IDENTIFICATION AND QUANTITATION OF 21 CANNABINOIDS IN NEAT ORAL FLUID BY UHPLC-MS/MS

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INTRODUCTION

Testing for cannabinoid use has been driven by concerns about consumer health, unregulated product content, and workplace safety. The use of oral fluids has been accepted by the US Department of Health and Human Services as a matrix for the Federal Drug Testing Program and allows for noninvasive sample collection. The method developed by our laboratory provides a detailed analysis of neat oral fluid specimens, evaluating the presence of 21 cannabinoids including Cannabidiol (CBD) and CBD metabolites at concentrations from 0.025 to 10 ng/mL.

OBJECTIVE

Develop an analytical method for extraction, detection, and quantitation of (-)-Δ9-THC, Δ9-Carboxy-THC (Δ9-COOH-THC), 11-Hydroxy-THC (11-OH-THC), 8β-Hydroxy-Δ9-THC (8β-OH-Δ9-THC), Δ9-Tetrahydrocannabivarin (Δ9-THCV), Δ9-Carboxy-Tetrahydrocannabivarin (Δ9-COOH-THCV), (-)-Δ8-THC, Δ8-Carboxy-THC (Δ8-COOH-THC), Δ8-Tetrahydrocannabivarin (Δ8-THCV), Cannabidiol (CBD), 7-Hydroxy-Cannabidiol (7-OH-CBD), 7-Carboxy-Cannabidiol (7-COOH-CBD), Cannabidiolic Acid (CBDA), Cannabinol (CBN), Cannabinolic Acid (CBNA), Cannabichromene (CBC), Cannabichromenic Acid (CBCA), Cannabigerol (CBG), Cannabigerolic Acid (CBGA), Cannabicyclol (CBL), and Cannabicyclolic Acid (CBLA) in oral fluid by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for a controlled dosing research study.

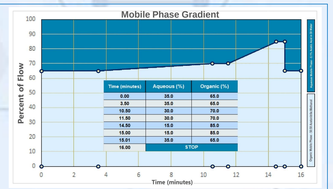
EXTRACTION METHOD

500 µL of oral fluid sample was mixed well with 100 µL of internal standard solution and 600 µL of 0.1M Ammonium Bicarbonate (pH 10.5) in appropriately labeled silanized glass culture tubes. A liquid-liquid extraction was performed by adding 2.5 mL of 80:20 tert-Butyl Methyl Ether:Isopropyl Alcohol, vortexing for 5 minutes, and separation by centrifugation. Samples were then frozen and the organic layer was collected and subsequently dried and reconstituted with 300 µL of 50:50 0.1% Acetic Acid in DIH20:Acetonitrile.

INSTRUMENT PARAMETERS

Table 1: UHPLC-MS/MS Parameters

UHPLC System	Shimadzu Nexera	LC-30AD Pumps
		SIL-30 AC Auto Sampler
	Shimadzu Prominence	CBM-20A Communications Bus Module
		CTO-20A Column Oven
		DGU-20As Degasser
Injection Volume	30 µL	
Analytical Column	Phenomenex Kinetex 2.6 µm C18 100 Å, 150 x 2.1 mm (Phenomenex Part No. 00F-4462-AN)	
Guard Column	Phenomenex SecurityGuard ULTRA Cartridge, UHPLC C18 2.1mm ID Column (Phenomenex Part No. AJ0-8782)	
Column Temp.	45°C	
Mobile Phase	Aqueous	0.1% Acetic Acid in DI H2O
	Organic	50:50 Acetonitrile:Methanol
Flow Rate	0.750 mL/min	
Run Time	16.00 minutes	
Mass Spectrometer	Sciex API7500 Triple Quad	
Ionization	ESI	Positive and Negative
Source Temp.	550°C	
Scheduled MRM	80-sec detection window	
	Target cycle time	2000 milliseconds



Positive Ionization					
Analyte	Internal Standard	Transitions (±0.3 amu)			Retention Time (± 0.8 minute)
		Precursor	Quant	Qual	
7-OH-CBD	7-OH-CBD-D3	331.2	313.2	193.1	1.52
	7-OH-CBD-D3	334.3	316.1	196.1	1.51
8-β-OH-Δ9-THC	11-OH-THC-D3	331.2	201.1	271.2	2.29
	11-OH-THC-D3	334.2	196.1	201.0	3.51
11-OH-THC	11-OH-THC-D3	331.2	193.1	201.0	3.55
Δ8-THCV	11-OH-THC-D3	287.2	165.1	123.1	5.30
CBD	CBD-D3	315.2	193.1	259.1	5.77
Δ9-THCV	11-OH-THC-D3	287.2	165.1	123.1	5.75
CBG	CBG-D3	317.2	123.0	193.1	6.26
	CBG-D3	320.2	123.0	196.1	6.22
CBN	CBN-D3	311.2	223.1	195.1	8.86
	CBN-D3	314.2	223.1	195.1	8.80
Δ9-THC	Δ9-THC-D3	315.2	193.1	123.0	10.63
	Δ9-THC-D3	318.2	196.1	123.0	10.57
Δ8-THC	Δ8-THC-D9	315.2	193.1	123.0	11.27
	Δ8-THC-D9	324.2	202.1	123.0	11.09
CBL	11-OH-THC-D3	315.2	235.1	193.0	11.87
	11-OH-THC-D3	334.2	196.1	201.0	3.51
CBC	CBC-D9	315.2	193.1	123.0	13.70
	CBC-D9	324.2	193.1	123.0	13.58

Negative Ionization					
Analyte	Internal Standard	Transitions (±0.3 amu)			Retention Time (± 0.8 minutes)
		Precursor	Quant	Qual	
7-COOH-CBD	7-COOH-CBD-D3	343.2	231.1	299.2	1.45
	7-COOH-CBD-D3	346.2	234.2	302.2	1.44
THCVA	Δ9-THCA-D9	315.2	163.0	271.1	1.92
	Δ9-THCA-D9	352.2	308.2	254.2	4.10
Δ8-THCA	Δ8-THCA-D6	343.2	299.2	245.1	3.92
	Δ8-THCA-D6	349.2	305.2	254.1	3.84
Δ9-THCA	Δ9-THCA-D9	343.2	299.2	245.1	4.22
CBDA	Δ9-THCA-D9	357.2	245.1	227.1	6.38
CBGA	Δ9-THCA-D9	359.2	341.2	315.2	8.17
CBNA	Δ9-THCA-D9	353.2	309.1	222.0	13.05
CBLA	Δ8-THCA-D6	357.2	339.1	191.1	15.08
CBCA	Δ8-THCA-D6	357.2	191.1	339.1	15.34

RESULTS / DISCUSSION

A single-point calibrator at 2.0 ng/mL was used for quantitation. A low control at 0.8 ng/mL (40% of calibrator), two positive controls at 2.5 ng/mL (125% of calibrator), and two negative controls, with one of the negative controls and one of the positive controls injected at the end of the batch to bracket donor samples. In addition to the low and positive controls, a conversion control was included in every batch. The conversion control was used for monitoring the potential conversion of CBD and its metabolites to Δ9 THC and Δ8-THC and corresponding metabolites, and contained CBD, 7-OH-CBD, 7-COOH-CBD, and CBDA at 5.0 ng/mL.
Linearity was evaluated by spiking synthetic oral fluid with various concentrations of (-)-Δ9-THC, Δ9-COOH-THC, 11-OH-THC, 8β-OH-Δ9-THC, Δ9-THCV, Δ9-COOH-THCV, (-)-Δ8-THC, Δ8-COOH-THC, Δ8-THCV, CBD, 7-OH-CBD, 7-COOH-CBD, CBDA, CBN, CBNA, CBC, CBCA, CBG, CBGA, CBL, and CBLA over the analytical range of 25.0 pg/mL to 10 ng/mL. Assay limits of detection and quantitation (LOD/LOQ) and upper limit of linearity (ULOL) were established through the assessment of accuracy and precision data from the analysis of 5 replicates of each of 13 concentration levels, which included 40%, 50%, 100%, 125%, 150%, and 200% of the calibrator. At the 0.025 ng/mL level, all analytes had replicates that met quantitative acceptability criteria of within ±20 of target and met qualitative acceptance criteria (see Table 6), except for 8β-OH-Δ9-THC, which was acceptable at 0.05 ng/mL. At the upper limit of linearity, replicates for all analytes were within ±20% of target and met all chromatographic acceptance criteria at 5.0 ng/mL. Replicates for 8β-OH-Δ9-THC, 11-OH-THC, Δ8-THCV, CBD, Δ9-THCV, Δ9-THC, Δ8-THC, CBC, 7-COOH-CBD, Δ9-COOH-THCV, Δ8-COOH-THC, Δ9-COOH-THC, and CBDA met all quantitative and qualitative acceptance criteria at 10.0 ng/mL.

Figure A: Representative Chromatogram and Structures of Method Analyte Components in Positive and Negative Ionization Modes

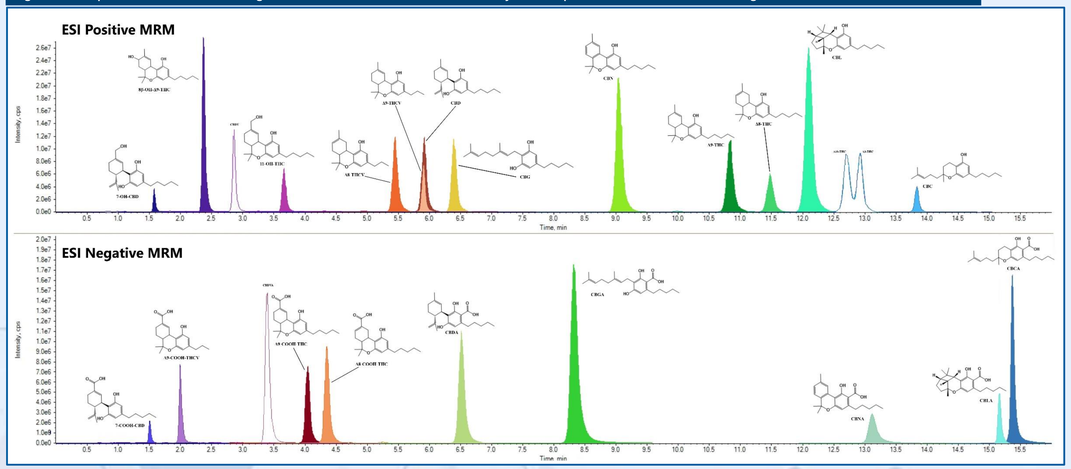


Figure B: Analyte Linearities

Table 2: Analyte LOD/LOQ and ULOL Accuracy and Precision

Analyte	LOD/LOQ (ng/mL)	Mean at LOD/LOQ	% Mean Accuracy	%CV	ULOL (ng/mL)	Mean at ULOL	% Mean Accuracy	%CV
7-OH-CBD	0.025	0.021	84.8	2.11	5.0	4.86	97.2	3.29
8-βOH-Δ9-THC	0.050	0.048	96.000	5.311	10.0	8.481	84.808	3.354
11-OH-THC	0.025	0.023	91.2	5.72	10.0	9.382	93.820	3.866
Δ8-THCV	0.025	0.024	96.8	5.39	10.0	9.289	92.9	5.02
CBD	0.025	0.025	100.8	9.47	10.0	9.463	94.6	4.09
Δ9-THCV	0.025	0.024	96.8	6.79	10.0	10.546	105.5	6.06
CBG	0.025	0.026	102.4	3.49	5.0	4.737	94.7	4.10
CBN	0.025	0.025	99.2	3.37	5.0	4.520	90.4	3.35
Δ9-THC	0.025	0.024	96.0	2.95	10.0	9.501	95.0	2.33
Δ8-THC	0.025	0.026	102.4	5.24	10.0	10.063	100.6	3.45
CBL	0.025	0.024	96.0	5.89	5.0	4.710	94.2	3.25
CBC	0.025	0.024	96.8	8.96	10.0	9.306	93.1	7.24
7-COOH-CBD	0.025	0.024	94.4	7.70	10.0	8.896	89.0	2.14
Δ9-COOH-THCV	0.025	0.024	96.0	8.33	10.0	9.636	96.4	2.03
Δ8-COOH-THC	0.025	0.025	100.4	1.16	10.0	9.937	99.4	3.04
Δ9-COOH-THC	0.025	0.024	95.2	1.88	10.0	9.416	94.2	2.21
CBDA	0.025	0.025	100.0	2.83	10.0	8.742	87.4	1.56
CBGA	0.025	0.026	103.2	3.24	5.0	4.125	82.5	2.00
CBNA	0.025	0.026	103.2	3.24	5.0	4.233	84.7	1.64
CBLA	0.025	0.028	112.8	1.59	5.0	4.033	80.7	0.19
CBCA	0.025	0.026	104.0	4.71	5.0	4.075	81.5	1.84

Table 3: Evaluation of Carryover

Analytes	Matrix Samples Spiked at 0.8ng/mL		
	Carryover Check	Analyte Peak Area	% of Calibrator Area
7-OH-CBD	Negative after 5.0 ng/mL	81789.13	0.123
8β-OH-Δ9-THC	Negative after 10.0 ng/mL	45072.41	0.067
11-OH-THC	Negative after 10.0 ng/mL	7760.43	0.025
Δ8-THCV	Negative after 10.0 ng/mL	13250.26	0.022
CBD	Negative after 10.0 ng/mL	3950.95	0.013
Δ9-THCV	Negative after 10.0 ng/mL	506.84	0.004
CBG	Negative after 5.0 ng/mL	547.98	0.001
CBN	Negative after 5.0 ng/mL	14452.37	0.011
Δ9-THC	Negative after 10.0 ng/mL	13258.12	0.018
Δ8-THC	Negative after 10.0 ng/mL	14624.84	0.038
CBL	Negative after 5.0 ng/mL	5577.93	0.003
CBC	Negative after 10.0 ng/mL	5293.60	0.032
7-COOH-CBD	Negative after 10.0 ng/mL	5638.59	0.096
Δ9-THCV-COOH	Negative after 10.0 ng/mL	794.67	0.021
Δ8-THC-COOH	Negative after 10.0 ng/mL	2076.65	0.020
Δ9-THC-COOH	Negative after 10.0 ng/mL	21144.98	0.170
CBDA	Negative after 10.0 ng/mL	2407.51	0.009
CBGA	Negative after 5.0 ng/mL	23936.05	0.016
CBNA	Negative after 5.0 ng/mL	22268.35	0.016
CBLA	Negative after 5.0 ng/mL	52579.92	0.128
CBCA	Negative after 5.0 ng/mL	39575.25	0.063

Carryover was tested by injecting negative samples after the ULOL (5.0 ng/mL and 10.0 ng/mL) spiked samples. Carryover passed for all analytes, which were lacking acceptable peak shape, did not have acceptable ion ratios, and had analyte peak area counts less than 10% of the calibrator. Based on these results, the carryover limit was set equal to the ULOL for each analyte.

Table 4: Evaluation of Matrix Effect

Analytes	Matrix Samples Spiked at 0.8ng/mL		
	Mean Calculated Concentration (ng/mL)	Mean Accuracy	Standard Deviation
7-OH-CBD	0.785	98.1%	0.045
8β-OH-Δ9-THC	0.731	91.3%	0.038
11-OH-THC	0.716	89.6%	0.033
Δ8-THCV	0.812	101.5%	0.101
CBD	0.748	93.5%	0.034
Δ9-THCV	0.854	106.7%	0.126
CBG	0.713	89.1%	0.036
CBN	0.735	91.8%	0.025
Δ9-THC	0.753	94.1%	0.033
Δ8-THC	0.723	90.4%	0.049
CBL	0.830	103.7%	0.077
CBC	0.767	95.9%	0.021
7-COOH-CBD	0.707	88.4%	0.027
Δ9-THCV-COOH	0.700	87.5%	0.047
Δ8-THC-COOH	0.704	87.9%	0.025
Δ9-THC-COOH	0.760	95.0%	0.016
CBDA	0.864	108.0%	0.034
CBGA	0.900	112.5%	0.044
CBNA	0.787	98.4%	0.053
CBLA	0.800	100.0%	0.068
CBCA	0.856	107.0%	0.067

The potential of sample matrix components to interfere with the analytical method was evaluated by testing ten random negative oral fluid samples that were extracted unaltered and with cannabinoid analytes spiked at 40% of the cutoff concentration (0.8 ng/mL). Results showed no indication of methodic ion suppression or enhancement, as component recovery was consistent and spiked samples passed with analyte concentrations within ±20% of target. All samples passed with acceptable chromatography as no qualitative issues were observed, and no interfering peaks were present in the negative samples that could be problematic in quantitation or identification.

Table 5: Quantitative Acceptance Criteria

Relative Retention Time (RRT)	±2% of expected RRT of the analyte/internal standard pair established by the batch calibrator
Internal Standard (IS) Response	Total IS peak area = ±10% of calibrator IS peak area
Symmetry / Peak Shape	Gaussian peaks; asymmetry at 10% of peak height = <3.0 for IS and quant peaks
Resolution	Adjacent peaks ≥50% resolved (≤ 10% valley/peak height ratio)
Ion Ratios (Qualifiers)	Ratio of abundance of quantitative to qualifier ion = ±20% of target ratio established by batch calibrator

Table 6: Interference Compounds Investigated (500 ng/mL)

Normeperidine	Pheniramine	Nortriptyline
Tramadol	Chlorpheniramine	Norsertraline
ODM-Tramadol	Brompheniramine	Norfluoxetine
Dextromethorphan	Diphenhydramine	Fluoxetine
Pentazocine	Gabapentin	Butalbital
7-Aminonitrazepam	Quetiapine Sulfoxide	Secobarbital
7-Aminoclonazepam	Fentanyl	Phenobarbital
7-Aminoflunitrazepam	Alfentanil	Butabarbital
Hydroxytriazolam	Sufentanil	Amobarbital
Estazolam	Norfentanyl	Pentobarbital
Hydroxyalprazolam	Methadone	Propoxyphene
Nordiazepam	EDDP	Ketamine
Lorazepam	Codeine	Norketamine
2-Hydroxyethylflurazepam	Morphine	Methaqualone
Hydroxymidazolam	Oxycodone	Phenylpropanolamine
Lormetazepam	Oxymorphone	Ephedrine
Oxazepam	Hydrocodone	Pseudoephedrine
Bromazepam	Hydromorphone	Phenylephrine
Temazepam	Norhydrocodone	Phenethylamine
Halazepam	Noroxycodone	Phentermine
Diazepam	6-AM	Acetaminophen
Clonazepam	Dihydrocodeine	Aspirin
Alprazolam	Naltrexone	Ibuprofen
Triazolam	Naloxone	Naproxen
Flurazepam	Nalbuphine	Caffeine
Prazepam	Tapentadol	Hydroxycotinine
Phendimetrazine	Butorphanol	Cotinine
Phenmetrazine	Norbuprenorphine	ETG
Diethylpropion	Buprenorphine	ETS
Ritalinic acid	Cyclobenzaprine	Amphetamine
Meprobamate	Promethazine Sulfoxide	Methamphetamine
Zolpidem	Lamotrigine	MDA
Naltrexol	Aripiprazole	MDMA
Doxylamine	Amitriptyline	MDEA

Interference was assessed for the compounds listed in Table 6 at 500 ng/mL, which includes over-the-counter, illicit, and commonly prescribed drugs. The compounds were spiked in groups into a negative oral fluid sample as well as an oral fluid sample containing the cannabinoid analytes at 40% of calibrator concentration (0.8 ng/mL). Negative samples met acceptance criteria for a negative control, lacking acceptable analyte peak shape and ion ratios, and having analyte peak area counts less than 10% of the calibrator. The 0.8 ng/mL spiked samples passed all qualitative acceptance criteria. Quantitatively, all analytes were within ±20% of target concentration with the exception of 7-OH-CBD. Ion suppression was observed for 7-OH-CBD and 7-OH-CBD-D3 in samples containing Halazepam, yielding lower concentrations. Throughout the interference study, no peaks were observed that were greater than the assay LOQ (0.025 or 0.050 ng/mL, analyte dependent), which could create possible quantitation or identification issues. All results were considered acceptable for validation.

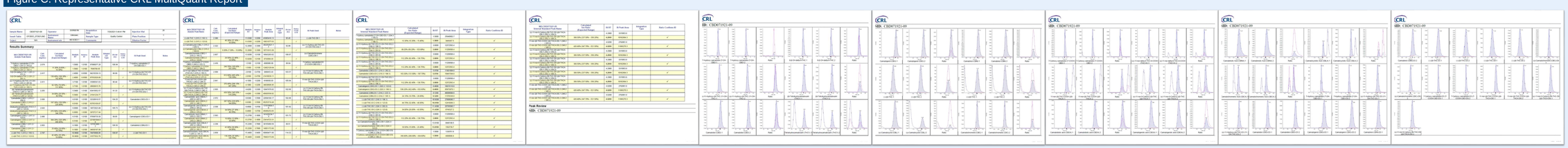
CONCLUSION

Designed for a research study using oral fluid, the alkaline sample extraction favored recovery of parent drug compounds; with the utilization of polarity switching, the sensitivity of the API7500 MS/MS was able to offset reduced recovery of acidic metabolites. For evaluation of the cannabinoid elimination phase, the instrument method was optimized for low-level analyte detection; samples with concentrations greater than the linear range were reanalyzed with a diluted preparation.
The analytical method reliably identified and quantitated 21 cannabinoids in oral fluid in low pg/mL levels, adding to scientific knowledge of cannabinoid metabolism and distribution in oral fluid. This method demonstrated selectivity, accuracy, and reproducibility for federally-sponsored research studies.

DISCLOSURE

No relevant financial or nonfinancial relationships to disclose.

Figure C: Representative CRL MultiQuant Report



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