CANNABINOID SCREENING BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

42.1 METHOD

This test method may be used to identify Δ^9-THC (THC) and its metabolite, 11-nor-9-carboxy-Δ^9-THC (THCCOOH), in biological specimens and other submitted evidence. The targeted compounds and internal standards are isolated from biological specimens or evidence by the use of liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

42.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, tissue homogenate and non-biological aqueous solutions. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Matrix-matching of all calibrators and the negative and positive control is required for analysis of liver (tissue) homogenate or serum/plasma specimens (see 42.4.2 and 42.4.3).

When analyzing non-biological evidence (e.g., infused beverages), care should be taken to prevent carryover on the instrument (e.g., analyze at dilution, include solvent blanks following injection, run at the end of the batch). Analysis of urine specimens is performed using the cannabinoids confirmation test method, TCc12727.

42.3 REAGENTS, MATERIALS AND EQUIPMENT

42.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used, unless otherwise specified.

- 10% Acetic acid

  Add 10 mL of concentrated acetic acid to approximately 50 mL DI H_2O in a 100 mL flask and mix. Dilute to 100 mL with DI H_2O and mix. The solution is stored in a glass bottle at room temperature and expires one year from the date of preparation.

- Acetic acid (glacial)
- Acetonitrile (ACN), reagent grade and LC-MS grade
- Certified blank blood (specified for THC) and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade H_2O (or equivalent from a high-purity filtration system)
- Ethyl acetate (EtAC)

- Extraction solvent; hexanes:ethyl acetate 9:1

  Add 90 mL hexanes to a glass flask. Add 10 mL ethyl acetate and mix. Store the solvent in a glass flask/bottle at room temperature. Use on date of preparation only.

- Formic acid, concentrated
- 0.1% Formic acid in LC-MS grade H_2O

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade H_2O and mix. The solution is stored in an amber glass bottle at room temperature and expires one year from the date of preparation.

  NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.

- Hexanes
- Methanol (MeOH), reagent grade and HPLC grade
- Reconstitution solution, 50:50 LC-MS grade ACN:LC-MS grade H_2O

  Add 2 mL of LC-MS grade ACN to 2 mL of LC-MS grade H_2O in a glass tube, cap and mix. Use on date of preparation only.

- Sodium hydroxide (NaOH), concentrated, 10N

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

42.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Disposable glass transfer pipettes
- Glass autosampler vials with integrated conical inserts and caps
- HPLC column (Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7 µM particle size, or equivalent)
- Laboratory glassware (graduated cylinders, flasks)

42.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS-MS with API-ES source (6420 or equivalent)
- Shimadzu HPLC, or equivalent
- Sciex API 3200 MS-MS, or equivalent
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, rotary mixer, vortex mixer)

42.4 STANDARDS, CALIBRATORS AND CONTROLS

42.4.1 STANDARDS

- Working standard (WS): 0.1/0.5 ng/µL
- Working control standard (QC): 0.1/0.5 ng/µL
- Stock internal standard: 1/5 ng/µL
- Working internal standard: 0.1/0.5 ng/µL

42.4.2 SEMI-QUANTITATIVE (SEMI-QUANT) CALIBRATORS

Semi-quant calibrators are prepared in certified blank blood at the time of analysis, as detailed in 42.5 SAMPLE PREPARATION.

Analysis of liver (tissue) homogenate or serum/plasma specimens requires that all semi-quant calibrators be prepared in blank alternate matrix. If testing only an alternate matrix, a blood semi-quant curve is not required.

42.4.3 CONTROLS

42.4.3.1 At least one negative blood control and one positive blood control are tested with each batch. For analysis of liver (tissue) homogenate or serum/plasma specimens only, whole blood controls are not required.

42.4.3.2 Samples with known target concentrations (semi-quant calibrators and positive/negative controls) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive known samples (semi-quant calibrators or positive control). When the batch contains more than 20 specimens, a positive known sample must be analyzed mid-run.

42.4.3.3 For analysis of liver (tissue) homogenate or serum/ plasma specimens, matrix-matching of all semi-quant calibrators and the negative and positive controls (must meet 10% for that matrix) is required.

42.5 SAMPLE PREPARATION

42.5.1 Label a clean extraction tube (16 x 100 mm recommended) for each member of the test batch (i.e., semi-quant calibrator, control, case sample).

42.5.2 Add 1 mL DI H_2O to each tube.

42.5.3 Using a calibrated pipette, add 0.5 mL of certified blank blood into each of the semi-quant calibrator tubes, and negative and positive control tube(s).

42.5.4 Prepare a 1:10 dilution of the working standard. (0.01, 0.05 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after semi-quant calibrator preparation.

42.5.5 Using a calibrated pipette, spike the semi-quant calibrators according to the following table, using the working standard and prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 1/5 ng/mL	50	0.01/0.05 ng/µL	1:10
Calibrator 2 – 2/10 ng/mL	100	0.01/0.05 ng/µL	1:10
Calibrator 3 – 10/50 ng/mL	50	0.1/0.5 ng/µL	WS

42.5.6 Prepare a 1:10 dilution of the working control standard. (0.01, 0.05 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the control working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

42.5.7 Using a calibrated pipette, add 150 µL of the 1:10 dilution of the working control standard to the positive control tube. The target concentration for the control is 3.0 ng/mL THC and 15 ng/mL THCCOOH.

42.5.8 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

42.5.9 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL THC-d_3 and 50 ng/mL THCCOOH-d_3.

42.5.10 Add 400 µL of 10% acetic acid and vortex-mix.

42.5.11 Add 4 mL extraction solvent (hexanes:ethyl acetate, 9:1) to each tube.

42.5.12 Cap the tubes and place on a rotary mixer for 30 minutes.

42.5.13 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes) to achieve separation.

42.5.14 Transfer the organic layer to a clean, labeled centrifuge or screw-cap tube.

42.5.15 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

42.5.16 Reconstitute samples with 50 µL of reconstitution solvent (50:50 LC-MS grade ACN:LC-MS grade H_2O). Briefly vortex, then centrifuge the tubes for 10 minutes at 3500 rpm to collect the extracts at the bottom of the tubes.

42.5.17 Transfer the extracts to labeled glass autosampler vials with integrated inserts and cap.

42.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – THC (instrumental parameters in Appendix B)
- Calibration curve – linear, $1/a^2$ weighting factor
- Updating calibrator (retention times ±2%, transition ratios ±20%) – Cal 3
- Result comparisons – all units in ng/mL

  Cal 1: truncated to two decimal places (acceptable range ±25%; 0.75 – 1.25 ng/mL for THC; 3.75 – 6.25 ng/mL for THCCOOH).

  Cals 2-3, Pos Ctl: truncated to one decimal place for target concentrations ≤10 ng/mL; truncated, whole integer values for target concentrations >10 ng/mL (acceptable range for all ±20%).

  NOTE: The positive control must meet acceptability criteria for chromatography, retention time and ratios. Where control values for THC or THCCOOH are not within ±20% of target, qualitative results are still reportable from this screen; however, this should be considered when comparing screening values to those from confirmation/quantitative testing (TCc12727).

42.7 REPORTING

Results at or above the cutoff concentrations (Cal 1) are reported as positive for all matrices, provided that the specimen results meet all criteria for acceptance (e.g., retention time, chromatography, transition ratios), including results >Cal 3 concentrations (10 ng/mL THC, 50 ng/mL THCCOOH).

42.8 METHOD PERFORMANCE

- Limit of detection (for qualitative reporting): 1.0 ng/mL THC
  5.0 ng/mL THCCOOH
- Dynamic range: 1.0 – 10 ng/mL THC, 5.0 – 50 ng/mL THCCOOH

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

11-nor-9-carboxy-Δ^9-THC (THCCOOH)
11-nor-9-carboxy-Δ^9-THC-d_3 (THCCOOH-d_3)
Δ^9-THC (THC)
Δ^9-THC-d_3 (THC-d_3)

APPENDIX B
INSTRUMENTAL PARAMETERS

Shimadzu/Sciex LC-MSMS System

SHIMADZU LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid in LC-MS grade H_2O
Solvent B	ACN (LC-MS grade)
Initial composition	60% A, 40% B
0 – 1.0 min	40% B
1.0 – 7.0 min	95% B
7.0 – 10.0 min	95% B
10.1 – 12.5 min	40% B
Post time	2.5 min
Column temp	50°C
Autosampler	
Injection volume	10 µL
Rinsing volume	1000 µL
Rinsing solvent	75:25 HPLC grade MeOH:LC-MS grade H_2O
Cooler temperature	25ºC

SCIEX MASS SPECTROMETER

Scan type	(+) MRM	Curtain/collision gas	Nitrogen
Ion mode	ESI	Curtain gas flow	40 L/min
Resolution (Q1)	Unit	Collision gas flow	4 L/min
Resolution (Q3)	Unit	Gas 1 temp	40ºC
Valve position A	To waste	Gas 2 temp	80ºC
Valve position B (all transitions)	To MS	Ion voltage	5.5 kV
Valve position A	To waste	Interface temp	650ºC

Compound	MRM Transitions	Dwell Time
THCCOOH-d_3	348.3→330.0, 302.0	50 msec
THCCOOH	345.4→299.2, 193.3	50 msec
THC-d_3	318.3→196.3, 123.1	100 msec
THC	315.2→193.3, 123.2	100 msec

Agilent LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.5 mL/min
Solvent A	0.1% Formic acid in LC-MS H_2O
Solvent B	ACN (LC-MS grade)
Initial Composition	60% A, 40% B
Hold time	1 min (40% B)
1-7 min	% B increased to 95%
Hold time	3 min (95% B)
10-10.5 min	% B decreased to 40%
Re-equilibration	2.0 minutes
Column Temp	50°C
Autosampler	
Injection Volume	10.0 µL
Injection flush-port	Active
Flush-port time	5 sec
Flush-port solvent	75:25 HPLC MeOH:LC-MS H_2O

MASS SPECTROMETER

Ion mode	(+) MRM	Nebulizer gas	Nitrogen
Peak width	0.05 min	Nebulizer pressure	40 psi
Dwell time (Time Segment 2)	50 msec	Drying gas	Nitrogen
Dwell time (Time Segment 3)	100 msec	Drying gas flow	10.0 L/min
Time segment 1	To Waste	Drying gas temp	350°C
Time segment 2 (THCCOOH/THCCOOH-d_3)	To MS (EMV +400)		
Time segment 3 (THC/THC-d_3)	To MS (EMV +400)		
Time segment 4	To Waste		

Signals	MRM Transitions
THCCOOH-d_3	348.2→330.2, 302.2
THCCOOH	345.2→299.2, 193.1
THC-d_3	318.2→196.1, 123.0
THC	315.2→193.1, 123.0

LIST OF CHANGES

Revision Date	Description	Page Number
6/19/19	Method approved by Washington State Toxicologist. See DRA dated 5/17/19. Method released for use in evidentiary testing on 6/19/19.	All
5/11/20	Supplemental method verification performed to increase sample volume from 0.25 mL to 0.5 mL, with sample prep in 42.5 adjusted accordingly. Removed requirement to filter 0.1% formic acid when LC-MS grade water is used in preparation in section 42.3.1. Moved target compound/IS descriptions from 42.4.1 to APPENDIX A, instrument parameters moved to APPENDIX B. Added use of mid-run control in 42.4.3.2. Acquisition method listed in 42.6 is THC, as the screening data is now acquired with the same instrument settings as the cannabinoids confirmation method. Changed references for "LC-MS grade DI H_2O" to "LC-MS grade H_2O." Method verification included use of the Agilent LC-MSMS system; instrumental parameters for the Agilent LC-MSMS system added to APPENDIX B. Other minor edits throughout.	All