Determination of 18 Cannabinoids in Urine with Separation of 11-OH-THC Metabolites by UHPLC-MS/MS

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INTRODUCTION

With current legal ambiguity concerning both Δ9-Tetrahydrocannabinol and Δ8-Tetrahydrocannabinol, the importance of complete and specific toxicological analysis for parent drugs and metabolites is paramount. While the significance of testing for Δ9- and Δ8-Carboxy (COOH) THC metabolites cannot be understated, changes in employer drug-testing regulations have emphasized the need for the detection and separation of the psychoactive Hydroxy (OH) metabolites of Δ9- and Δ8-THC. The method developed by our laboratory allows for the quantitative determination of 18 different cannabinoids in urine, including 11-OH-Δ9-THC and 11-OH-Δ8-THC.

OBJECTIVE

Develop an analytical method for the extraction, detection, and quantitation of (-)-Δ9-THC, Δ9-Carboxy-THC (Δ9-COOH-THC), 11-Hydroxy-Δ9-THC (11-OH-Δ9-THC), Δ9-Tetrahydrocannabivarin (THCV), Δ9-Carboxy-Tetrahydrocannabivarin (Δ9-COOH-THCV), (-)-Δ8-THC, 11-Hydroxy-Δ9-THC, (11-OH-Δ9-THC), Δ8-Carboxy-THC (Δ8-COOH-THC), 11-Hydroxy-Δ8-THC (11-OH-Δ8-THC), Δ8-Tetrahydrocannabivarin (THCV), Δ8-Carboxy-Tetrahydrocannabivarin (Δ8-COOH-THCV), Cannabidiol (CBD), 7-Hydroxy-Cannabidiol (7-OH-CBD), 7-Carboxy-Cannabidiol (7-COOH-CBD), Cannabidiolic Acid (CBDA), Cannabinol (CBN), Cannabichromene (CBC), Cannabigerol (CBG), and Cannabicyclol (CBL) in urine by LC-MS/MS for a controlled dosing research study.

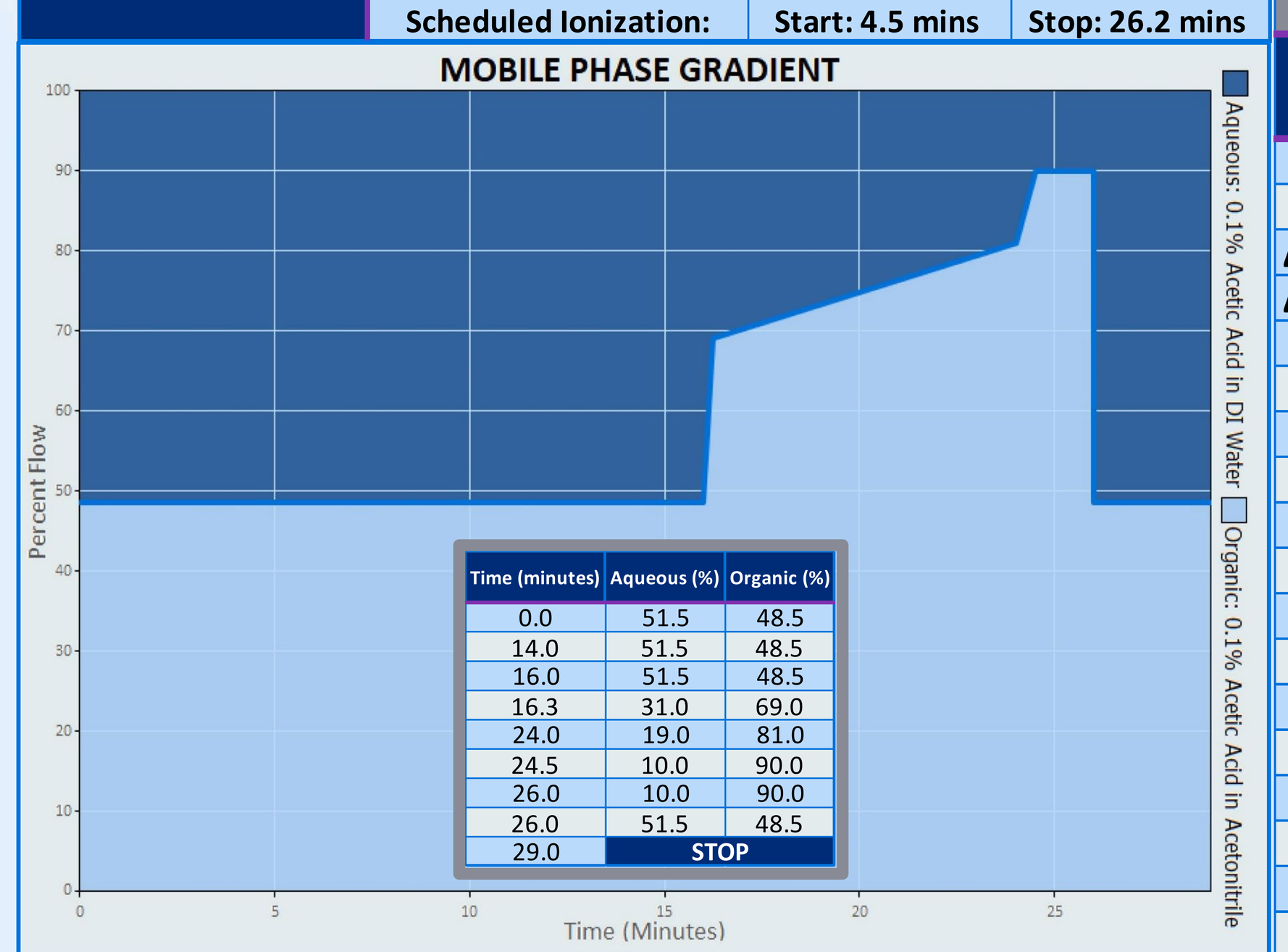
EXTRACTION METHOD

A 500 µL aliquot of urine specimen and 100 µL of internal standard were combined with 200 µL of Kura BG Turbo β-glucuronidase/0.1M phosphate buffer (pH 6.8) solution in a silanized glass culture tube. Samples were then incubated at 50°C for 30 minutes for hydrolysis. Following this initial hydrolysis step, a secondary hydrolysis was performed with the addition of 125 µL of 5N Potassium Hydroxide to each tube. Samples were vortexed to mix and hydrolyzed at room temperature for 10 minutes, and subsequently 100 µL 5N Formic Acid was added to each tube for neutralization. 1 mL of salt-saturated 0.1M Sodium Phosphate buffer pH 6.8, 1 mL of Acetonitrile, and 3 ml of 9:1 Hexanes: Ethyl Acetate were added to each tube. Samples were vortex-mixed for 5 minutes, centrifuged to separate, and placed in a dry ice bath to freeze the aqueous layer. The organic layer was decanted into a silanized glass culture tube and evaporated to dryness under a steady stream of nitrogen at 60°C. For reconstitution, 300 µL of 0.1% Formic Acid in 50:50 DI H_2O:Methanol was added to each sample and tubes were vortexed for a minimum of 15 seconds.

INSTRUMENT PARAMETERS

Table 1: UHPLC-MS/MS Parameters

UHPLC System	Shimadzu Nexera	LC-40D X3 Pumps SIL-40C X3 Auto Sampler SCL-40 System Controller CTO-40C Column Oven DGU-405 Degassing Unit
Injection Volume	30 µL	
Analytical Column	(2) Waters CORTECS C18+, 90Å, 2.7 µm, 2.1mm x 150mm (Waters Part No. 186007398)	
Guard Column	Waters CORTECS C18+ VanGuard, 90Å, 2.7 µm, 2.1mm x 5mm (Waters Part No. 186007685)	
Column Temperature	40°C	
Mobile Phase	Aqueous:	0.1% Acetic Acid in DI H2O
	Organic:	0.1% Acetic Acid in Acetonitrile
Flow Rate	0.700 mL/min	
Run Time	29.00 minutes	
Mass Spectrometer	Sciex API6500+ Triple Quad	
Ionization	ESI	Positive and Negative
Source Temperature	650°C	
Scheduled MRM	100-sec detection window; 240 seconds for 11-OH-THC and THC-COOH	
	Positive Target scan time:	0.100 seconds
	Negative Target scan time:	0.050 seconds
	Scheduled Ionization: Start: 4.5 mins	Stop: 26.2 mins



Time (minutes)	Aqueous (%)	Organic (%)
0.0	51.5	48.5
14.0	51.5	48.5
16.0	51.5	48.5
16.3	31.0	69.0
24.0	19.0	81.0
24.5	10.0	90.0
26.0	10.0	90.0
26.0	51.5	48.5
29.0	STOP	

Positive Ionization

Analyte	Internal Standard	Transitions (±0.3 amu) Precursor	Quant	Qual	Retention Time (± 0.8 minutes)
11-OH-Δ9-THC	11-OH-Δ9-THC-D3	331.2	193.1	201.1	14.20
11-OH-Δ9-THC-D3		334.2	196.1	201.1	14.05
11-OH-Δ8-THC	11-OH-Δ8-THC-D3	331.2	193.1	201.1	14.60
11-OH-Δ8-THC-D3		334.2	196.1	201.1	14.45
Δ9-THCV	CBC-D9	287.3	135.0	123.0	19.35
Δ8-THCV	CBD-D3	287.3	135.0	123.0	19.60
CBD	CBD-D3	315.2	193.2	135.2	19.70
CBD-D3		318.2	196.2	123.1	19.65
CBN	CBD-D3	311.1	241.0	208.0	21.45
CBN-D3		314.1	241.0	208.0	21.40
Δ9-THC	Δ9-THC-D3	315.1	193.2	123.0	22.55
Δ9-THC-D3		318.1	196.2	123.0	22.50
Δ8-THC	Δ8-THC-D9	315.1	193.2	135.3	22.80
Δ8-THC-D9		324.1	202.2	135.1	22.65
CBL	Δ8-THC-D9	315.2	193.0	123.0	23.50
CBC	CBC-D3	315.2	193.1	123.1	23.90
CBC-D9		324.2	202.1	123.1	23.80

Negative Ionization

Analyte	Internal Standard	Transitions (±0.3 amu) Precursor	Quant	Qual	Retention Time (± 0.8 minutes)
7-COOH-CBD	7-COOH-CBD-D3	343.2	297.3	179.0	5.55
7-COOH-CBD-D3		346.2	300.3	182.0	5.50
Δ9-COOH-THCV	7-COOH-CBD-D3	315.2	163.1	217.1	5.90
Δ9-COOH-THCV	7-COOH-CBD-D3	315.2	163.1	217.1	6.35
7-OH-CBD	7-OH-CBD-D3	329.2	268.1	179.0	6.00
7-OH-CBD-D3		332.2	271.1	182.0	6.05
11-OH-Δ9-THC	11-OH-Δ9-THC-D3	329.1	268.1	173.0	14.20
11-OH-Δ9-THC-D3		332.1	271.1	173.0	14.05
11-OH-Δ8-THC	11-OH-Δ8-THC-D3	329.1	268.1	173.0	14.60
11-OH-Δ8-THC-D3		332.1	271.1	173.0	14.45
Δ8-COOH-THC	Δ8-COOH-THC-D6	343.1	245.1	191.2	15.25
Δ8-COOH-THC-D6		349.1	251.1	191.1	14.90
Δ9-COOH-THC	Δ9-COOH-THC-D9	343.1	245.2	191.1	15.85
Δ9-COOH-THC-D9		352.1	254.1	194.1	16.40
CBG	CBG-D3	315.0	191.1	177.2	19.75
CBG-D3		318.0	194.0	177.0	19.70
CBDA	CBDA-D3	357.2	245.1	227.1	20.20
CBDA-D3		360.2	248.1	230.1	20.15

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

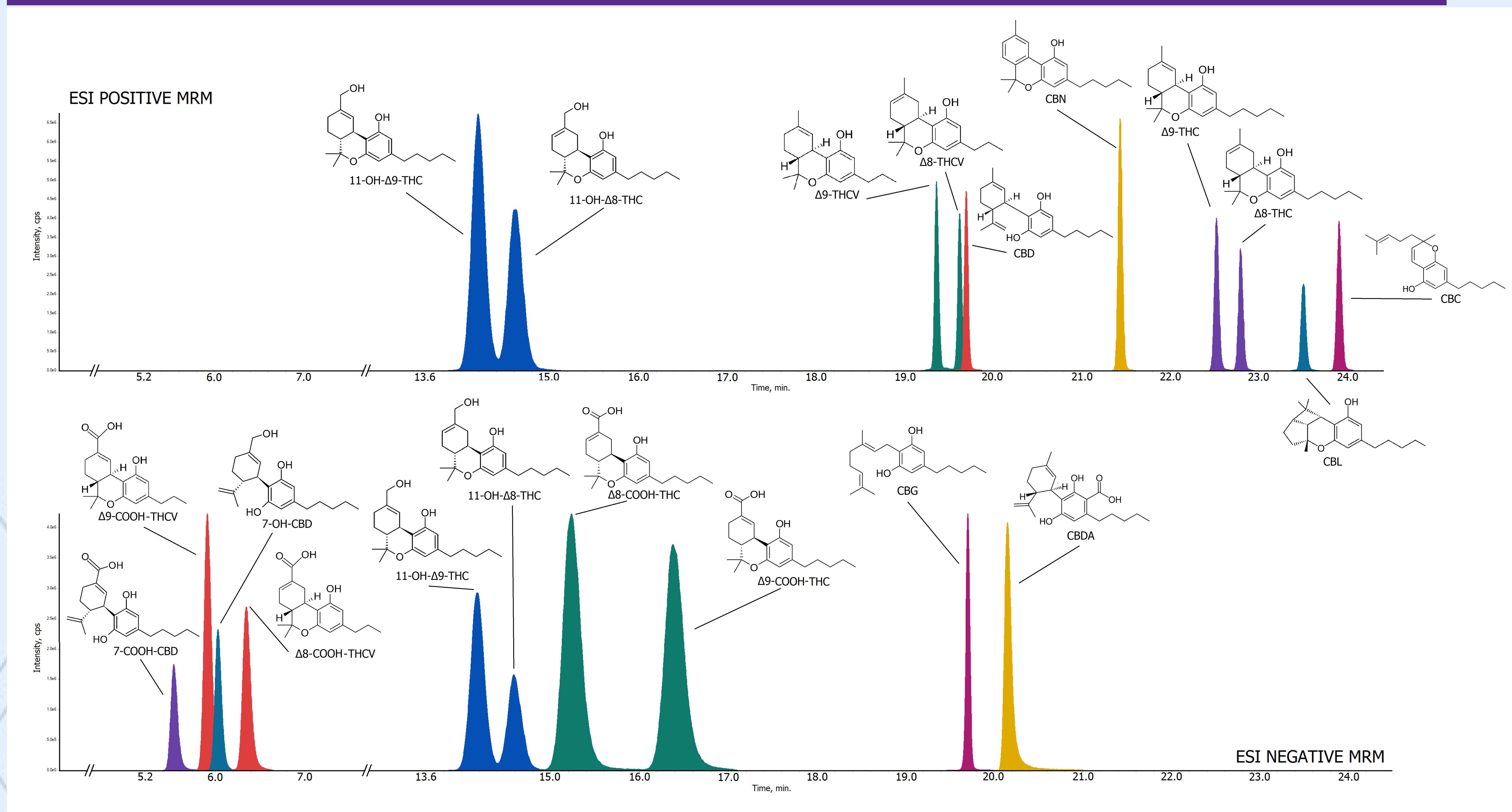
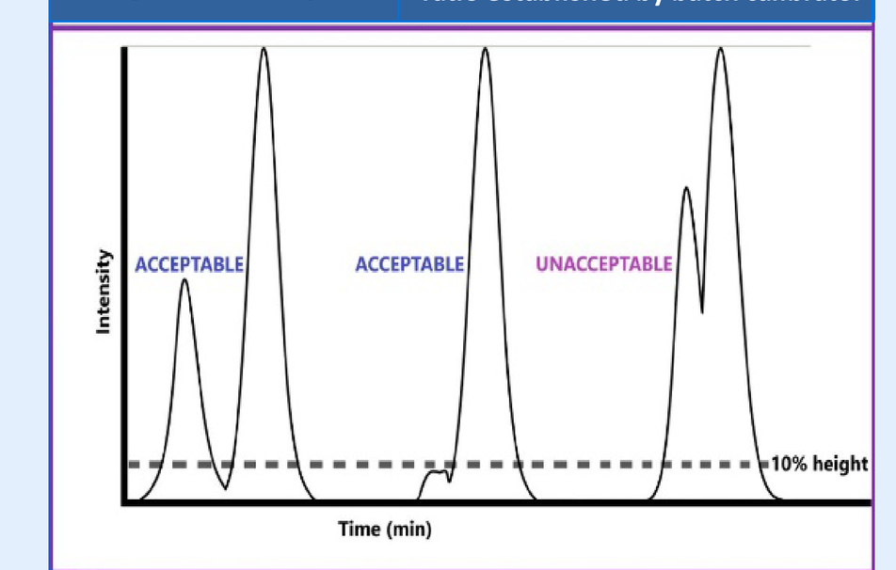
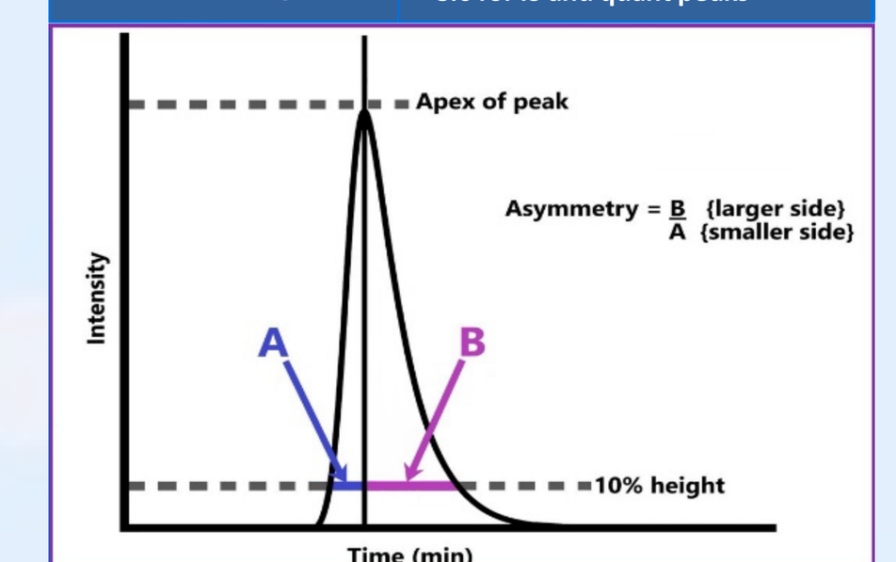


Table 3: Acceptance Criteria

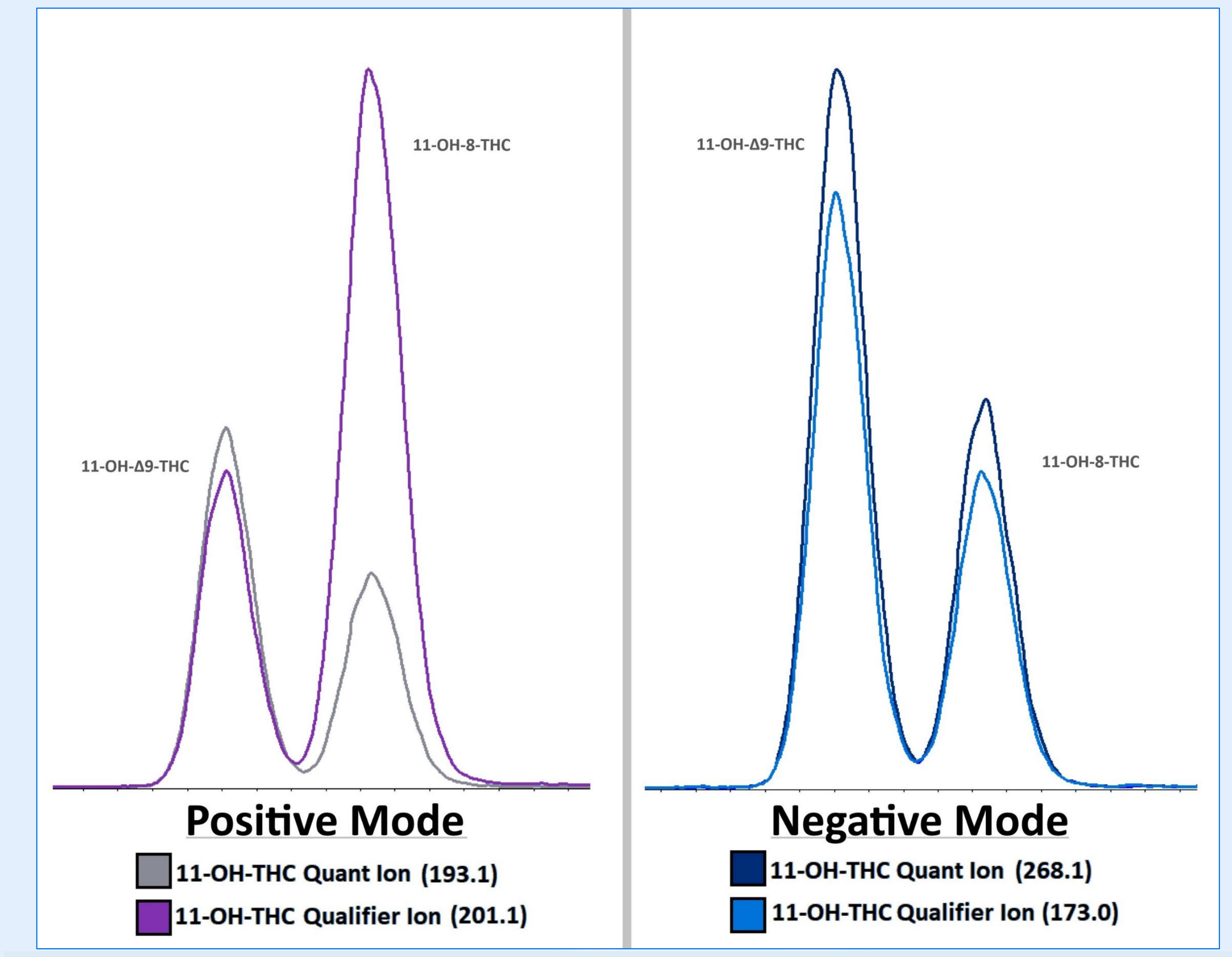
Quantitative Acceptance Criteria	
Relative Retention Time (RRT)	±2% of expected RRT of the analyte/internal standard pair established by the batch calibrator
Internal Standard (IS) Response	Total IS peak area = ≥10% of calibrator IS peak area
Ion Ratios (Qualifiers)	Ratio of abundance of quantitative to qualifier ion = ±20% of target ratio established by batch calibrator
Symmetry / Peak Shape	Gaussian peaks; asymmetry at 10% of peak height = <3.0 for IS and quant peaks
Resolution	Adjacent peaks ≥90% resolved (≤ 10% valley/peak height ratio)





11-OH-THC POSITIVE MODE vs NEGATIVE MODE

Figure C: 11-OH-THC in Positive and Negative Ionization modes



During development, it was noticed that the ion ratios of 11-OH-Δ8-THC behave differently than the ion ratios of 11-OH-Δ9-THC when running in positive mode versus negative mode. In positive mode, the 201.1 qualifier ion has significantly higher intensity for 11-OH-Δ8-THC than 11-OH-Δ9-THC, while the intensity of the 193.1 quant ion remains higher for 11-OH-Δ9-THC in either mode. In negative mode, the 268.1 quant ion and the 173.0 qualifier ion do not exhibit this behavior, and the 11-OH-Δ9-THC ions have greater intensity. In terms of ion ratios, both isomers in negative mode and 11-OH-Δ9-THC in positive mode exhibited Q1:Q3 ratios of less than 1, while the ion ratio for 11-OH-Δ8-THC in positive mode was 3.6. For both 11-OH-THC isomers, positive mode provides more sensitivity than negative mode, making it preferential to analysis. However, in methods that do not differentiate between the 11-OH-Δ8-THC and 11-OH-Δ9-THC metabolites, the increased intensity of the 11-OH-Δ8-THC qualifier ion could cause problems with ion ratio failures in samples containing 11-OH-Δ8-THC. For our laboratory's method, both positive and negative mode were validated for the quantitation of 11-OH-Δ8-THC and 11-OH-Δ9-THC, ensuring proper separation and identification of each isomer.

Table 4: 11-OH-THC Ionization

Positive Mode				Negative Mode			
Analyte	Ion	Area	Ion Ratio	Analyte	Ion	Area	Ion Ratio
11-OH-Δ9-THC	193.1	6427144	0.8946	11-OH-Δ9-THC	268.1	3722339	0.7853
	201.1	7184662			173.0	2923272	
11-OH-Δ8-THC	193.1	4429250	3.7239	11-OH-Δ8-THC	268.1	1828677	0.8142
	201.1	16494010			173.0	1488874	

RESULTS / DISCUSSION

Normal human urine fortified with bovine serum albumin was spiked with the 18 cannabinoid analytes at known concentrations and analyzed to establish method linearity and evaluate assay interference and matrix effects. For assay quantitation, a single-point calibrator at 10.0 ng/mL was used. A low control at 4.0 ng/mL (40% of calibrator), two positive controls at 12.5 ng/mL (125% of calibrator), and two negative controls were run with each analytical batch, 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, every batch included a conversion control and a hydrolysis control. 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. The hydrolysis control was used to verify that the drug-glucuronide conjugates were sufficiently and consistently hydrolyzing during the extraction process. Because commercially manufactured standards were not available, this control was formulated by pooling specimens that confirmed for the presence of 7-OH-CBD and 7-COOH-CBD by LC-MS/MS; the pooled urine was diluted with certified negative urine to yield CBD-metabolite concentrations within assay linearity, and was then spiked with 11-nor-9-carboxy-Δ9-THC glucuronide to ensure a minimum concentration of 50.0 ng/mL of Δ9-COOH-THC after hydrolysis.

Linearity was determined and assay limits of detection and quantitation (LOD/LOQ) and upper limit of linearity (ULOL) were established through the analysis of analyte-spiked samples ranging from 0.500 to 500.0 ng/mL. Accuracy and precision were assessed for 3 replicates of each of 13 concentration levels, including 40%, 50%, 100%, 125%, 150%, and 200% of the calibrator. For assay LOD/LOQ, all analytes met quantitative acceptability criteria with values within ±20% of target, and met all qualitative acceptance criteria (see Table 3) at the 0.5 ng/mL level. At the upper limit of linearity, replicates for all analytes met quantitative and qualitative acceptance criteria at 100.0 ng/mL; 11-OH-Δ8-THC and 11-OH-Δ9-THC replicates met all criteria at 250 ng/mL; and 7-OH-CBD, Δ9-COOH-THC, and Δ8-COOH-THC replicates were fully acceptable at 500.0 ng/mL.

Table 2: Analyte LOQ/ULOL

Analyte	LOD/LOQ (ng/mL)	ULOL (ng/mL)
7-COOH-CBD	0.500	100.0
Δ9-COOH-THCV	0.500	100.0
7-OH-CBD	0.500	500.0
Δ8-COOH-THCV	0.500	100.0
11-OH-Δ9-THC	0.500	250.0
11-OH-Δ8-THC	0.500	250.0
Δ8-COOH-THC	0.500	500.0
Δ8-COOH-THC	0.500	500.0
CBG	0.500	100.0
CBDA	0.500	100.0
Δ9-THCV	0.500	100.0
Δ8-THCV	0.500	100.0
CBD	0.500	100.0
CBN	0.500	100.0
Δ9-THC	0.500	100.0
Δ8-THC	0.500	100.0
CBL	0.500	100.0
CBC	0.500	100.0

Figure B: Analyte Linearities

CONCLUSION

The analytical method reliably identified and quantitated 18 cannabinoids in urine at concentrations from 0.50 to 500 ng/mL, contributing to the scientific knowledge of cannabinoid metabolism and distribution in urine. This method was able to separate the 11-OH-THC isomers with >90% resolution and shows viability for 11-OH-THC for both positive and negative ionization. This method demonstrated selectivity, accuracy, and reproducibility for federally-sponsored research studies.

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DISCLOSURE

No relevant financial or nonfinancial relationships to disclose.