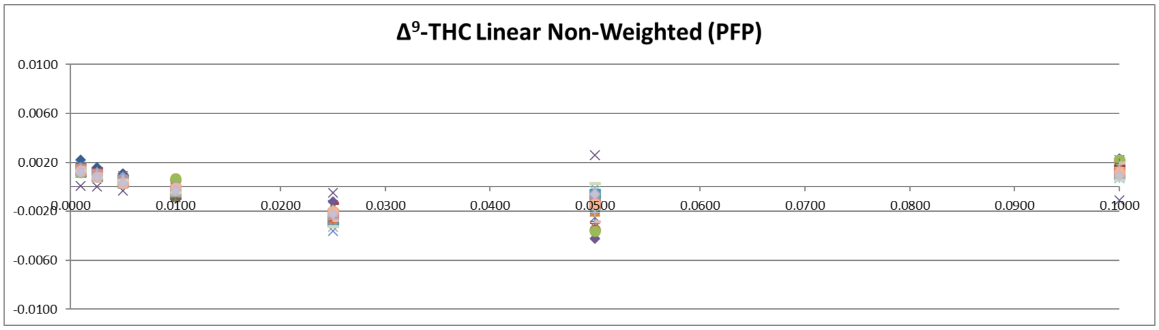


Chart 30

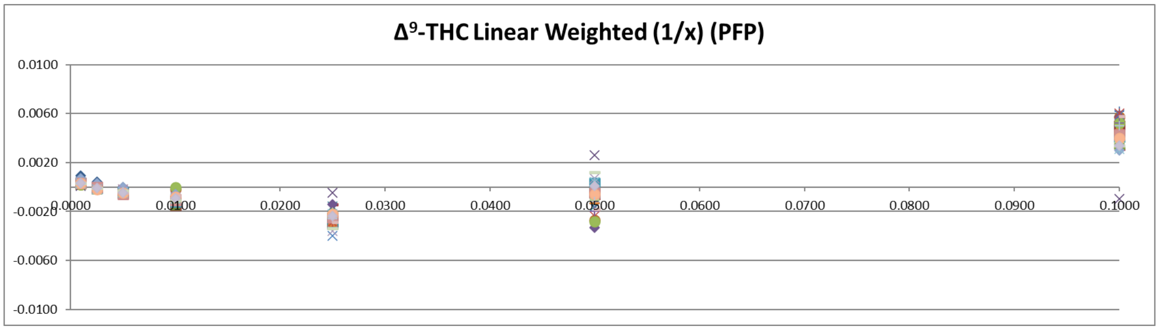


Chart 31

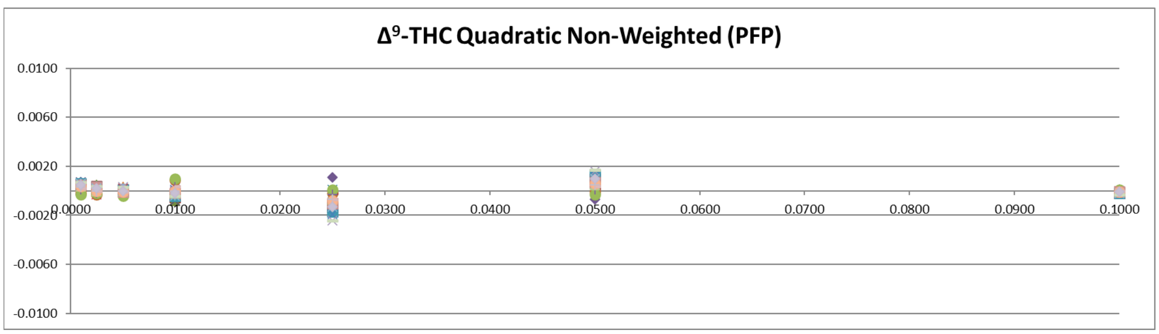


Chart 32

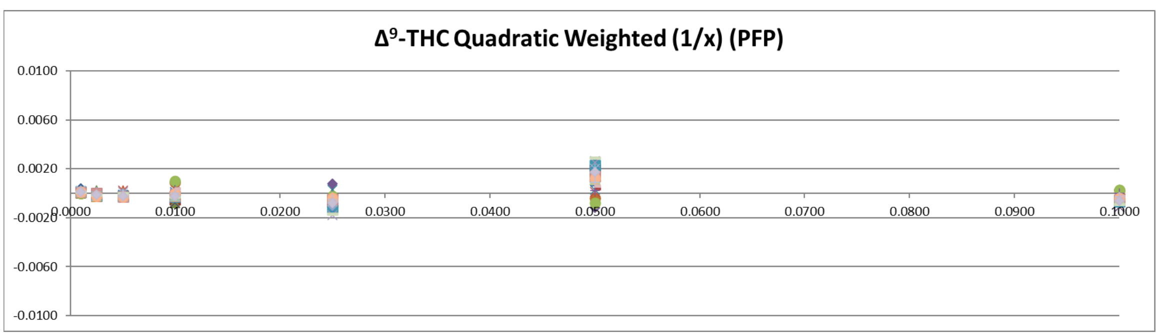


Figure 8 represents a quadratic weighted (1/x) calibration curve for Δ^9-THC on the PFP analytical column with a dynamic range of 1 ng/mL to 100 ng/mL.

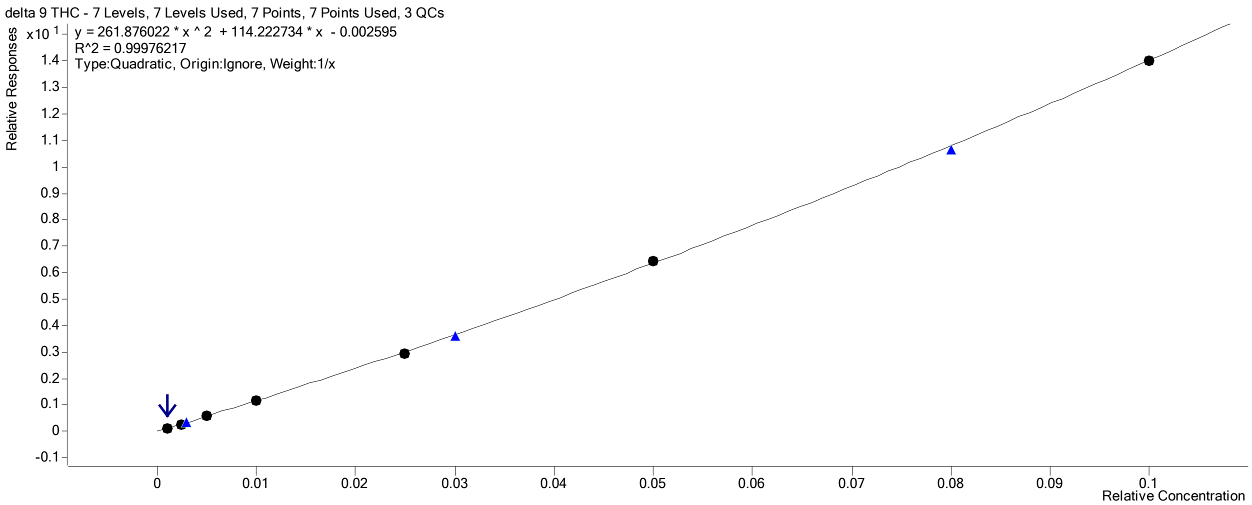


Figure 8 Δ^9-THC calibration curve with PFP analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99976217 and the origin was ignored.

Δ^8-THC: quadratic-weighted (1/x) calibration model using C18 analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = 0.0083 < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 7.4471 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for Δ^8-THC on the C18 analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = 3.2906×10^{-10} < 0.05 and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for Δ^8-THC using the C18 analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.3258 and non-weighted 0.8189).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for Δ^8-THC. Charts 33-36 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for Δ^8-THC respectively.

Chart 33

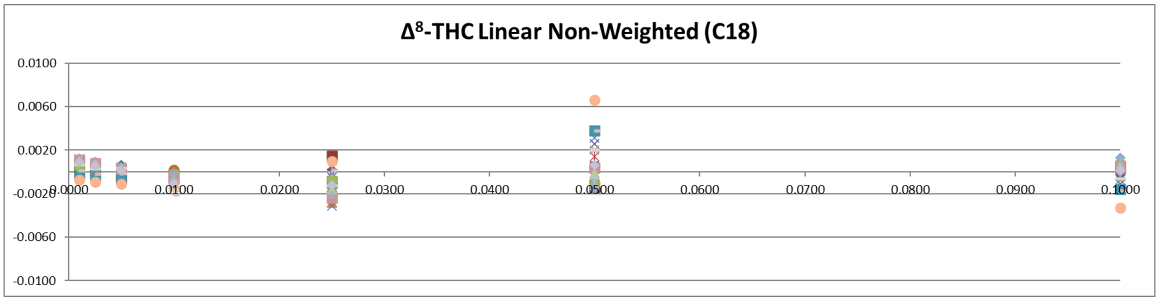


Chart 34

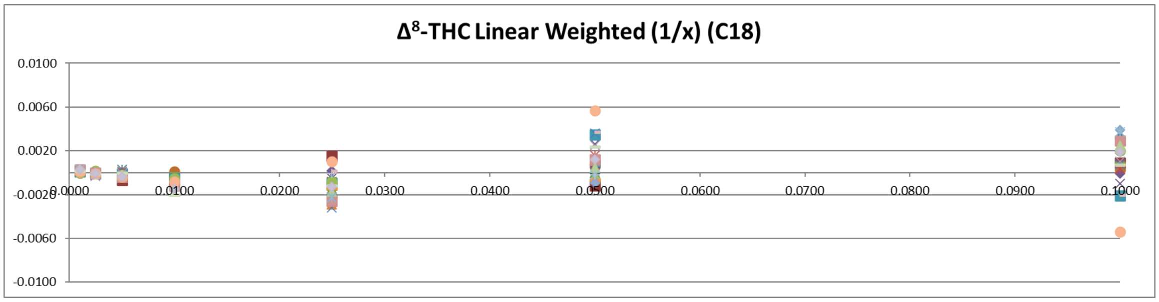


Chart 35

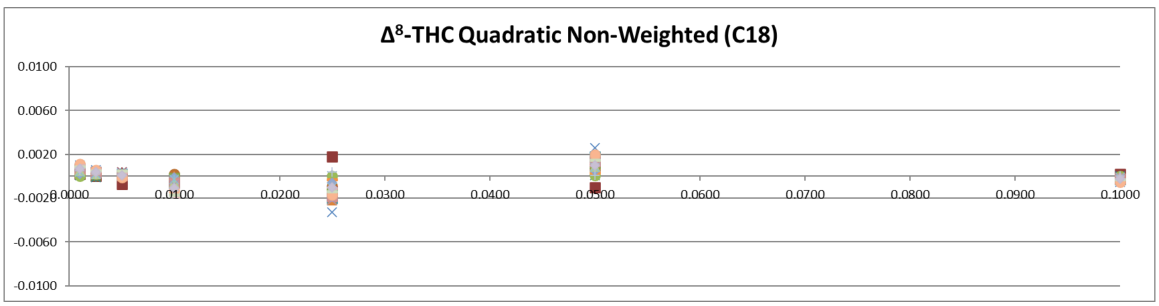


Chart 36

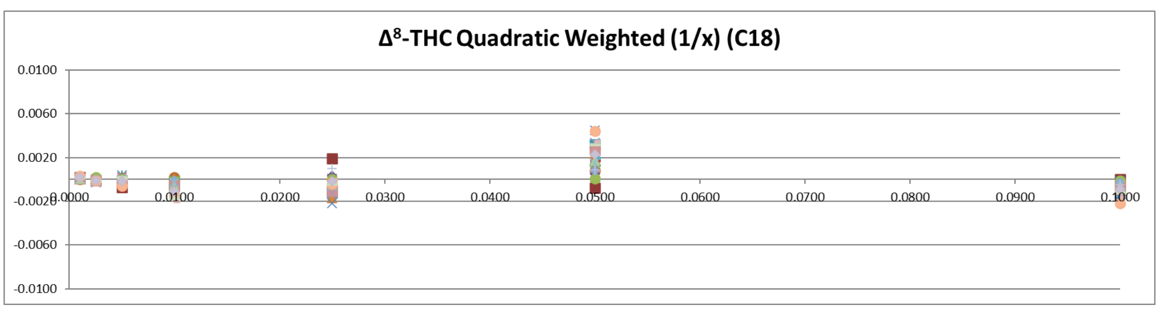


Figure 9 represents a quadratic weighted (1/x) calibration curve for Δ^8-THC on the C18 analytical column with a dynamic range of 1 ng/mL to 100 ng/mL.

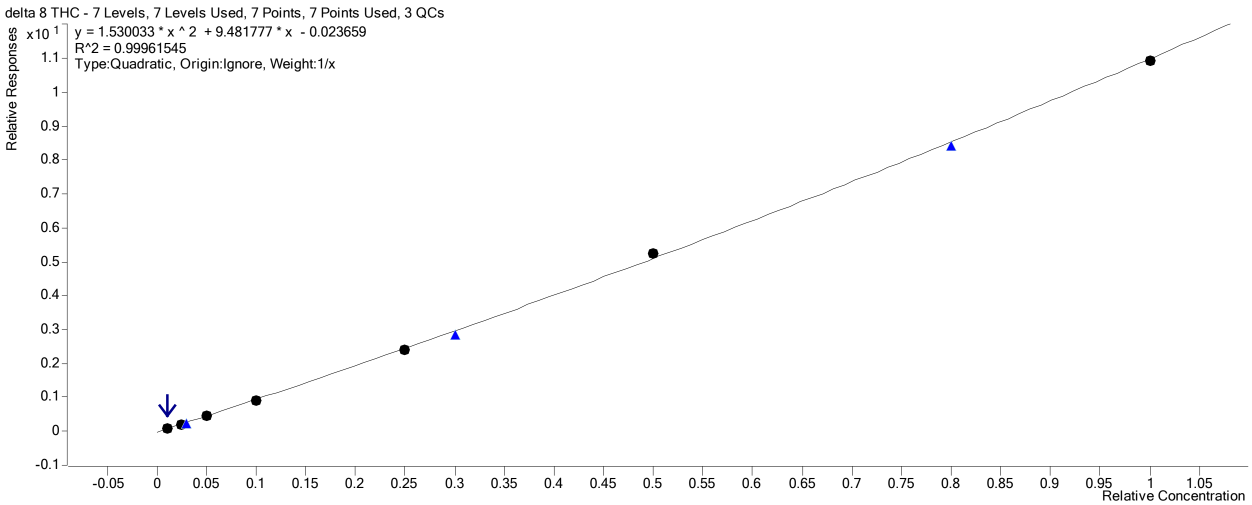


Figure 9 Δ^8-THC calibration curve with C18 analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99961545 and the origin was ignored.

Δ^8-THC: quadratic-weighted (1/x) calibration model using PFP analytical column

Comparison of Linear Weighted (1/x) and Quadratic Weighted (1/x) using ANOVA

The standard deviation of the residuals was used to determine the linear/quadratic nature of the calibration model.

P-value = $3.4843 \times 10^{-17} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

F = 137.8328 > 4.0012 (F_{crit}) and therefore the null hypothesis was rejected and the variances were determined to not be equal.

From these results, it was determined that there was significant difference between a linear weighted calibration model and a quadratic weighted calibration model. Therefore, the quadratic calibration model will be utilized for Δ^8-THC on the PFP analytical column.

Comparison of Quadratic Non-weighted and Quadratic Weighted (1/x) using a t-test

The sum of errors of the residuals was used to determine the weighting for the calibration model.

P-value = $3.1692 \times 10^{-7} < 0.05$ and therefore the null hypothesis was rejected and the groups were determined to be statistically significantly different.

The quadratic weighted (1/x) model was determined to be the most appropriate calibration model for Δ^8-THC using the PFP analytical column. The t-test for the comparison of weighted and non-weighted quadratic models indicated a significant difference between the two groups and therefore the quadratic weighted model was selected as the best fit. This was also due to the average sum of relative error for the residuals being lower for the weighted model than the non-weighted model (weighted 0.2849 and non-weighted 0.5682).

Residual plots were also used to help visually assist in the evaluation of the best fit calibration model for Δ^8-THC. Charts 37-40 show the linear non-weighted, linear weighted (1/x), quadratic non-weighted, and quadratic weighted (1/x) residual plots for Δ^8-THC respectively.

Chart 37

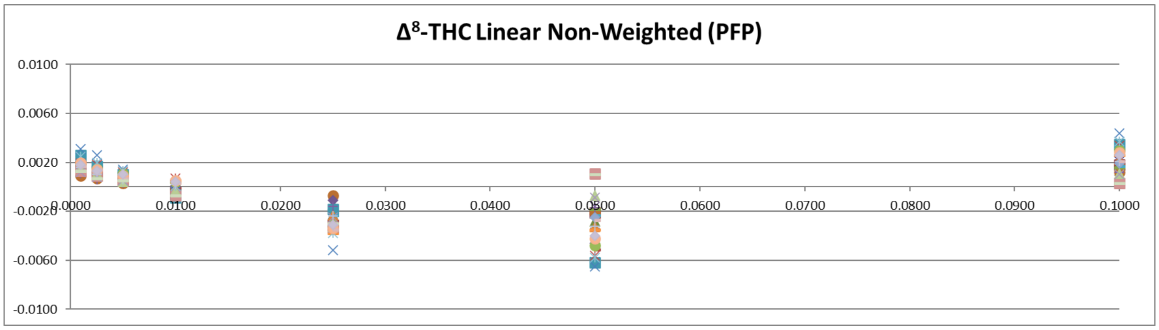


Chart 38

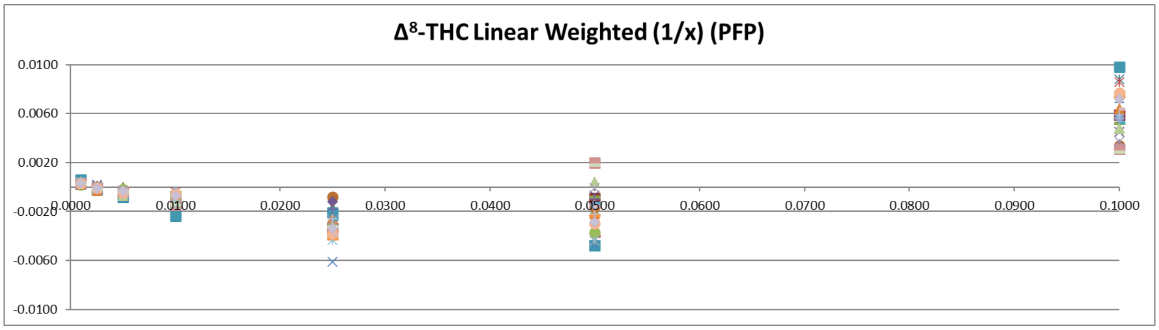


Chart 39

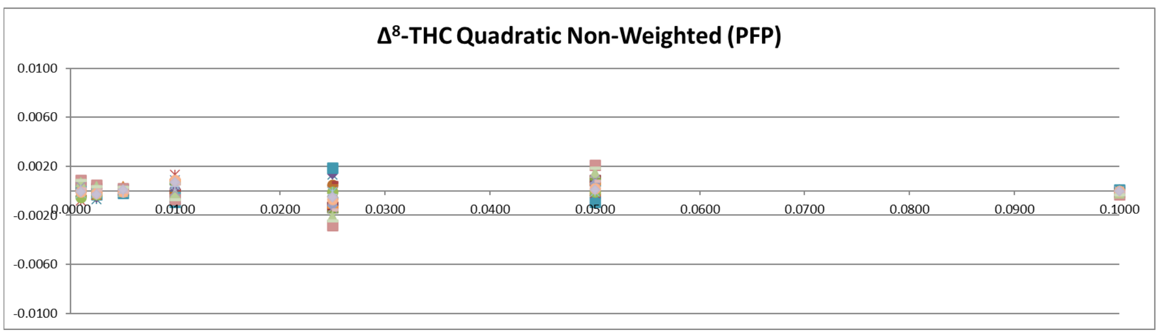


Chart 40

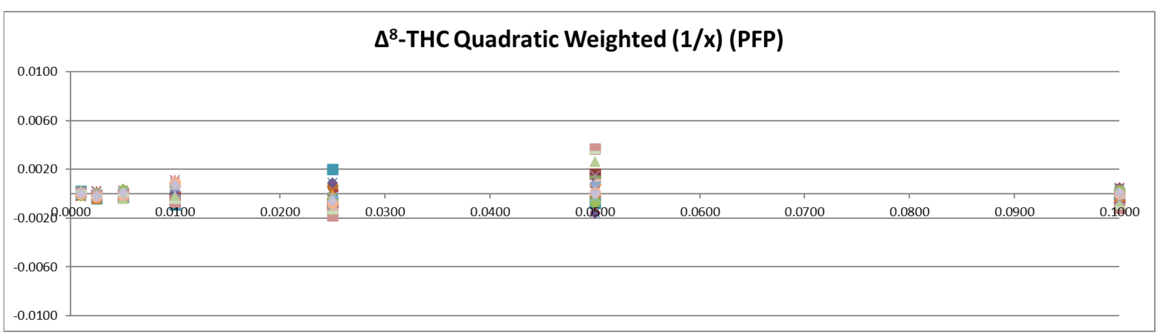


Figure 10 represents a quadratic weighted (1/x) calibration curve for Δ^{8}-THC on the PFP analytical column with a dynamic range of 1 ng/mL to 100 ng/mL.

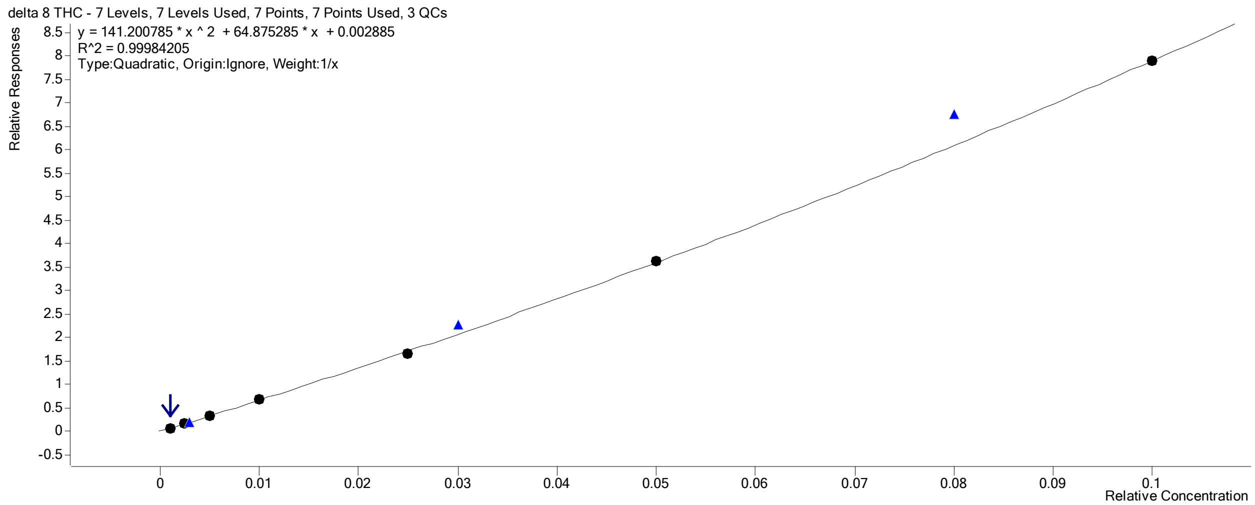


Figure 10 Δ^{8}-THC calibration curve with PFP analytical column

The relative response of the calibrators was represented with black circles while the control response was represented with the blue triangles on the calibration curve. The r^2 value was 0.99984205 and the origin was ignored.

During the validation, an interferent with the qualifier transition of Δ^{8}-THC was identified. The interference was initially observed when evaluating Δ^{8}-THC on the C18 analytical column. Additional column lot numbers were obtained to investigate the prevalence of the interferent. Given the prevalence of the interference with various lots of SLE columns, a collaboration with Biotage was initiated to further identify the interferent. With the structural information obtained from the cannabinoids SLE method, Biotage was able to identify and isolate the interferent. The interferent was removed from the column chemistries and the cartridges are undergoing internal review prior to sending samples for testing using the cannabinoids method. Biotage SLE columns are comprised of diatomaceous earth and have a natural variability between lot numbers and within individual lots of columns.

2. Validation of the Qualitative Analysis of Cannabinoids using LCMSMS

Method Validation Summary:

Note: Published internally as an independent document (Figures and Tables are chronological)

Method Validation Summary for the Qualitative Analysis of Cannabinoids by Supported Liquid Extraction using LCMSMS

The validation of "Qualitative Analysis of Cannabinoids by Supported Liquid Extraction Using LCMSMS" was conducted pursuant to the validation plan. The compounds included in the qualitative validation include (±)11-Hydroxy-Δ^8-tetrahydrocannabinol (Δ^8-OH-THC), 11-nor-9-carboxy- Δ^8-tetrahydrocannabinol (Δ^8-THC-COOH), 9S-hexahydrocannabinol, 9R-hexahydrocannabinol, and 9R-$\Delta^{6a,10a}$-tetrahydrocannbinol (9R-$\Delta^{6a,10a}$-THC). It should be noted that 9R-$\Delta^{6a,10a}$-THC and Δ^{10}-THC co-elute on both analytical columns with the method and have the same qualifier ratios. Therefore, these compounds cannot be distinguished from one another within the method and can be evaluated as 9R-$\Delta^{6a,10a}$-THC/Δ^{10}-THC. The validation included the following:

1. Sensitivity: Estimated Limit of Detection (LOD)
2. Ionization Suppression/Enhancement
3. Carryover
4. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
5. Stability
6. Robustness
7. Summary
8. References

An Agilent Technologies 1260 binary pump liquid chromatograph coupled independently to both an Agilent Technologies 6460 and 6470 tandem mass spectrometer was used during validation. Validation experiments were performed in accordance with the approved validation plan. The biological matrices evaluated during the validation included blank blood, antemortem blood, and postmortem blood, and urine.

1. Sensitivity: Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation was defined as an administratively defined decision point (threshold concentration). The limit of detection was evaluated on all instrumentation models and is understood to be an estimate based on the condition of the instruments at the time of the evaluation. The administrative threshold was established to be 2/5 ng/mL (9S-hexahydrocannabinol, 9R-$\Delta^{6a,10a}$-THC/ 9R-hexahydrocannabinol, Δ^8-OH-THC, Δ^8-THC-COOH) in blank blood and 5 ng/mL for all qualitative target compounds in antemortem blood, postmortem blood, and urine. Nine blank matrix sources, per matrix type were utilized in the determination of the estimated limit of detection per matrix type. The matrix sources were fortified with the compounds of interest over three batch analyses. A single replicate was evaluated for each matrix source within each batch analysis. The peak shape, retention time, qualifier ratio, and signal-to-noise ratio were evaluated for each compound at each concentration. The predetermined identification criteria included a retention time within ±3%, a qualifier ratio within ±20%, and a signal-to-noise ratio ≥3.3.

The estimated limit of detection was determined for the C18 and PFP analytical columns for each target compound. When evaluating blank blood, antemortem blood, postmortem blood, and urine, all compounds passed the predetermined requirements for limit of detection at 2/5 ng/mL in blank blood and 5 ng/mL in each additional matrix source using the C18 analytical column. When evaluating 9-R-hexahydrocannabinol there was one postmortem source replicate out of the 81 replicates evaluated for postmortem blood that had a qualifier ratio outside of the ±20% acceptance criteria.

When evaluating the estimated limit of detection in blank blood, antemortem blood, postmortem blood, and urine using the PFP analytical column a variety of failures were observed. The observed results are described in Table 1.

Table 1 PFP analytical column LOD results

Estimated Limit of Detection PFP Analytical Column				
Target Compound	Blank Blood	Antemortem Blood	Postmortem Blood	Urine
Δ^8-OH-THC	1/81 Replicates Failed Qualifier Ratio	All Replicates Pass	All Replicates Pass	2/81 Replicates Failed Qualifier Ratio
Δ^8-THC-COOH	2/81 Replicates Failed Peak Shape	2/81 Replicate Failed Peak Shape	2/81 Replicates Failed Peak Shape 1/81 Replicates Failed Qualifier Ratio	1/81 Replicates Failed Qualifier Ratio
9S-hexahydrocannabinol	All Replicates Pass	All Replicates Pass	All Replicates Pass	All Replicates Pass
9R-hexahydrocannabinol	All Replicates Pass	All Replicates Pass	All Replicates Pass	All Replicates Pass
9R-$\Delta^{6a,10a}$-THC	All Replicates Pass	All Replicates Pass	All Replicates Pass	All Replicates Pass

Some replicates did not meet all predetermined acceptance criteria for each matrix source. 9S-hexahydrocannabinol, 9R-hexahydrocannabinol, and 9R-$\Delta^{6a,10a}$-THC had all replicates meet the predetermined acceptance criteria for all four matrix types. When evaluating Δ^{8}-OH-THC, one (1) replicate out of 81 replicates for blank blood did not meet the qualifier ratio acceptance criteria of ±20%. Additionally, when evaluating the estimated limit of detection for urine, there were two (2) qualifier ion ratio failures out of the 81 replicates. Both antemortem blood and postmortem blood met the predetermined acceptance criteria for estimated limit of detection for all replicates. When evaluating Δ^{8}-THC-COOH, two (2) out of 81 blank blood replicates failed peak shape requirements. This was also observed in antemortem blood, and postmortem blood where two (2) out of 81 replicates failed peak shape criteria. In addition to the peak shape failures, postmortem blood had one (1) replicate out of 81 that had a qualifier ratio outside of ±20%. During the evaluation of urine, there was one (1) replicate out of 81 that had a qualifier ratio outside of ±20%.

Although acceptance criteria failures were observed, each compound in each matrix type had over 95% of the replicates meet the predetermined acceptance criteria. Therefore, the estimated limit of detection for all compounds on both analytical columns was determined to be 5 ng/mL.

3. Ionization Suppression/Enhancement

Ionization suppression and enhancement was evaluated by assessing the instrumental response of post-extraction fortified samples and neat standards. Post-extraction fortified samples were prepared from blank matrix that was subject to the supported liquid extraction protocol. After extraction, the blank samples were fortified with both target and internal standard. The neat samples were prepared by spiking an appropriate volume of the target analyte and internal standard in methanol directly into the autosampler vial. Neat samples were not dried down during preparation.

Equation 1 was used to calculate the ionization suppression/enhancement for the target compounds and the internal standards. The ionization suppression/enhancement was assessed at two different concentrations: 5/10/25 ng/mL and 50/100/250 ng/mL (9R-$\Delta^{6a,10a}$-THC, 9S-hexahydrocannabinol, 9R-hexahydrocannabinol/ Δ^{8}-OH-THC/ Δ^{8}-THC-COOH).

Equation 1

$$\text{Ion Suppression/Enhancement}=\left(\frac{\text{Average Post-Extraction Fortified Sample}}{\text{Average Neat Sample}}\right)\times 100$$

To fully evaluate the impact of ionization suppression/enhancement, duplicate determinations of each concentration for each matrix source were evaluated. A total of ten different sources per matrix type was used in the evaluation. The post-extraction fortified samples were compared to six replicate injections of neat standards. The overall ionization suppression or enhancement was calculated for both the C18 analytical column and the PFP analytical column. Table 2 shows the data associated with the C18 analytical column whereas Table 3 shows the data associated with the PFP analytical column.

Table 2 Ionization suppression and enhancement C18 analytical column

Ionization Suppression and Enhancement (C18) % Suppression/Enhancement ± Standard Deviation (%CV)				
Target Compound	Blank Blood (n=36)	Antemortem Blood (n=36)	Postmortem Blood (n=36)	Urine (n=36)
Δ^8-OH-THC	84.4±24.6(29)	63.2±12.8(20)	66.1±15.5(23)	55.5±14.7(26)
Δ^8-THC-COOH	90.7±18.2(20)	62.0±14.0(23)	78.5±18.9(24)	71.9±17.2(24)
9S-Hexahydrocannabinol	89.7±7.3(8)	85.9±9.4(11)	105.2±12.5(12)	58.3±8.0(14)
9R-Hexahydrocannabinol	84.7±8.1(10)	75±5.9(8)	81.1±8.1(10)	60.2±8.2(14)
9R-$\Delta^{6a,10a}$-THC	84.2±6.4(8)	70.9±6.9(10)	88.0±10.5(12)	59.5±7.4(12)
Δ^9-OH-THC-D_3	83.8±23.9(29)	63.7±12.5(20)	71.3±17.1(24)	66.8±18.0(27)
Δ^9-THC-COOH-D_3	86.5±22.4(26)	52.4±10.5(20)	86.0±23.3(27)	72.0±16.1(22)
Δ^9-THC-D_3	79.2±6.7(8)	71.7±5.3(7)	79.4±8.2(10)	55.1±8.5(15)

Table 3 Ionization suppression and enhancement PFP analytical column

Ionization Suppression and Enhancement (PFP) % Suppression/Enhancement ± Standard Deviation (%CV)				
Target Compound	Blank Blood (n=36)	Antemortem Blood (n=36)	Postmortem Blood (n=36)	Urine (n=36)
Δ^8-OH-THC	100.6±19.5(19)	79.8±5.5(7)	98.8±9.5(10)	111.2±13.9(13)
Δ^8-THC-COOH	114.8±25.7(22)	87.3±5.1(6)	100.2±14.2(14)	123.3±13.6(11)
9S-Hexahydrocannabinol	94.5±10.4(11)	103.4±6.3(6)	105.6±6.9(7)	97.5±8(8)
9R-Hexahydrocannabinol	93.2±8.1(9)	95.9±5.9(6)	100.4±6.1(6)	95.5±7.6(8)
9R-$\Delta^{6a,10a}$-THC	100.0±12.0(12)	71.3±5.5(8)	77.7±8.6(11)	99.4±8.9(9)
Δ^9-OH-THC-D_3	91.8±30.2(33)	61.4±4.3(7)	69±15.2(22)	107.3±12.5(12)
Δ^9-THC-COOH-D_3	103.9±20.8(20)	85.2±4.6(5)	104.9±9.5(9)	117.9±12.2(10)
Δ^9-THC-D_3	88.9±8.7(10)	97±6.2(6)	100.6±6.8(7)	97.7±7.4(8)

The values of 100% are indicative of no ionization suppression or enhancement in the samples. Values greater than 100% indicate ionization enhancement and values less than 100% indicate ionization suppression. Values greater than ±25% are indicative of significant ionization suppression or enhancement. The ionization enhancement did not exceed 25% for either analytical column. Ionization suppression was noted in several instances. The C18 analytical column demonstrated the most ionization suppression between the two column types evaluated. Blank blood, antemortem blood, postmortem blood, and urine all had indications of significant ionization suppression when using the C18 column and less notably with the PFP analytical column. No ionization suppression was noted with the PFP column and urine.

In addition to the average ionization suppression or enhancement, the variability between the matrices was also evaluated by assessing the %CV. The %CV was calculated for each matrix type and should not exceed ±20%. Significant variability (%CV >20%) was noted for several matrix types for several compounds when using the C18 analytical column. 9R-Hexahydrocannabinol, 9S-hexahydrocannabinol, 9R-$\Delta^{6a,10a}$-THC, Δ^9-THC-D_3 all had %CVs within ±20%. The PFP analytical column demonstrated less variability when compared to the C18 analytical column. Δ^8-THC-COOH failed to meet the predetermined acceptance criteria for %CV in blank blood. Δ^9-OH-THC-D_3 failed to meet the predetermined acceptance criteria for %CV in blank blood and postmortem blood.

Given the significant ionization suppression noted with the C18 and PFP analytical columns, and the variability between matrices exceeding a %CV of 20%, additional matrices were evaluated for the estimated limit of detection.

4. Carryover

Carryover was evaluated by analyzing blank matrix samples immediately following progressively higher concentrations of fortified matrix within the injection sequence. Three concentrations, 0.1/0.2/0.5 mg/L, 0.2/0.4/1.0 mg/L, and 0.4/0.8/2.0 mg/L (9R-$\Delta^{6a,10a}$-THC, 9S-hexahydrocannabinol, 9R-hexahydrocannabinol/ Δ^8-OH-THC/ Δ^8-THC-COOH), were evaluated in three sources each of blank blood, antemortem blood, postmortem blood, and urine. The blank sample immediately following the fortified matrix sample was evaluated for an instrumental response greater the 10% of the administratively established threshold (2/5 ng/mL [9S-hexahydrocannabinol, 9R-$\Delta^{6a,10a}$-THC/ 9R-hexahydrocannabinol, Δ^8-OH-THC, Δ^8-THC-COOH]). No blank matrix samples immediately following any fortified matrix sample had indications of carryover.

5. Interferences

To assess for interference, the qualifier and quantifier ions for the target compounds and internal standards were monitored. If an instrumental response was noted and was less than 10% of the administratively established threshold response for the qualifier and quantifier ions, the impact of the instrumental response was deemed insignificant.

a. Endogenous Compounds

To evaluate samples for endogenous interferences, a total of ten matrix sources per matrix type (blank blood, antemortem blood, postmortem blood, and urine) were extracted and evaluated without the addition of internal standard. The samples were evaluated for the presence of instrumental response for the analyte and internal standard. No endogenous interferences were identified.

b. Internal Standard

To evaluate potential interferences of internal standard by a high concentration of analyte, samples were fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single matrix sample, per matrix type was evaluated. During validation it was noted that high concentrations (0.1 mg/L) of 9S-hexahydrocannabinol and 9R-hexahydrocannabinol interfered with the qualifier ion transition for Δ^9-THC-D_3 on the PFP analytical column. No other interferences from a high concentration of analyte were detected.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, a single matrix sample, per matrix type was fortified with an appropriate concentration of internal standard (10/20/50 ng/mL) without the analyte of interest and analyzed for the absence of response for the analyte. No interferences from internal standard were detected.

c. Commonly Encountered Analytes

Interferences from commonly encountered compounds were evaluated by analyzing blank matrix fortified with high concentrations of commonly encountered drugs and metabolites. Table 4 depicts the compounds that were assessed for interference.

Table 4 Commonly encountered analytes

Drug Class	Drug	Concentration
Opioids and Cocaine	Oxymorphone, Hydromorphone, 6-Monoacetylmorphine, Acetylfentanyl, Fentanyl, Benzoylecgonine, Meperidine, Tramadol, Methadone, Morphine, Codeine, Oxycodone, Hydrocodone, Cocaethylene, Cocaine	0.2/2.0/1.0 mg/L
Anti-Epileptic Drugs	Gabapentin, Levetiracetam, Lamotrigine, Zonisamide, 10,11-dihydro-10-hydroxycarbamazepine, Oxcarbazepine, Topiramate, Carbamazepine, Phenytoin, Pregabalin, Lacosamide	0.01 mg/mL
Benzodiazepines	Alprazolam, Clonazepam, Lorazepam, Diazepam, Nordiazepam, Oxazepam, Temazepam, Zolpidem	0.002 mg/mL
NPS	Dibutylone, N-ethyl Pentylone, Tenocyclidine, Clonazolam, 4-Chloro-alpha-PVP, PV8, 6-MAPB, SDB-006, 3-Fluoro AMB, 4-Fluoro AMB, MMB-FUBINACA, MMB-CHMICA, 5F-AB-PINACA, MAB-CHMINICA, ADB-FUBICA, 4F-ADB, 4-APDB, 5-APDB, 6-APDB, MDMB-FUBINACA, 25I-NBOMe, 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOH, 25I-NBF, 25I-NBMD, Pentylone, 3-Methoxy-PCP, Methoxphenidine, Mitragynine, Methiopropamine, 5-DBFPV, 5F-PB-22, AB-FUBINACA, AB-PINACA, 3-Fluoro-phenmetrazine, PB-22	1.0 mg/L
Carisoprodol and Meprobamate	Carisoprodol, Meprobamate	0.1 mg/mL
Fentanyls	3-Fluorofentanyl, 4-Methoxybutyrylfentanyl, Acetylfentanyl, Acrylfentanyl, alpha-Methylacetylfentanyl, alpha-Methylfentanyl, Benzodioxolefentanyl, beta-Hydroxythiofentanyl, Butyrylfentanyl, Carfentanil, cis-3-Methylfentanyl, Cyclopropylfentanyl, Despropionylfentanyl, Fentanyl, Furanylfentanyl, Methoxyacetylfentanyl, Ocfentanil, ortho-Fluoroacrylfentanyl, ortho-Fluorobutyrylfentanyl, ortho-Fluorofentanyl, ortho-Fluoroisobutyrylfentanyl, para-Fluoroacrylfentanyl, para-Fluorobutyrylfentanyl, para-Fluorofentanyl, para-Fluoroisobutyrylfentanyl, Phenylfentanyl, Tetrahydrofuranfentanyl, trans-3-Methylfentanyl, U-47700, U-49900, Valerylfentanyl	0.05/0.1 mg/L
Acidic/Neutral Drugs	Acetaminophen, Carbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, Glutethimide, Ibuprofen, Levetiracetam, Oxcarbazepine, Phenytoin, Salicylic Acid	0.006 mg/mL
Basic Drugs	Amitriptyline, Citalopram, Cyclobenzaprine, Diphenhydramine, Nortriptyline, PCP, Trazodone, Dextromethorphan	0.006 mg/mL
Amphetamines	Amphetamine, Methamphetamine, MDA, MDMA, Bupropion, Phentermine	0.002 mg/mL
Barbiturates	Butalbital, Phenobarbital, Butabarbital Pentobatbital, Secobarbital	0.04 mg/mL

No interferences from commonly encountered compounds were noted. Individual cannabinoids were extracted and evaluated for an instrumental response for the target compounds and internal standards within the analytical methods. Table 5 lists the cannabinoid interferences evaluated during the validation. Additionally, each compound within the method was evaluated individually for interferences with other compounds within the method.

Table 5 Cannabinoid interferent analysis

Cannabinoids	
(±) Cannabicyclol (CBL)	Cannabinolic Acid (CBNA)
(6aR,9R)-Δ^{10}-THC	Cannabivarin (CBV)
(6aR,9S)-Δ^{10}-THC	exo-THC
±cis-Δ^{9}-THC	Tetrahydrocannabivarin (THCV)
Δ^{9}-OH-THC	Tetrahydrocannabivarinic Acid (THCVA)
Δ^{9}-THC-COOH	Δ^{8}-Iso-THC
Δ^{8}-THC	Δ^{8}-THC Acetate (Δ^{8}-THC-O-Acetate)
9R-Δ^{7}-THC	Δ^{8}-Tetrahydrocannabiphorol (Δ^{8}-THCP)
9S-Δ^{6a,10a}-THC	Δ^{9}-Tetrahydrocannabinolic Acid A
9S-Δ^{7}-THC	Δ^{9}-THC Acetate (Δ^{9}-THC-O-Acetate)
Cannabichromene (CBC)	Δ^{9}-Tetrahydrocannabutol (Δ^{9}-THCB)
Cannabichromenic Acid (CBCA)	Δ^{9}-Tetrahydrocannabihexol (Δ^{9}-THCH)
Cannabicyclolic Acid (CBLA)	Δ^{9}-Tetrahydrocannabiorcol (Δ^{9}-THCO)
Cannabidiol (CBD)	Δ^{9}-Tetrahydrocannabiphorol (Δ^{9}-THCP)
Cannabidiolic Acid (CBDA)	8(S)-hydroxy-9(S)-Hexahydrocannabinol
Cannabidivarin (CBDV)	8(R)-hydroxy-9(R)-Hexahydrocannabinol,
Cannabidivarinic Acid (CBDVA)	(±)-9β-hydroxy Hexahydrocannabinol
Cannabigerol (CBG)	(±)-9α-hydroxy Hexahydrocannabinol
Cannabigerolic Acid (CBGA)	(±)-9-nor-9α-hydroxy Hexahydrocannabinol
Cannabigerovarinic Acid (CBGVA)	(±)-9-nor-9β-hydroxy Hexahydrocannabinol
Cannabinol (CBN)	

Potential interferences were identified and noted with each analytical column. Table 6 describes compounds that provided an instrumental response (either quantifier or qualifier transitions) within the dynamic MRM window of the specified compound.

Table 6 Interfering compound evaluation

Interference Summary		
Compound	Poroshell PFP 3.0 x 100 mm, 2.7 µm	Poroshell 120 EC-C18 3.0 x 50 mm, 2.7 µm
Δ^{8}-OH-THC	Δ^{9}-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC	Δ^{9}-OH-THC, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC
Δ^{8}-THC-COOH	Δ^{9}-THC-COOH	Δ^{9}-THC-COOH
9S-Hexahydrocannabinol	9S-Δ^{7}-THC, Δ^{8}-Iso-THC, 9R-Hexahydrocannabinol, Δ^{9}-THC	Δ^{8}-THC, CBL, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC, 9R-Δ^{6a,10a}-THC, 9S-Δ^{6a,10a}-THC, 9S-Δ^{7}-THC, CBCA, CBN, exo-THC, Δ^{8}-Iso-THC, 9R-Hexahydrocannabinol
9R-Hexahydrocannabinol	9S-Δ^{7}-THC, CBN, 9S-Hexahydrocannabinol, Δ^{9}-THC	Δ^{8}-THC, CBL, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC, 9R-Δ^{6a,10a}-THC, 9S-Δ^{6a,10a}-THC, 9S-Δ^{7}-THC, CBCA, CBN, exo-THC, Δ^{8}-Iso-THC, 9S-Hexahydrocannabinol
9R-Δ^{6a,10a}-THC	(6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC, 9S-Δ^{6a,10a}-THC, CBC, Δ^{8}-THC-O-Acetate	Δ^{8}-THC, CBL, (6aR,9R)-Δ^{10}-THC, (6aR,9S)-Δ^{10}-THC, 9R-Δ^{7}-THC, 9S-Δ^{6a,10a}-THC, 9S-Δ^{7}-THC, CBC, CBCA, CBN, exo-THC, THCVA, Δ^{8}-Iso-THC, Δ^{8}-THC-O-Acetate, Δ^{9}-THC-O-Acetate, Δ^{9}-THCB, Δ^{9}-THC

The compounds listed in Table 6 are compounds that produced an instrumental response for either the quantifier or qualifier transition within the Dynamic MRM window of the analyte of interest. All the compounds listed within Table 6 except for Δ^{10}-THC either did not meet the predetermined acceptance criteria for retention time (±3%) or did not produce a qualifier ratio within ±20% of the target compound. On the C18 analytical column (6aR,9R)-Δ^{10}-THC produced a significant instrumental response with acceptable retention time and qualifier ratios. On the PFP analytical column (6aR,9S)-Δ^{10}-THC produced a significant instrumental response with acceptable retention time and qualifier ratios. One additional note is that 9S-hexahydrocannabinol and 9R-hexahydrocannabinol produced instrumental responses within each Dynamic MRM window with acceptable retention time and qualifier ratios. These compounds are chromatographically resolved and should employ relative retention time for retention time evaluation.

6. Stability

The stability of extracted samples that were not analyzed immediately was evaluated at two concentrations for each matrix type (blank blood, antemortem blood, postmortem blood, and urine). The low concentration included Δ^{8}-OH-THC, 9R-hexahydrocannabinol, 9R-Δ^{6a,10a}-THC at 10 ng/mL and Δ^{8}-THC-COOH and 9S-hexahydrocannabinol at 25 ng/mL. The high concentration included Δ^{8}-OH-THC at 100 ng/mL and 9R-hexahydrocannabinol, 9S-hexahydrocannabinol, and 9R-Δ^{6a,10a}-THC at 50 ng/mL with Δ^{8}-THC-COOH at 250 ng/mL. The samples were extracted and injected immediately in triplicate to establish the Day 1 instrumental response. Both concentration levels were subsequently injected in triplicate every twenty-four hours over a six-day period. It was intended to evaluate the stability for a seven-day period but due to sample evaporation the stability study ended after five days. The stability study was performed in a cooled autosampler that was maintained at approximately 4°C to minimize evaporation.

The instrumental response was compared for each time point. If the average instrumental response decreased below 80% or increased above 120% of the average Day 1 response, then the target was considered unstable after that time. Table 7 shows the stability for both analytical columns at low and high concentrations for Δ^{8}-OH-THC in each matrix type.

Table 7 Δ^{8}-OH-THC injection stability

Stability Study Δ^{8}-OH-THC										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	110	134	137	137	100	188	145	206	143
Antemortem Blood	100	136	138	116	125	100	97	112	79	115
Postmortem Blood	100	109	110	110	116	100	97	105	86	118
Urine	100	107	91	101	114	100	130	110	121	157
100 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	102	136	111	142	100	106	111	104	103
Antemortem Blood	100	110	141	158	112	100	101	116	98	110
Postmortem Blood	100	114	109	112	117	100	96	110	104	80
Urine	100	124	80	122	113	100	124	119	124	128

When evaluating the stability of the C18 column, Δ[8]-OH-THC was stable for one day in antemortem blood and urine, two days in blank blood, and five days in postmortem blood. The stability of the PFP column was slightly different than the C18 analytical column. At the high concentration, all matrices were stable for five days with the exception of urine. At the low concentration, postmortem blood was the only matrix source that was stable for five days. Blank blood and urine were stable for one day while antemortem blood was stable for three days. The stability of Δ[8]-THC-COOH is shown in Table 8.

Table 8 Δ[8]-THC-COOH injection stability

Stability Study Δ[8]-THC-COOH										
25 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	130	137	120	115	100	191	157	221	155
Antemortem Blood	100	140	159	128	138	100	102	114	79	117
Postmortem Blood	100	99	107	101	107	100	99	114	106	139
Urine	100	108	93	102	112	100	127	116	124	158
250 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	108	132	100	123	100	104	113	105	103
Antemortem Blood	100	116	153	170	112	100	103	110	91	104
Postmortem Blood	100	114	111	114	121	100	103	107	97	88
Urine	100	118	80	115	110	100	118	116	120	124

When evaluating the stability of the C18 column, Δ[8]-THC-COOH was stable for one day in blank blood and antemortem blood, four days in postmortem blood, and five days in urine. The stability of the PFP column was slightly different than the C18 analytical column. At the high concentration, all matrices were stable for five days with the exception of urine which was stable for four days. At the low concentration, blank blood and urine were stable for one day, antemortem blood was stable for three days, and postmortem blood was stable for four days. The stability of 9S-hexahydrocannabinol is shown in Table 9.

Table 9 9S-Hexahydrocannabinol injection stability

Stability Study 9S-Hexahydrocannabinol										
25 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	136	140	147	148	100	131	110	127	125
Antemortem Blood	100	111	120	118	126	100	111	111	115	133
Postmortem Blood	100	103	107	110	114	100	110	100	106	117
Urine	100	102	103	103	107	100	136	127	142	167
250 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	110	110	116	100	106	98	101	105
Antemortem Blood	100	102	112	115	105	100	110	106	109	116
Postmortem Blood	100	99	104	111	114	100	107	102	109	121
Urine	100	103	93	99	96	100	120	115	123	127

When evaluating the stability of the C18 column, 9S-hexahydrocannabinol was stable for five days in all matrices at the high concentration. At the low concentration, postmortem blood and urine were stable for five days, antemortem blood was stable for two days, and blank blood was stable for one day. The stability of the PFP column was slightly different than the C18 analytical column. Blank blood and urine were determined to be stable for one day while antemortem blood and postmortem blood was determined to be stable for four days. The stability of 9R-hexahydrocannabinol is shown in Table 10.

Table 10 9R-Hexahydrocannabinol injection stability

Stability Study 9R-Hexahydrocannabinol										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	136	138	145	147	100	135	106	124	128
Antemortem Blood	100	111	119	120	128	100	115	112	121	138
Postmortem Blood	100	103	108	111	117	100	112	104	110	120
Urine	100	103	105	105	109	100	135	129	145	167
50 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	109	112	118	100	110	94	102	108
Antemortem Blood	100	103	113	115	105	100	114	108	114	121
Postmortem Blood	100	101	108	117	125	100	111	107	115	127
Urine	100	103	94	101	98	100	119	115	123	126

The stability of 9R-hexahydrocannabinol was similar to 9S-hexahydrocannabinol. When evaluating the stability of the C18 column, 9R-hexahydrocannabinol was stable for five days in all matrices at the high concentration with the exception of postmortem blood that was determined to be stable for four days. At the low concentration, postmortem blood and urine were stable for five days, antemortem blood was stable for four days, and blank blood was stable for one day. The stability of the PFP column was slightly different than the C18 analytical column. Blank blood and urine were determined to be stable for one day while antemortem blood was stable for three days. Postmortem blood was determined to be stable for four days. The stability of 9R-$\Delta^{6a,10a}$-THC is shown in Table 11.

Table 11 9R-$\Delta^{6a,10a}$-THC injection stability

Stability Study 9R-$\Delta^{6a,10a}$-THC										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	138	141	149	150	100	134	105	119	124
Antemortem Blood	100	110	115	118	124	100	98	105	103	105
Postmortem Blood	100	102	106	109	113	100	98	99	96	92
Urine	100	103	105	107	110	100	133	124	138	163
50 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	110	112	119	100	110	97	101	107
Antemortem Blood	100	103	113	114	107	100	100	101	100	101
Postmortem Blood	100	100	105	112	113	100	95	98	93	91
Urine	100	103	94	101	99	100	120	115	121	124

When evaluating the stability of the C18 column, 9R-$\Delta^{6a,10a}$-THC was stable for five days in all matrices at the high concentration. At the low concentration, postmortem blood and urine were stable for five days, antemortem blood was stable for four days, and blank blood was stable for one day. The stability of the PFP column was slightly different than the C18 analytical column. Blank blood and urine were determined to be stable for one day while antemortem blood and postmortem blood was determined to be stable for five days.

Given the variability of instrumental responses observed from day-to-day injections, the internal standard variability was also assessed. The internal standards provided similar variability as compared to the target analytes. Therefore, the average relative response of analyte to internal standard was evaluated to assess the impact on the quantitative results from the change in raw instrumental response. The percent response from the Day 1 ratioed response is show in Tables 12, 13, 14, 15, and 16.

Table 12 Δ^{8}-OH-THC ratioed injection stability

Ratioed Stability Study Δ^{8}-OH-THC										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	90	94	93	92	100	95	100	97	97
Antemortem Blood	100	102	99	101	100	100	94	100	102	104
Postmortem Blood	100	99	99	100	99	100	110	91	87	88
Urine	100	103	104	105	102	100	101	100	100	102
100 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	100	99	99	97	100	98	99	99	100
Antemortem Blood	100	102	107	108	104	100	95	105	108	108
Postmortem Blood	100	96	98	100	98	100	92	104	108	95
Urine	100	102	102	103	102	100	98	99	98	98

Table 13 Δ[8]-THC-COOH ratioed injection stability

Ratioed Stability Study Δ[8]-THC-COOH										
25 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	100	95	85	82	100	102	105	106	105
Antemortem Blood	100	101	118	134	142	100	101	92	93	98
Postmortem Blood	100	91	94	97	96	100	112	105	108	108
Urine	100	102	103	103	104	100	100	102	100	99
250 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	107	96	92	88	100	100	102	101	101
Antemortem Blood	100	105	112	111	109	100	98	95	95	101
Postmortem Blood	100	99	101	110	117	100	101	102	98	100
Urine	100	99	100	99	100	100	100	99	100	100

Table 14 9S-Hexahydrocannabinol ratioed injection stability

Ratioed Stability Study 9S-Hexahydrocannabinol										
25 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	103	102	103	100	98	102	101	100
Antemortem Blood	100	108	104	104	104	100	107	107	109	112
Postmortem Blood	100	99	98	101	98	100	108	107	111	115
Urine	100	97	100	96	95	100	102	102	103	102
250 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	103	101	102	98	100	99	102	101	100
Antemortem Blood	100	96	98	97	95	100	109	108	111	115
Postmortem Blood	100	96	100	98	98	100	105	105	112	114
Urine	100	100	100	97	99	100	100	100	102	102

Table 15 9R-Hexahydrocannabinol ratioed injection stability

Ratioed Stability Study 9R-Hexahydrocannabinol										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	102	100	102	100	101	98	99	102
Antemortem Blood	100	108	103	106	106	100	111	108	115	116
Postmortem Blood	100	99	100	102	101	100	110	112	116	118
Urine	100	97	101	98	97	100	101	104	105	103
50 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	99	103	100	100	103	98	102	103
Antemortem Blood	100	96	98	97	96	100	113	110	116	119
Postmortem Blood	100	98	103	104	107	100	109	111	118	119
Urine	100	100	101	99	102	100	99	100	102	102

Table 16 9R-$\Delta^{6a,10a}$-THC ratioed injection stability

Ratioed Stability Study 9R-$\Delta^{6a,10a}$-THC										
10 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	106	104	103	104	100	99	98	94	100
Antemortem Blood	100	107	99	104	103	100	95	100	98	88
Postmortem Blood	100	97	98	100	97	100	97	106	102	90
Urine	100	97	101	100	99	100	108	111	112	109
50 ng/mL										
	C18 Analytical Column					PFP Analytical Column				
Matrix Type	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Blank Blood	100	104	101	103	101	100	103	101	101	102
Antemortem Blood	100	97	98	96	98	100	99	102	102	99
Postmortem Blood	100	96	100	99	97	100	94	101	95	86
Urine	100	100	100	99	103	100	104	103	105	106

When evaluating the stability of the ratio of analyte to internal standard for all compounds were found to be stable for five days with the exception of Δ^8-THC-COOH using the C18 analytical column. The ratio of analyte to internal standard deviated greater than ±20% of the Day 1 response on Day 4 and Day 5. Therefore, Δ^8-THC-COOH was determined to be stable for three days post extraction. All other compounds met the predetermined acceptance criteria when using either the C18 or PFP analytical columns.

7. Robustness

Analysis for the validation was completed on two instrument models to capture the variability between instrumentation. Agilent Technologies 6460 and 6470 LCMSMS instruments were utilized during validation. Critical experiments such as limit of detection samples were evaluated using all instrument models.

8. Summary

The cannabinoids evaluated within the qualitative validation include Δ^8-OH-THC, Δ^8-THC-COOH, 9S-Hexahydrocannabinol, 9R-Hexahydrocannabinol, and 9R-$\Delta^{6a,10a}$-THC. Prior to validation it was noted that 9R-$\Delta^{6a,10a}$-THC and Δ^{10}-THC co-eluted and shared the same qualifier ion ratios. Therefore, these two compounds cannot be distinguished from each other within this method. 9R-$\Delta^{6a,10a}$-THC was utilized as the certified reference material during validation.

The estimated limit of detection was evaluated for each compound in blank blood, antemortem blood, postmortem blood, and urine. The estimated limit of detection was determined to be a lower concentration (2 ng/mL) for blank blood compared to other matrices evaluated within the method. This was specifically observed for 9S-hexahydrocannabinol and 9R-$\Delta^{6a,10a}$-THC. Although the estimated limit of detection was lower for these two compounds in blank blood, the estimated limit of detection for all other matrices and all other compounds was determined to be 5 ng/mL.

Ionization suppression/enhancement was evaluated for the target compounds and internal standards within the method. Significant ionization suppression was noted when evaluating both the C18 analytical column and the PFP analytical column. Given the significant ionization suppression noted, additional matrix sources were evaluated when assessing the estimated limit of detection. Carryover was assessed during validation and no carryover was detected.

When evaluating interferences within the method, no endogenous interferences were noted. During the assessment of the contribution of analyte to internal standard response and internal standard response to analyte, it was noted that 9S-hexahydrocannabinol and 9R-hexahydrocannabinol interfered with the qualifier ion transition for Δ^9-THC-D_3 on the PFP analytical column. No other interferences from a high concentration of analyte were detected. The assessment of interferences from commonly encountered compounds included an evaluation of phytocannabinoids, semi-synthetic cannabinoids, and synthetic cannabinoids. Although several cannabinoids produced an instrumental response in the dynamic MRM window for both the C18 and PFP analytical columns, the response was either outside of the retention time acceptance criteria or the qualifier ratio and therefore would not be misidentified as the compound of interest. The only compound that produced an interference that could not be distinguished from the target compound was Δ^{10}-THC.

When evaluating the stability of the compounds within the method, the raw instrumental response was highly variable for most compounds. Therefore, the ratioed response of analyte to internal standard was assessed. All compounds, in all matrices were determined to be stable for five days with the exception of Δ^8-THC-COOH in antemortem blood using the C18 analytical column which was stable for three days. The comprehensive validation demonstrates that the supported liquid extraction method with analysis using LCMSMS is fit for the purpose of evaluating Δ^8-OH-THC, Δ^8-THC-COOH, 9S-Hexahydrocannabinol, 9R-Hexahydrocannabinol, and 9R-$\Delta^{6a,10a}$-THC qualitatively.

All data from the validation has been stored on the Toxicology Validation SharePoint.

9. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 27, **2023**.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 29, **2023**.

Siddiqi, A., Wagner, R. Validation of Cannabinoids Quantitation and Confirmation by LCMSMS. Virginia Department of Forensic Science. **2023.**

Herr, D., Siddiqi, A., Wagner, R. Cannabinoids quantitation and confirmation by LCMSMS method development. Virginia Department of Forensic Science. **2022**.

ANSI/ASB Standard 036, *Standard Practices for Method Validation in Forensic Toxicology*. 1st Edition. **2019**.

ANSI/ASB 098 Standard, *Standard for Mass Spectral Analysis in Forensic Toxicology.* 1st Edition. **2023**.

OUTCOMES

10. Activities/Accomplishments

Within the research project, a method was developed for the quantitative and qualitative evaluation of cannabinoids in biological matrices using supported liquid extraction. The methodology employed LCMSMS with two analytical columns of different stationary phases to enhance the confirmation of cannabinoids. Two methods (quantitative and qualitative) were validated in accordance with ANSI/ASB 036) 036, *Standard Practices for Method Validation in Forensic Toxicology*.

11. Results of Findings

Given the structural similarities of cannabinoids, specifically tetrahydrocannabinol isomers, it is imperative to have chromatographic separation for proper identification and quantitation. To enhance the selectivity of LCMSMS, a two-column chromatographic method was developed to enable additional confirmation regarding the identity of a compound. Within the validations, the evaluation of interferences from other cannabinoids was critical in the assessment of the method and its validity.

12. Limitations

Currently, the method is not in an automated format which limits the number of samples able to be evaluated within a given analytical run. Additionally, the method is unable to distinguish 9R-$\Delta^{6a,10a}$-THC, (6aR,9R)-Δ^{10}-THC, and (6aR,9S)-Δ^{10}-THC.

ARTIFACTS

Oral Presentation: Quantitative Analysis of Δ^{9}-Tetrahydrocannabinol (THC) in the presence of THC Isomers in Biological Specimens using Liquid Chromatography Tandem Mass Spectrometry. 2022 NIJ Forensic Science Research and Development (R&D) Symposium.

Oral Presentation: Analytical Challenges Associated with the Quantitative Analysis of Tetrahydrocannabinol Isomers in Biological Matrices. Society of Forensic Toxicologists (SOFT) Conference, Cleveland, OH. 2022.

Poster Presentation: Evaluation of a Quantitative Analysis Method for Tetrahydrocannabinol Isomers in Biological Matrices. Society of Forensic Toxicologists (SOFT) Conference, Denver, CO. 2023.