CONFIRMATION OF CANNABINOIDS BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

27.1 METHOD

This test method may be used to confirm the presence of 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.

27.2 SPECIMENS

The specimen volume is 1 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, tissue homogenate and non-biological aqueous solutions. Dilutions of specimens may be analyzed at the Forensic Scientists discretion.

NOTE: Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in liver (tissue) homogenate or serum/plasma specimens (see 27.4.2 and 27.4.3).

27.3 REAGENTS, MATERIALS AND EQUIPMENT

27.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H_2O) is specified, laboratory general-use DI H_2O is used in reagent preparation. Organic solvents are reagent grade 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. 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 (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

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade DI H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade DI H_2O and mix. Filter this solution prior to use on the HPLC. The solution is stored in an amber glass bottle at room temperature and expires one year from the date of preparation.
- Hexanes
- Methanol (MeOH)
- Reconstitution solution, 50:50 LC-MS grade ACN:LC-MS grade DI H_2O

  Add 2 mL of LC-MS grade acetonitrile to 2 mL of LC-MS grade DI 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.

27.3.2 MATERIALS

- Disposable extraction tubes (16 x 125 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, 2.7µM particle size, or equivalent)
- Laboratory glassware (graduated cylinders, flasks)
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

27.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series), Shimadzu HPLC, or equivalent
- Agilent MS-MS with API-ES source (6410/6420), 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, heating block, pH indicator paper, rotary mixer, solvent filtration apparatus, vortex mixer)

27.4 STANDARDS, CALIBRATORS AND CONTROLS

27.4.1 STANDARDS

- Working standard (WS): 10 ng/µL THC/50 ng/µL THCCOOH
- Working control standard (QC): 10 ng/µL THC/50 ng/µL THCCOOH
- Stock internal standard: 1 ng/µL THC-d_3/5 ng/µL THCCOOH-d_3
- Working internal standard: 0.1 ng/µL THC-d_3/0.5 ng/µL THCCOOH-d_3

27.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 27.5 SAMPLE PREPARATION. For urine analysis, preparation is described in 27.6.

Quantitation in liver (tissue) homogenate or serum/plasma specimens requires that a calibration curve be prepared in blank alternate matrix. If testing only an alternate matrix, a whole blood calibration curve is not required.

27.4.3 CONTROLS

27.4.3.1 At least one negative whole blood control and three positive whole blood controls are tested with every batch, prepared as described in 27.5. For quantitative analysis of liver (tissue) homogenate or serum/plasma specimens only, whole blood controls are not required.

27.4.3.2 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls.

27.4.3.3 For qualitative analysis, one positive and one negative control must be included for each alternate matrix type tested in the batch.

27.4.3.4 For quantitative analysis of liver (tissue) homogenate or serum/plasma specimens, matrix matching of the full calibration curve, negative control and all positive controls (to meet 10% and bracket specimens in that matrix) is required.

27.4.3.5 For urine analysis, calibrators 1 and 5 and the glucuronide process control (included to verify successful hydrolysis of glucuronides) serve as positive known samples for that matrix.

27.5 SAMPLE PREPARATION (BLOOD, SERUM, PLASMA, TISSUE HOMOGENATE)

NOTE: Laboratory general-use DI H_2O is used in sample preparation. LC-MS grade DI H_2O (or equivalent) and LC-MS grade ACN are used in reconstitution (27.5.18). Organic solvents used in sample preparation are reagent grade.

27.5.1 Label a clean extraction tube (16 x 125 mm recommended) for each member of the test batch (i.e., calibrator, control, case sample).

27.5.2 Add 2 mL DI H_2O to each tube.

27.5.3 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

27.5.4 Prepare a 1:10 dilution of the working standard. (1, 5 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.

b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

27.5.5 Prepare a 1:100 dilution of the working standard. (0.1, 0.5 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

27.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the dilutions prepared from the working standard.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 1/5 ng/mL	10	0.1/0.5 ng/µL	1:100
Calibrator 2 – 2/10 ng/mL	20	0.1/0.5 ng/µL	1:100
Calibrator 3 – 5/25 ng/mL	50	0.1/0.5 ng/µL	1:100
Calibrator 4 – 10/50 ng/mL	100	0.1/0.5 ng/µL	1:100
Calibrator 5 – 25/125 ng/mL	25	1/5 ng/µL	1:10
Calibrator 6 – 50/250 ng/mL	50	1/5 ng/µL	1:10
Calibrator 7 – 100/500 ng/mL	100	1/5 ng/µL	1:10

27.5.7 Prepare a 1:10 dilution of the control working standard. (1, 5 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

27.5.8 Prepare a 1:100 dilution of the control working standard. (0.1, 0.5 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

27.5.9 Using a calibrated pipette, spike the controls according to the following table, using the dilutions prepared from the control working standard.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Control 1 – 3/15 ng/mL	30	0.1/0.5 ng/µL	1:100
Control 2 – 20/100 ng/mL	20	1/5 ng/µL	1:10
Control 3 – 80/400 ng/mL	80	1/5 ng/µL	1:10

27.5.10 Using a calibrated pipette, sample 1 mL of each case specimen into its respective tube.

27.5.11 Using a calibrated pipette or verified repeater-pipette, add 100 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.

27.5.12 Add 800 µL of 10% acetic acid and vortex-mix.

27.5.13 Add 8 mL extraction solvent (hexanes:ethyl acetate, 9:1) to each tube.

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

27.5.15 Centrifuge the tubes for 15 minutes at 2500 rpm (recommended for 16 x 125 mm tubes) to achieve separation.

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

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

27.5.18 Reconstitute samples with 100 µL of reconstitution solvent (50:50 LC-MS grade ACN:LC-MS grade DI H_2O) and briefly vortex-mix. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

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

27.6 SAMPLE PREPARATION (URINE)

Urine specimens require hydrolysis of glucuronide conjugates prior to sample preparation, according to the following procedure:

27.6.1 Label a clean extraction tube for each member of the urine test batch (i.e., calibrator, control, case sample).

27.6.2 Using a calibrated pipette, add 1 mL negative urine to each of the calibrator and control tubes.

27.6.3 Using a calibrated pipette, prepare urine calibrators at calibrator 1 and calibrator 5 concentrations, as described in 27.5.6.

27.6.4 Prepare a 1:10 dilution (10 ng/µL) of the 0.1 mg/mL THCCOOH glucuronide stock CRM.

a. Using a calibrated pipette, combine 0.1 mL of the glucuronide CRM with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

27.6.5 Prepare a 1:100 dilution (1 ng/µL) of the THCCOOH glucuronide stock CRM.

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

27.6.6 Using a calibrated pipette, add 100 µL of the prepared 1 ng/µL glucuronide THCCOOH glucuronide dilution to the glucuronide (process) control tube.

27.6.7 Using a calibrated pipette, add 1 mL of each urine case specimen to its respective tube.

27.6.8 Using a calibrated pipette or verified repeater-pipette, add 100 µL working internal standard solution to each tube. Final concentration is 50 ng/mL THCCOOH-d_3.

27.6.9 Cap tubes and vortex briefly.

27.6.10 Add 40 µL 10N NaOH to each tube.

27.6.11 Verify pH of each tube is >10 using pH indicator paper.

27.6.12 Cap tubes and vortex briefly.

27.6.13 Incubate tubes for 20 minutes at 60°C.

27.6.14 Remove from heat and cool to room temperature.

27.6.15 Add 25 µL glacial acetic acid to neutralize pH.

27.6.16 Vortex briefly.

27.6.17 Proceed with sample preparation at 27.5.13.

27.7 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – THC (instrumental parameters in Appendix A)
- Calibration curve – linear, $1/a^2$ weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4

  Updating calibrator – urine (retention times ±2%, ion ratios ±20%) – Cal 5
- Result comparisons – all units in ng/mL

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

  Cals 2-7, Pos Ctl 1-3: truncated to one decimal place for target concentrations ≤10 ng/mL; truncated, whole integer values for target concentrations >10 ng/mL.
- The glucuronide conjugate (process) control is used to evaluate the effectiveness of urine hydrolysis, and is considered acceptable if recovery of THCCOOH is demonstrated and general criteria for acceptance are met.

- Urine specimens with a calculated concentration of ≥ 5 ng/mL (THCCOOH cal 1) are suitable for qualitative reporting if urine calibrators and glucuronide process control meet acceptability criteria.

27.8 REPORTING

- THC results, and associated measurement uncertainties, are reported to two significant figures, in units of nanograms per milliliter (ng/mL).

- The full THC result from the data report (to two decimal places) is used to calculate the associated measurement uncertainty (with coverage factor k=3, 99.7% confidence level).

  a. Example: THC is measured at 7.85 ng/mL.

  b. Multiply the full result of 7.85 ng/mL by 0.26 (26%), to obtain an uncertainty of 2.041 ng/mL.

  c. The THC result is truncated to 7.8 ng/mL, and the associated uncertainty is rounded to 2.0 ng/mL (both two significant figures) for reporting.

     NOTE: When inputting THC results in LIMS, the full result from the data report (to two decimal places) is entered (LIMS calculates the associated uncertainty from this full result). The final THC result and associated uncertainty that appear on the final test report are verified independently (at time of issue and at time of technical review), as described in the example above.

- THCCOOH results are reported to two significant figures, in units of ng/mL. Measurement uncertainty for THCCOOH is not included on the test report.

- Additional information on measurement uncertainty is found in the document *Estimation and Reporting of Measurement Uncertainty (PQ12706)*.

- THCCOOH results from urine specimens are reported qualitatively.

27.9 METHOD PERFORMANCE

- Limit of detection: 0.5 ng/mL THC, 2.5 ng/mL THCCOOH
- Lower limit of quantification: 1.0 ng/mL THC, 5.0 ng/mL THCCOOH
- Dynamic range: 1.0 – 100 ng/mL THC, 5.0 – 500 ng/mL THCCOOH
- Upper limit of quantitation: 100 ng/mL THC, 500 ng/mL THCCOOH

27.10 REFERENCES

- A. Black, B.E. O'Reilly, in-house development.

- D.M. Schwope, K.B. Scheidweiler, M.A. Huestis. Direct quantification of cannabinoids and cannabinoid glucuronides in whole blood by liquid chromatography – tandem mass spectrometry. *Analytical Bioanalytical Chemistry*. 401(4):1273-1283 (2011).

- J. Hudson, J. Hutchings, C. Harper, R. Wagner, Validation of a Cannabinoid Quantitation Method Using an Agilent 6430 LC/MS/MS, *Agilent Application Note 5991-2554EM*, June 2013.
- Pat Friel, Agilent Technologies, Inc.
- Virginia Department of Forensic Sciences, Cannabinoid Quantitation/Confirmation method.

APPENDIX A
INSTRUMENTAL PARAMETERS

Agilent LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.5 mL/min
Solvent A	0.1% Formic acid
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 MeOH:DI 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

Shimadzu/Sciex LC-MSMS System

SHIMADZU LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
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 MeOH:DI 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 (Sciex 3/Sciex 4)	40ºC/60ºC
Valve position A	To waste	Gas 2 temp (Sciex 3/Sciex 4)	80ºC/50ºC
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

LIST OF CHANGES

Revision Date	Description	Page Number
5/8/14	Method approved by Washington State Toxicologist. See DRA dated 05/5/14. Method released for use in evidentiary testing on 5/8/14.	All
12/5/14	Instrumental parameters for liquid chromatograph updated to reflect an increase in the temperature of the analytical column from 40ºC to 50ºC. See DRA dated 12/5/14.	11
10/07/15	Edited 27.1 for deviation approval by a member of TLD Management. Added 27.10.1.4 to indicate that no points may be dropped from the THC calibration curve in order for the batch to be acceptable for reporting THC. Edited 27.8 for use of either the Agilent or the Sciex instruments/methods and added MultiQuant to 27.9.1 for batch data analysis. Included Shimadzu/Sciex LCMSMS instrument parameters in Appendix A. See DRA dated 9/30/15.	1, 7-8, 12
4/6/16	Added clarification to 27.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM or stock standard expiration dates in 27.6.1.3, 27.6.1.4 and 27.6.1.5. Added instructions for calculation of THC measurement uncertainty, including the example and note in 27.12.1.1. Other minor edits throughout.	4, 9-10
7/24/17	Wording added to 27.4.3 regarding dilution and standard volume testing. Preparation of the control working standard was added to section 27.6.3.2. Specified use of calibrated pipettes for measurement of blank blood, specimens, and standards throughout section 27.7 SAMPLE PREPARATION. Edited 27.10.10.2.e to indicate all positive controls must meet acceptability criteria to report quantitative results. Other minor edits throughout.	1-10
11/26/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 27.2 SPECIMENS and control descriptions expanded in 27.4.3. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Formatting and minor edits throughout.	All