CONFIRMATION OF CANNABINOIDS BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY

7.1 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 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 either the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

7.2 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.

7.3 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.

7.4 SPECIMENS

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

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

7.4.3 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.

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

7.5 REAGENTS, MATERIALS AND EQUIPMENT

7.5.1 REAGENTS

7.5.1.1 Acetonitrile

7.5.1.2 BSTFA + 1% TMCS (N,O-bis-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane)

7.5.1.3 Certified blank blood

7.5.1.4 Chloroform (HPLC grade only)

7.5.1.5 Deionized water (DI H_2O)

7.5.1.6 Ethyl acetate

7.5.1.7 Hydrochloric acid (HCl), concentrated (approximately 37%)

7.5.1.8 Methanol

7.5.1.9 Monobasic potassium phosphate (KH_2PO_4)

7.5.1.10 Phosphate buffer, saturated

In a 1 L flask, dissolve 250 g KH_2PO_4 in approximately 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix thoroughly (will require low heating). Store the solution in a glass bottle at room temperature for up to two years. Adjustments to final volume are permitted as long as proportions are maintained.

7.5.1.11 10N Potassium hydroxide

In a 1 L flask, dissolve 561.1 g KOH in approximately 800 mL DI H_2O. Dilute to 1 L and mix until dissolved. Store the solution in a glass bottle at room temperature for up to two years. Adjustments to final volume are permitted as long as proportions are maintained.

7.5.1.12 Potassium hydroxide (KOH), pellets

7.5.2 MATERIALS

7.5.2.1 Autosampler vials, inserts and caps

7.5.2.2 Disposable 16 x 150mm tubes

7.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

7.5.2.4 Disposable pipette tips

7.5.2.5 Disposable safety closures for 16 x 150mm tubes

7.5.2.6 Disposable glass transfer pipettes

7.5.2.7 GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 μm film thickness, or equivalent)

7.5.2.8 Laboratory glassware (graduated cylinders, flasks)

7.5.2.9 Volumetric glassware (flasks)

7.5.3 EQUIPMENT

7.5.3.1 Agilent GC (6890 or equivalent)

7.5.3.2 Agilent MS (5973 or equivalent)

7.5.3.3 Calibrated, adjustable air-displacement pipettes

7.5.3.4 Centrifuge

7.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

7.5.3.6 Oven, dry bath or wet bath

7.5.3.7 Rotary mixer

7.5.3.8 Vacuum aspirator

7.5.3.9 Vortex mixer

7.6 STANDARDS, CALIBRATORS AND CONTROLS

7.6.1 STANDARDS

7.6.1.1 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.

7.6.1.2 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-D_3: 0.1 mg/mL
c. 11-nor-9-carboxy-Δ^9-THC: 1.0 mg/mL
d. 11-nor-9-carboxy-Δ^9-THC-D_9: 0.1 mg/mL

7.6.1.3 Working standards

a. Working standards are prepared by adding the volumes of stock standards listed below to individual 50 mL class-A volumetric flasks and filling to volume with methanol.

	Working Std 1		Working Std 2		Working Std 3	
	THC	THCCOOH	THC	THCCOOH	THC	THCCOOH
Volume (µL)	*50	25	25	125	100	400
Final (ng/µL)	0.1	0.5	0.5	2.5	2	8

*1:10 dilution of stock standard used (0.1 mg/mL)

b. Working standards are stored in the freezer in amber bottles and expire one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

7.6.1.4 Working internal standard

a. Using a calibrated pipette, measure 250 µL THC-d_3 and 500 µL THCCOOH-d_9 stock internal 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 internal standard is 0.5 ng/µL THC-d_3 and 1 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.

7.6.2 CALIBRATORS

7.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standards. The preparation of the calibrators is detailed in 7.7 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.

7.6.3 CONTROLS

7.6.3.1 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.)

7.6.3.2 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 must be either a different lot number or from a different supplier to those used in producing the working standard.
d. Control working standards are prepared by adding the volumes of stock standards listed below to individual 100 mL class-A volumetric flasks and filling to volume with methanol. Control working standards are stored in the freezer in amber bottles and expire one year from date of preparation. Volumes may be adjusted provided that proportions remain constant.

	THC Working Control Std	THCCOOH Working Control Std
Volume (µL)	20	200
Final (ng/µL)	0.2 ng/µL	2 ng/µL

e. The preparation of the positive whole blood controls is detailed in 7.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

7.7 SAMPLE PREPARATION

7.7.1 Label a clean 16 x 150mm tube for each member of the test batch. (i.e. Calibrator, control, case sample)

7.7.2 Add 2 mL of certified blank whole blood into each of the six calibrator tubes, the two positive control tubes and the negative control tube(s).

7.7.3 Using the working standards, spike the calibrators according to the following table.

Calibrator Description (THC/THCCOOH)	Volume (µL) Added	Working Standard
Calibrator 1 (1.0/5.0 ng/mL)	20	1
Calibrator 2 (2.0/10 ngm/L)	40	1
Calibrator 3 (5.0/25 ng/mL)	20	2
Calibrator 4 (10/50 ng/mL)	40	2
Calibrator 5 (25/100 ng/mL)	25	3
Calibrator 6 (50/200 ng/mL)	50	3

7.7.4 Using the control working standards, spike the positive controls according to the following table.

Control Description (THC/THCCOOH)	THC Volume (µL) Added	THCCOOH Volume (µL) Added
Control 1 (4.0/25 ng/mL)	40	25
Control 2 (16/75 ng/mL)	160	75

7.7.5 If in-house positive controls are being used, transfer 2 mL of each into their labeled tubes.

7.7.6 Sample 2 mL of each case sample into its respective tube.

7.7.7 Add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 12.5 ng/mL THC-d_3 and 25 ng/mL THCCOOH-d_9.

7.7.8 Slowly add 5 mL acetonitrile to each tube while vortexing.

7.7.9 Centrifuge the tubes for 5 minutes at 2000-2500 rpm to achieve separation.

7.7.10 Transfer the acetonitrile layer to clean, labeled 10 mL centrifuge or screw cap tubes.

7.7.11 Transfer the tubes to the evaporator and evaporate the extracts to approximately 1 mL at 50°C.

7.7.12 Add 2 mL saturated KH_2PO_4 to each tube and vortex briefly.

7.7.13 Add 8 mL chloroform to each tube.

7.7.14 Cap the tubes and place on a rotary mixer for a minimum of 20 minutes.

7.7.15 Centrifuge the tubes for 5 minutes at 2000-2500 rpm to achieve separation.

7.7.16 Aspirate the top (aqueous) layer into the appropriate waste container.

7.7.17 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Extracts must be completely dry for efficient chemical derivatization.

Note: After evaporation, tubes may be transferred to a 60°C oven for 5 minutes to ensure dryness.

7.7.18 Remove from the evaporator and allow tubes to cool.

7.7.19 In a fume hood, add 50 µL ethyl acetate and 50 µL BSTFA + 1% TMCS to each tube and immediately cap and vortex briefly.

7.7.20 Incubate the tubes for a minimum of 20 minutes at 60-70°C.

7.7.21 Remove from heat and centrifuge the tubes for 2 minutes at 2000 rpm to cool and collect the extracts at bottom of tubes.

7.7.22 Transfer the extracts to labeled glass autosampler vials and cap.

FOR 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 30 µL for target concentration of 60 ng/mL THCCOOH).
c. Sample 1 mL of each case sample into its respective tube.
d. Add 50 µL of the working internal standard solution to each tube.
e. Add 100 µL 10N KOH to each tube.
f. Cap the tubes and incubate for 10 minutes at 60°C to hydrolyze conjugated THCCOOH.
g. Remove from heat and add 300 µL concentrated HCl.
h. Vortex briefly and allow tubes to cool to room temperature
i. Continue with sample preparation at 7.7.12.

7.8 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 CANNAB.M for each line.

7.9 DATA ANALYSIS

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

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

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

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

7.10 CRITERIA FOR BATCH ACCEPTANCE

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

7.10.1 Calibrators and calibration curves

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

7.10.1.2 Retention times shall be within ±2% of those in calibrator 4 and ion ratios shall be within ±20% of the average calculated from the acceptable calibrators. These are inclusive ranges.

Average ion ratio calculations shall be checked as part of the technical review. Completion of the technical review is recorded on the QC log.

7.10.1.3 Quantitative results for THC and THCCOOH in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of their targets. These are inclusive ranges. Result comparisons will use values truncated after the first decimal place in units of ng/mL.

7.10.1.4 The calibration curves for THC and THCCOOH shall have correlation coefficients ≥0.99.

7.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

7.10.2 Controls

7.10.2.1 The negative blood control(s) shall not identify THC or THCCOOH above its limit of quantitation. 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 quantitation, based on above criteria.

7.10.2.2 Positive controls

a. Chromatographic peaks for THC, THCCOOH and internal standards shall appear symmetrical.
b. Retention times shall be within ±2% of those in calibrator 4 and ion ratios shall be within ±20% of the average calculated from the acceptable calibrators. These are inclusive ranges.
c. Quantitative results for THC and THCCOOH in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparisons will use values truncated after the first decimal place in units of ng/mL.
d. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. At least one positive control must meet these criteria for all compounds for the batch to be acceptable.
f. For positive urine control(s), retention times shall be within ±2% of those in calibrator 4 and ion ratios shall be within ±20% of the average calculated from the acceptable blood calibrators.

7.11 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.

7.11.1 Any chromatographic peak for THC or THCCOOH shall appear symmetrical.

7.11.2 The retention times for THC and THCCOOH are ±2% of those in calibrator 4 and the ion ratios are within ±20% of the average calculated from the acceptable calibrators. These are inclusive ranges.

7.11.3 The quantitative results for each identified compound must be within the dynamic range of the test method.

7.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

7.11.5 Urine samples are suitable for qualitative reporting if criteria in 7.11.1 and 7.11.2 are met.

7.12 REPORTING

7.12.1 Results are reported in units of nanograms per milliliter (ng/mL).

7.12.2 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).

7.12.3 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

7.12.4 When confirmed using this assay, urine results are reported qualitatively.

7.13 METHOD PERFORMANCE

7.13.1 Lower limit of quantification:	THC	1.0 ng/mL
	THCCOOH	5.0 ng/mL
7.13.2 Dynamic range:	THC	1.0 ng/mL – 50 ng/mL
	THCCOOH	5.0 ng/mL – 200 ng/mL
7.13.3 Upper limit of quantitation:	THC	50 ng/mL,
	THCCOOH	200 ng/mL

7.14 TRACEABILITY

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

APPENDIX A
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Temperature	260° C
Split Ratio	30:1
Gas Type	Helium
Gas Saver	On
Gas Saver Flow	15.0 mL/min
Gas Saver Time	2.0 min
Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	8 (Ethyl acetate)
Solvent Wash B	8 (Ethyl acetate)
Sample Pumps	2

Oven/Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.1 mL/min
Initial Temperature	175° C
Initial Time	0.50 min
Ramp Rate	15° C/min
Final Temperature	300° C
Final Time	4.00 min

MASS SPECTROMETER

Solvent Delay	6.00 min	MS Quad Temperature	150°C
EM Offset	600	MS Source Temperature	230°C
Resolution	Low	Dwell Time	40 msec
Signals	Ions	Ion Ratios	
THC	386.3, 371.2, 303.1	371.2/386.3, 303.1/386.3	
THC-D_3	389.3, 374.2	374.2/389.3	
THCCOOH	473.2, 488.3, 371.2	488.3/473.2, 371.2/473.2	
THCCOOH-D_3	479.2, 497.2	497.2/479.2	

LIST OF CHANGES

Revision Date	Description	Page Number
03/01/12	Method approved by Washington State Toxicologist. See DRA dated 02/22/12. Method released for use in evidentiary testing on 03/01/12.	All