4.3.2.1 Title

LC-Cannabinoid Confirmation in Blood

4.3.2.2 Purpose and Scope

The purpose of this confirmatory method is to utilize LC-MS/MS technology to provide quantitative results for Δ^9-tetrahydrocannabinol (Delta-9 THC), Δ^8-tetrahydrocannabinol (Delta-8 THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-Hydroxy Delta-9 THC), and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Delta-9 Carboxy THC). For ease, and space considerations, the analytes are abbreviated Δ9-THC, Δ8-THC, Δ9-THC-OH, and Δ9-THC-COOH throughout this SOP.

4.3.2.3 Limitations

Interference studies were conducted with a combination of approximately two-hundred-fifty over the counter, prescription, and illicit drugs. While this study included many drugs that are expected to be encountered in routine casework, it did not include every possible known substance that an individual could be exposed to. The drugs included in the interference study did not cause any false positive drug results for the analytes of interest.

The presence of Δ^8-hydroxy-tetrahydrocannabinol and Δ^8-carboxy-tetrahydrocannabinol may interfere with Δ^9-hydroxy-tetrahydrocannabinol and Δ^9-carboxy-tetrahydrocannabinol, respectively. The presence of exo-tetrahydrocannabinol may interfere with Δ^9-tetrahydrocannabinol. See 4.3.2.12 for additional details.

Carryover was assessed after analyzing standards that were ten times higher than standard six. No carryover existed in blank samples analyzed immediately afterward. To be conservative, analyte/internal standard area ratios higher than five times standard six shall result in the reinjection of the next casework sample.

4.3.2.4 Specimen Criteria

500 µL blood

4.3.2.5 Safety

blood – mitigate biohazard risk by employing universal biohazard precautions

acetonitrile – see MSDS, mitigate chemical hazard risk by taking appropriate precautions

formic acid – see MSDS, mitigate chemical hazard risk by taking appropriate precautions

methanol – see MSDS, mitigate chemical hazard risk by taking appropriate precautions

methyl tert-Butyl Ether (MTBE) – see MSDS, mitigate chemical hazard risk by taking appropriate precautions

4.3.2.6 Equipment

LCMSMS-21-1
Poroshell 120, EC-C18, 2.1x100mm, 2.7µm column
Poroshell 120, UHPLC Guard, EC-C18, 2.1mm
1 mL capacity Biotage ISOLUTE+ SLE cartridge
Centrifuge
Pipettes
Repeater pipette
Vortex mixer
Solvent evaporator
Vacuum pump
Glass block vacuum chamber
Extraction cartridge 12/24 manifold
Clean through extraction tip
16x100 mm glass culture tubes
Autosampler vial with glass insert
Autosampler vial cap
Formic acid – LCMS grade
Acetonitrile – LCMS grade
Deionized water – LCMS grade
MTBE

4.3.2.7 Sample Preparation and Procedure

Prepare extraction manifold by labeling a 1 mL capacity SLE cartridge and placing cartridge onto the extraction manifold equipped with disposable extraction tips. Place a collection tray with appropriately labeled 16x100 mm collection tube into the glass block vacuum chamber.

Note: ensure that each disposable extraction tip is lined up with the corresponding collection tube.

To all appropriately labeled 16x100 mm glass tubes, add 500 µL of 0.1% formic acid in water. Add 20 µL of internal standard solution to all culture tubes, including the negative control. Add 20 µL of standards 1-6, and the low, medium, and high control to appropriate culture tubes.

Prepare an interference standard by adding 20 µL of interference mix to 500 µL of blank blood. The interference mix includes:

- 1000 ng/mL CBD
- 2.5 ng/mL Δ8-THC-COOH
- 1 ng/mL Δ8-THC-OH

Prepare a matrix negative control by adding 500 µL of blank blood (no internal standard) to appropriate culture tube. A matrix negative is not necessary if the lot# of blank blood being used for calibrators and controls has previously been analyzed in this confirmatory assay. It is the responsibility of the individual performing the confirmatory analysis to verify this information.

Add 500 µL of blank blood to standard and control culture tubes.
Add 500 µL of unknown blood to culture tubes.

Vortex each calibrator, control, and unknown to mix sample with diluent.
Pipette 750 µL of each calibrator, control, and unknown into the labeled SLE cartridge.
Apply a short pulse of vacuum (approximately 1-2 seconds) to initiate the sample loading into the SLE cartridge. Allow gravity or slight individual positive pressure to load the samples into the SLE cartridge.

Note: wait for each sample to completely descend into the SLE cartridge before moving onto the next step.

Allow cartridge sorbent 5 minutes to absorb the sample.
Add 3 mL of MTBE to each SLE cartridge and allow 5 minutes for gravity to begin eluting the sample.
Add a second 3 mL aliquot of MTBE to each SLE cartridge and allow 5 minutes for gravity to begin eluting the sample.

Note: after the second 5 minute elution, use minimal vacuum pressure or pulse the vacuum pump to assist with the elution of the sample. Do not exceed approximately 1 mL elution flow per minute.

Place collection tubes into solvent evaporator (<40°C) and dry with nitrogen (approximately 4-6 psi) until dry (approximately 5-10 minutes).
Reconstitute by adding 40 µL of 0.1% formic acid in acetonitrile to each culture tube, vortex. Add 40 µL of 0.1% formic acid in water to each culture tube, vortex.
Transfer each sample into an appropriately labeled LC autosampler vial and cap.
Centrifuge for 10 minutes at 3500 rpm.
Run on instrument.

Note: validation demonstrated that the analytes in this method are stable for 3 days after extraction, when stored in 15 °C. Samples not analyzed within 3 days of extraction must be reextracted.

4.3.2.8 Performance Characteristics

Standards have been validated at the following concentrations (ng/mL):

Analyte	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
Δ9-THC	1	5	30	50	70	100
Δ8-THC	1	5	30	50	70	100
Δ9-THC-COOH	5	10	25	50	75	100
Δ9-THC-OH	1	4	8	12	16	20

Controls have been validated at the following concentrations (ng/mL):

Analyte	LOW Control	MEDIUM Control	HIGH Control
Δ9-THC	3	40	80
Δ8-THC	3	40	80
Δ9-THC-COOH	8	40	80
Δ9-THC-OH	2	10	18

Note: Instructions for calibrator and control preparation can be found in form FS-127.

Internal Standards have been validated at the following concentrations (ng/mL):

Analyte	Concentration
Δ9-THC-D3	50
Δ8-THC-D3	50
Δ9-THC-COOH-D3	50
Δ9-THC-OH-D3	10

Note: Instructions for Internal Standard preparation can be found in form FS-126.

Limits of Detection (LOD), Lower Reporting Limits (LRL), Lower Limits of Quantitation (LLOQ), and Upper Limits of Quantitation (ULOQ) have been validated at the following concentrations (ng/mL):

Analyte	LOD	LRL	LLOQ	ULOQ
Δ9-THC	0.4	0.5	1	100
Δ8-THC	0.4	0.5	1	100
Δ9-THC-COOH	2	2.5	5	100
Δ9-THC-OH	1	1	1	20

Note: the LOD for Δ9-THC, Δ8-THC, and Δ9-THC-COOH may be lower than what is listed in this table. As no concentrations below the LRL will be reported, validation studies did not go any lower.

Note: the LRL is determined as 50% of the area ratio between analyte and internal standard of calibrator 1. The concentrations provided in the above table are estimates of the LRL. The LRL for Δ9-THC-OH is equivalent to Standard 1.

4.3.2.9 Analytical Parameters

Instrument Operating Parameters are in TX-PM 5.15 Instrument Operating Parameters.

Instrument Maintenance

Check to ensure that the following maintenance has been completed:
Curtain plate has been cleaned within the last 30 days
Guard column has been changed within the last 2 months
Mobile phases have been prepared within the last month and are of sufficient volume to complete the analytical run
HPLC is connected to MS
Correct mobile phases are being used
Correct column is in place
Waste container is not full
Pump valves are closed

Needle rinse has sufficient volume

Calibration Models

Analyte	Curve Type	Weighting	Origin	Transition 1	Transition 2	Internal Standard
Δ9-THC	Quadratic	Inverse (1/x)	Ignore	315.1 / 193.1	315.1 / 123.0	Δ9-THC-D3 318.1 / 123.0
Δ8-THC	Quadratic	Inverse (1/x)	Ignore	315.1 / 193.1	315.1 / 123.1	Δ8-THC-D3 318.1 / 123.0
Δ9-THC-COOH	Linear	Inverse (1/x)	Ignore	343.0 / 299.1	343.0 / 191.0	Δ9-THC-COOH-D3 346.0 / 194.0
Δ9-THC-OH	Linear	Inverse (1/x)	Ignore	331.1 / 193.1	331.1 / 105.1	Δ9-THC-OH-D3 334.1 / 196.1

Note: The retention time order of the analytes is Δ9-THC-OH, Δ9-THC-COOH, Δ9-THC, and Δ8-THC.

4.3.2.10 Data Analysis

LCMSMS-21-1 Cannabinoid Flagging Rules and Calculated Columns

When processing a batch with Sciex OS the results table fields listed below shall comply with the following numbering format:

Column name	Number format
Accuracy %	0.00
Actual Concentration	0.00
Area	0.000e0
Area Ratio	0.0000
Calculated Concentration	0.000
Exp. IR High	0.000
Exp. IR Low	0.000
Exp. RRT High	0.000
Exp. RRT Low	0.000
Expected Ion Ratio	0.000
Expected RRT	0.000
Ion Ratio	0.000
ISTD Area	0.000e0
Mean ISTD Area	0.000e0

Relative RT	0.000
Reporting Limit	0.0000
Standard 1 Area Ratio	0.0000

Acceptance Criteria of Calibrators

Calibrator values shall be within ±20% of the target value. The r2 value for calibration curves shall be ≥0.990.

Method Validation did not evaluate the impact of dropping calibration points on the linear curves of Δ9-THC-OH or Δ9-THC-COOH. No calibration points may be dropped.

Acceptance Criteria of Controls

Control values shall be within ±20% of the target value. All quantitative controls are required to be within acceptance criteria. The negative control shall not contain the analytes of interest at a reportable concentration.

The interference standard shall not contain the analytes that are being reported in this confirmatory assay. The peak height of Δ8-THC-COOH shall be used as a threshold to determine the reportability of Δ9-THC metabolites.

Acceptance Criteria of Internal Standards

Internal Standard area counts shall be within acceptance criteria of the mean of the batch, specific to each IS. The following acceptance criteria shall be used:

Analyte	Acceptance
Δ9-THC-D3	± 30%
Δ8-THC-D3	± 40%
Δ9-THC-COOH-D3	± 25%
Δ9-THC-OH-D3	± 20%

Acceptance Criteria of Casework Samples

Chromatographic Quality Requirement

Chromatographic quality for LC/MS/MS transition data is defined as a reasonably symmetrical shaped peak consistent with those observed in calibrators and positive controls and can be differentiated from a negative control.

Retention Time Requirement

This assay uses a Relative Retention Time (RRT) model. The RRT is calculated by dividing the analyte retention time by the internal standard retention time. This is performed for all calibrators used in the calibration curve, averaged, and then given an acceptable value of ± 2.5%. Positive analytes must be within this range to be considered acceptable.

Ion Ratio Requirement

This assay utilizes one transition ion as a quantitative ion and a second transition ion as a confirmatory ion. The ion ratio is calculated by dividing the area count of transition 2 by the area count of transition 1. The ratio between the two ions must be within ± 20% of the average of all the calibrators used in the calibration curve to be acceptable.

NOTE: Chromatographic and Mass Spectral Quality Control shall be evaluated for all calibrators, controls, and unknowns in an analytical batch.

4.3.2.11 Corrective Measures

If acceptance criteria are not met, consult with the unit supervisor to determine whether re-extraction or qualitative reporting will be performed. The rationale for that decision shall be recorded in the electronic case file.

4.3.2.12 Reporting

Quantitative results will be truncated and reported in ng/mL to one decimal for values <10 ng/mL. Quantitative values ≥10 ng/mL will be truncated and reported as a whole number.

Analytes present at a concentration < (LOQ) with an area ratio of analyte to I.S. ≥50% of Standard 1 will be reported as "< (LOQ)".

Analytes present at a concentration < LOQ with an area ratio of analyte to internal standard <50% of Standard 1 will not be reported. If no analytes in this confirmatory assay are positive the report will state, "Not Detected: Cannabinoids".

If an analyte is present above the ULOQ, it will be reported as "> (ULOQ)" If an analyte is present above the ULOQ and the ion ratios fail acceptance criteria, the sample may be reinjected with 1 µL to demonstrate acceptable ion ratios. The impacted analyte will remain reported as "> (ULOQ)".

If Δ8-THC-COOH is present above the interference standard threshold, Δ9-THC metabolites cannot be reported. In those instances, the language on the reports shall state: "Δ9-THC-COOH and Δ9-THC-OH were not able to be reported due to an interfering substance. For additional information contact this laboratory."

Validation demonstrated that ≥10 ng/mL of exo-tetrahydrocannabinol interferes with Δ9-THC, at all calibration levels. When both analytes are present in the same sample, the report shall state: "Δ9-THC was not able to be reported due to an interfering substance. For additional information contact this laboratory."

4.3.2.13 References

LCMSMS-21-1 LC-Cannabinoid Final Validation Document

ANSI/ASB Standard 036

ANSI/ASB Standard 054

ANSI/ASB Standard 152

ANSI/ASB Standard 017