

CONFIRMATION OF CANNABINOIDS BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY

15.1 POLICY

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 samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

15.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of THC and THCCOOH present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compounds.

15.3 PRINCIPLE

The targeted compounds and internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, the specimens, now termed extracts, are injected into a gas chromatograph (GC) where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, selected-ion-monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any THC or THCCOOH identified in a sample is determined from its calibration curve.

15.4 SPECIMENS

15.4.1 The specimen volume is 2 mL for all specimen types except urine. The default volume for urine is 1 mL.

15.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

15.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

15.4.4 Analysis of larger specimen volumes must be approved and documented.

15.5 REAGENTS, MATERIALS AND EQUIPMENT

15.5.1 REAGENTS

- 15.5.1.1 Acetone
- 15.5.1.2 1% acetone in acetonitrile
Add 49.5 mL acetonitrile to a glass flask. Add 0.5 mL acetone and mix. Use on date of preparation only. Adjustments to final volume are permitted as long as proportions are maintained.
- 15.5.1.3 Acetonitrile
- 15.5.1.4 BSTFA + 1% TMCS (N,O-bis-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane)
- 15.5.1.5 Certified blank blood
- 15.5.1.6 Deionized water (DI H₂O)
- 15.5.1.7 Ethyl acetate
- 15.5.1.8 Extraction solvent (hexanes:ethyl acetate 9:1)
Add 90 mL hexanes to a glass flask. Add 10 mL ethyl acetate and mix. Use on date of preparation only. Adjustments to final volume are permitted as long as proportions are maintained.
- 15.5.1.9 Hexanes
- 15.5.1.10 1N Hydrochloric acid
Add 400 mL DI H₂O to a glass flask. Carefully add 42 mL concentrated HCl (12N). Dilute to 500 mL with DI H₂O and mix. Store in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.
- 15.5.1.11 Hydrochloric acid (HCl), concentrated 12N
- 15.5.1.12 Methanol
- 15.5.1.13 0.2N Sodium hydroxide
Add 800 mL DI H₂O to a glass flask. Add 20 mL concentrated NaOH (10N). Dilute to 1 L with DI H₂O and mix. Store the solution in a glass or plastic bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.
- 15.5.1.14 Sodium hydroxide (NaOH), concentrated 10N

15.5.2 MATERIALS

- 15.5.2.1 Autosampler vials, inserts and caps
- 15.5.2.2 Disposable 16 x 125mm tubes
- 15.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures
- 15.5.2.4 Disposable pipette tips

- 15.5.2.5 Disposable safety closures for 16mm tubes
- 15.5.2.6 Disposable glass transfer pipettes
- 15.5.2.7 GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 μ m film thickness, or equivalent)
- 15.5.2.8 Laboratory glassware (graduated cylinders, flasks)
- 15.5.2.9 Volumetric glassware (flasks)

15.5.3 EQUIPMENT

- 15.5.3.1 Agilent GC (6890 or equivalent)
- 15.5.3.2 Agilent MS (5973 or equivalent)
- 15.5.3.3 Calibrated, adjustable air-displacement pipette
- 15.5.3.4 Centrifuge
- 15.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)
- 15.5.3.6 Oven, dry bath or wet bath
- 15.5.3.7 Rotary mixer
- 15.5.3.8 Vortex mixer

15.6 STANDARDS, CALIBRATORS AND CONTROLS

15.6.1 STANDARDS

15.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards which in turn are used to produce calibrators, positive controls and the working internal standard.

15.6.1.2 Stock standards and stock internal standard (IS) are purchased from an approved reference material supplier and include the following:

- a. Δ^9 -THC: 1.0 mg/mL
- b. Δ^9 -THC-D₃: 0.1 mg/mL
- c. 11-nor-9-carboxy- Δ^9 -THC: 1.0 mg/mL
- d. 11-nor-9-carboxy- Δ^9 -THC-D₃: 0.1 mg/mL

15.6.1.3 Working standard

- a. Using calibrated pipettes, measure 500 μ L of THC and 2.5 mL of THCCOOH stock standards into a 50 mL class-A volumetric flask.
- b. Add methanol to the flask to the designated volume.
- c. The final concentration of the working standard is 10 ng/ μ L THC and 50 ng/ μ L THCCOOH. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

15.6.1.4 Working internal standard

- a. Using a calibrated pipette, measure 250 μL THC- d_3 and 1.25 mL THCCOOH- d_9 stock internal standards into a 25 mL class-A volumetric flask.
- b. Add methanol to the flask to the designated volume.
- c. The final concentration of the working internal standard is 1 ng/ μL THC- d_3 and 5 ng/ μL THCCOOH- d_9 . The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

15.6.2 CALIBRATORS

- 15.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standards. The preparation of the calibrators is detailed in 15.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

15.6.3 CONTROLS

15.6.3.1 Negative Control

- a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
- b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

15.6.3.2 Positive Controls

- a. Two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
- b. Control stock standards are obtained from an approved reference material supplier.
- c. The control stock standards should be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot or supplier must be used, the working control standard should be prepared by someone other than the person that prepared the working standard.
- d. The preparation of the positive whole blood controls is detailed in 15.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
- e. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

15.7 SAMPLE PREPARATION

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

- 15.7.2 Add 2 mL of certified blank whole blood into each of the five calibrator tubes, the matrix blank tube, the two positive control tubes and the negative control tube(s).
- 15.7.3 Prepare a 1:10 dilution of the working standard. (1.0, 5.0 ng/μL)
 - a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 15.7.4 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 acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 15.7.5 Using the working standards, spike the calibrators according to the following table.

Calibrator Description (THC/THCCOOH)	Volume (μL) Added	Working Standard
Calibrator 1 (2.0/10 ng/mL)	40	0.1/0.5 ng/μL
Calibrator 2 (4.0/20 ng/mL)	80	0.1/0.5 ng/μL
Calibrator 3 (10/50 ng/mL)	20	1.0/5.0 ng/μL
Calibrator 4 (20/100 ng/mL)	40	1.0/5.0 ng/μL
Calibrator 5 (40/200 ng/mL)	80	1.0/5.0 ng/μL

- 15.7.6 Prepare a 1:10 dilution of the control working standard. (1.0, 5.0 ng/μL)
 - a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after control preparation.
- 15.7.7 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 acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after control preparation.
- 15.7.8 Using the control working standards, spike the positive controls according to the following table.

Control Description (THC/THCCOOH)	Volume (μL)
	Added
Control Low (5.0/25 ng/mL)	100 (0.1/0.5 ng/μL)
Control High (15/75 ng/mL)	30 (1.0/5.0 ng/μL)

- 15.7.9 If in-house positive controls are being used, transfer 2 mL of each into their labeled tubes.
- 15.7.10 Sample 2 mL of each case sample into its respective tube.

- 15.7.11 Add 20 μ L of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL THC-d₃ and 50 ng/mL THCCOOH-d₉.
- 15.7.12 Add 3 mL acetonitrile with 1% acetone dropwise to each tube while vortexing.
- 15.7.13 Cap the tubes and centrifuge for 10 minutes at 3500 rpm to achieve separation.
- 15.7.14 Transfer the acetonitrile layer to clean, labeled 16 x 125mm tubes.
- 15.7.15 Add 2 mL 0.2N NaOH to each tube and vortex briefly.
- 15.7.16 Add 4 mL extraction solvent to each tube.
- 15.7.17 Cap the tubes and place on a rotary mixer for a 10 minutes.
- 15.7.18 Centrifuge the tubes for 5 minutes at 2000-2500 rpm to achieve separation.
- 15.7.19 Transfer the top layer (extraction solvent) to clean, labeled centrifuge or screw cap tubes. Retain the tubes with the aqueous layer for THCCOOH extraction.
- 15.7.20 Transfer tubes (THC fraction) to the evaporator and evaporate the extracts to dryness at 40°C. Extracts must be completely dry for efficient chemical derivatization.

NOTE: After drying down, proceed with sample preparation for THC fraction at 15.7.27.
- 15.7.21 Add 2 mL 1N HCl to each tube from 15.7.19 aqueous (THCCOOH fraction) and vortex briefly.
- 15.7.22 Add 4 mL extraction solvent to each tube.
- 15.7.23 Cap the tubes and place on a rotary mixer for 30 minutes.
- 15.7.24 Centrifuge the tubes for 5 minutes at 2000-2500 rpm to achieve separation.
- 15.7.25 Transfer the top layer (extraction solvent) to clean, labeled centrifuge or screw cap tubes.
- 15.7.26 Transfer tubes (THCCOOH fraction) to the evaporator and evaporate the extracts to dryness at 40°C. Extracts must be completely dry for efficient chemical derivatization.
- 15.7.27 In a fume hood, add 25 μ L ethyl acetate and 25 μ L BSTFA + 1% TMCS to each tube (THC and THCCOOH) and immediately cap and vortex briefly.
- 15.7.28 Incubate the tubes for a minimum of 30 minutes at 70°C.
- 15.7.29 Remove from heat and centrifuge the tubes for 2 minutes at 2000 rpm to cool and collect the extracts at bottom of tubes.
- 15.7.30 Transfer the extracts to labeled glass autosampler vials and cap.

URINE EXTRACTION

- a. Add 1 mL blank urine to negative and positive control tubes.

- b. Spike positive urine control using the working control standard (add 20 μL (0.5 $\text{ng}/\mu\text{L}$ dilution) for target concentration of 10 ng/mL THCCOOH).
- c. Sample 1 mL of each case sample into its respective tube.
- d. Prepare a 1:10 dilution (0.5 $\text{ng}/\mu\text{L}$ THCCOOH- d_9) of the working internal standard. Using a calibrated pipette, combine 0.1 mL of the working internal standard with 0.9 mL of acetonitrile or methanol in a labeled tube. Cap and vortex mix.
- e. Add 100 μL of the 1:10 dilution of the working internal standard to each tube. Final concentration of the internal standard is 50 ng/mL THCCOOH- d_9 .
- f. Add 100 μL 10N NaOH to each tube.
- g. Cap the tubes and incubate for 15 minutes at 60°C to hydrolyze conjugated THCCOOH.
- h. Vortex briefly and allow tubes to cool to room temperature.
- i. Continue with sample preparation at 15.7.21.

15.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is THC for each line.

NOTE: Rinse autosampler syringe thoroughly with isopropanol followed by ethyl acetate, verifying smooth movement of syringe plunger before starting run. If necessary, replace syringe prior to starting batch.

15.9 DATA ANALYSIS

15.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.

15.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a $1/x$ (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

15.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

15.9.4 Technical review of the batch is conducted according to the criteria listed below.

15.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

15.10.1 Calibrators and calibration curves

15.10.1.1 Chromatographic peaks for THC, THCCOOH and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

15.10.1.2 Retention times shall be within $\pm 2\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 4. These are inclusive ranges.

15.10.1.3 Quantitative results for THC and THCCOOH in each calibrator shall be within $\pm 20\%$ of their target values with the exception of calibrator 1 which shall be within $\pm 25\%$ of their targets. These are inclusive

ranges. Result comparisons will use values truncated after the first decimal place in units of ng/mL.

- 15.10.1.4 The calibration curves for THC and THCCOOH shall have correlation coefficients ≥ 0.99 .
- 15.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

15.10.2 Controls

- 15.10.2.1 The negative control(s) or blank matrix shall not identify THC or THCCOOH above its limit of detection (0.5 ng/mL THC, 4.0 ng/mL THCCOOH). Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios. Negative urine control(s) shall not identify THCCOOH above its limit of detection, based on above criteria.
- 15.10.2.2 Positive controls
 - a. Chromatographic peaks for THC, THCCOOH and internal standards shall appear symmetrical.
 - b. Retention times shall be within $\pm 2\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 4. These are inclusive ranges.
 - c. Quantitative results for THC and THCCOOH in each control shall be within $\pm 20\%$ of the target values. These are inclusive ranges. Result comparisons will use values truncated after the first decimal place in units of ng/mL.
 - d. All positive controls for a compound must meet these criteria for that compound to be reported from the batch.
 - e. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
 - f. Positive urine control(s) must meet criteria in 15.10.2.2.a above.

15.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

- 15.11.1 Any chromatographic peak for THC or THCCOOH shall appear symmetrical.
- 15.11.2 The retention times for THC and THCCOOH are $\pm 2\%$ and the ion ratios are within $\pm 20\%$ of those in calibrator 4. For urine samples, retention times for THCCOOH are within $\pm 2\%$ and ion ratios are within $\pm 20\%$ of those in the positive urine control. These are inclusive ranges.
- 15.11.3 The quantitative results for each identified compound must be within the dynamic range of the test method.
- 15.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.
- 15.11.5 Urine samples are suitable for qualitative reporting if criteria in 15.11.1 and 15.11.2 are met and the value is ≥ 10 ng/mL, based on the single-point curve generated from the positive control.

15.12 REPORTING

- 15.12.1 Results are reported in units of nanograms per milliliter (ng/mL).
- 15.12.2 Results are truncated to no more than two significant figures for reporting.
 - a. Example 1: THC is measured as 7.85 ng/mL.
 - b. The result is truncated to 7.8 ng/mL (two significant figures) and reported.
 - c. Example 2: THCCOOH is measured at 122.52 ng/mL.
 - d. The result is truncated to 122 ng/mL (three significant figures), but reported as 120 ng/mL (two significant figures).
- 15.12.3 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.
- 15.12.4 When confirmed using this assay, urine results are reported qualitatively.

15.13 METHOD PERFORMANCE

- 15.13.1 Lower limit of quantification:

THC	2.0 ng/mL
THCCOOH	10 ng/mL
- 15.13.2 Dynamic range:

THC	2.0 – 40 ng/mL
THCCOOH	10 – 200 ng/mL
- 15.13.3 Upper limit of quantitation:

THC	40 ng/mL
THCCOOH	200 ng/mL

15.14 TRACEABILITY

- 15.14.1 Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

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APPENDIX A
 INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Temperature	260° C
Split Ratio	Pulsed splitless
Pulse Pressure	45.0 psi
Pulse Time	0.40 min
Purge Flow	30.0 mL/min
Purge Time	0.50 min
Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	5 (Ethyl acetate)
Solvent Wash B	5 (Ethyl acetate)
Sample Pumps	2

Oven/Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.3 mL/min
Initial Temperature	170° C
Initial Time	0.50 min
Ramp Rate	15° C/min
Final Temperature	300° C
Final Time	4.83 min

MASS SPECTROMETER

Solvent Delay	6.00 min	MS Quad Temperature	150°C
EM Offset	Set in tune	MS Source Temperature	230°C
Resolution	Low	Dwell Time	40 msec
Signals	Ions	Ion Ratios	
THC	386, 371, 303	371/386, 303/386	
THC-D ₃	389, 374	374/389	
THCCOOH	473, 488, 371	488/473, 371/473	
THCCOOH-D ₉	479, 497	497/479	

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