4.3.2 Quantitative Confirmation of Cannabinoids in Blood

4.3.2.1 Analytes

11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC metabolite) and Δ9-tetrahydrocannabinol (parent THC)

4.3.2.2 General Description of Method

An internal standard GC/MS identification and optional quantitation of derivatized Δ9-tetrahydrocannabinol (THC) and the 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) metabolite using pentafluoropropionic anhydride (PFPA) and hexafluoro-isopropanol (HFIP). Extraction of the THC parent and metabolite from the blood matrix is accomplished by using a solid phase extraction (SPE) method that has been adapted from a Phenomenex, Inc. method.

4.3.2.3 Equipment and Reagents

- GC/MS equipped with a suitable column for separating THC compounds from other drugs and coextractives (i.e. 15 meter DB5 capillary column).
- SPE vacuum tank, manifold, vacuum source.
- SPE Columns intended for THC extraction, such as Phenomenex Strata XL-C columns or equivalent.
- Internal standards: 11-nor-9-carboxy-Δ9 tetrahydrocannabinol-D9 (D9-THC-COOH), Cerilliant T-007 or equivalent, and Δ9 tetrahydrocannabinol-D3 (D3-THC), Cerilliant T-003 or equivalent.
- Controls and calibrators: Δ9-THC, Cerilliant T-005 or equivalent; 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, Cerilliant T-006 or equivalent.
- The usual assortment of laboratory glassware, reaction vessels, pipettes, reagent grade chemicals, vortexers and shakers.
- Derivatizing reagents: pentafluoropropionic anhydride, Aldrich 252387, or equivalent; 1,1,1,3,3,3-hexafluoro-2-propanol, Aldrich 325244 or equivalent.

4.3.2.4 Sample Preparation

A homogeneous blood sample is assured by gently rocking the specimen on the Labquake Shaker for at least 5 minutes. If the specimen is clotted, homogenizing glassware can be used to obtain a liquid sample. All sample handling is performed in the biological safety cabinet using the universal biohazard handling techniques.

4.3.2.4.1 Standard Calibration Curve

- Prepare a standard curve of 1 - 50 ng/mL THC and 5 - 100 ng/mL THC-COOH by pipetting 2 mL blank blood and 20 µL of the appropriate standard into clean, labeled culture tubes:

Standard	Analyte	Concentration (ng/µL)	Final Concentration (ng/mL)
1	THC-COOH	0.5	5
	THC	0.1	1
2	THC-COOH	1	10
	THC	0.25	2.5
3	THC-COOH	2.5	25
	THC	0.5	5
4	THC-COOH	5	50
	THC	1	10
5	THC-COOH	7.5	75
	THC	2.5	25
6	THC-COOH	10	100
	THC	5	50

4.3.2.4.2 Controls
Prepare low, medium and high positive controls by pipetting 2 mL blank blood and 20 µL each of the appropriate control into clean, labeled culture tubes:

Control	Analyte	Concentration (ng/µL)	Final Concentration (ng/mL)
Low	THC-COOH	1	10
	THC	0.3	3
Medium	THC-COOH	4	40
	THC	2	20
High	THC-COOH	8	80
	THC	4	40

- Prepare a UTAK THC 10 ng/mL Whole Blood Toxiclogy Control:
    - Remove from freezer and allow to thaw at room temperature with cap on prior to use.
    - Control should not be thawed in a water bath or by heating.
    - Swirl gently by hand for 3 - 4 minutes to ensure a homogeneous mixture.

- Pipet 2 mL into a clean, labeled culture tube.

- Prepare a negative control by pipetting 2 mL of blank blood into a clean, labeled culture tube.

4.3.2.4.3 Unknowns

- Pipette 2 mL sample blood into a clean, labeled culture tube.
- Smaller sample volume is allowable if sample quantity is limited. Add distilled water to bring sample volume to 2 mL.

4.3.2.4.4 Additional Preparation of all Standards, Controls and Unknowns

- Add 20 µL D9-THC-COOH (10 ng/µL)/D3-THC (1 ng/µL) as the internal standard.
- Add 200 µL methanol. Vortex briefly.
- Add dropwise 2.5 mL cold acetonitrile while vortexing sample at moderate speed.
- Continue vortexing at least 60 seconds.
- Centrifuge for 10 minutes at 3500 rpm.
- Decant supernatant into clean, labeled 5 mL culture tube.
- Discard tubes containing pellets.
- Dry under nitrogen at 35ºC until volume is 100 - 200 µL (~ 25 minutes).
- Add 2.5 mL of 100x diluted glacial acetic acid (pH 3.4 - 4.0). Vortex.

4.3.2.5 Sample Extraction

4.3.2.5.1 Condition Strata Extraction Columns

To appropriately labeled extraction columns placed on a manifold pass the following reagents individually through all columns under gravity or low vacuum (<3 in Hg):

- 2 mL methanol, allow to flow through under gravity.
- 2 mL 100 mM hydrochloric acid, allow to flow through under gravity.

4.3.2.5.2 Apply Sample

- Carefully decant the sample mixture into its respective extraction column.
- Allow the sample to flow through the column under gravity.

4.3.2.5.3 Wash Column

- 2 mL 100 mM HCl, allow to flow through under gravity.
- 2 mL 100 mM HCl/acetonitrile (70:30); allow to flow through under gravity.
- DRY COLUMN ≥ 10 INCHES Hg FOR APPROXIMATELY 7 MINUTES

4.3.2.5.4 Elute Cannabinoids

- 2 mL of acetonitrile/ glacial acetic acid (98/2). Allow to flow through under gravity.
- Use vacuum pump to remove the last of the eluate from column.

4.3.2.5.5 Evaporate Eluates

- Evaporate to ≤ 1 mL at < 40°C. Transfer to autosampler vial and continue evaporating to dryness.

4.3.2.5.6 Unextracted Control

- An unextracted control is prepared by pipetting 20 µL Std 1 and 20 µL D9-THC-COOH/D3-THC internal standard into a clean, labeled autosampler vial.
- Evaporate to dryness at ≤40° C in turboVap.
- Unextracted control is prepared in the same manner as all standards, controls and unknowns from this point forward.

4.3.2.5.7 Derivatize

To each vial add:

- 10 µL ethyl acetate
- 40 µL hexafluoroisopropanol (HFIP). Each molecule of HFIP adds 150 amu to the derivatizable analyte.
- 60 µL perfluoropropionic anhydride (PFPA). Each molecule of PFP adds 146 amu to the derivatizable analyte.
- Cap vial.
- Mix / vortex.
- Incubate 45 minutes at 70º C.

4.3.2.5.8 Evaporate Derivatives

- Evaporate the solvent in each vial to dryness (≤ 40ºC), until the pungent smell of the anhydride is absent.
- Add 30 µL ethyl acetate (EtOAc). Higher concentration samples (e.g., Std 4) may require a larger volume of solvent.
- Mix/vortex.
- Run on appropriate GC/MS.

4.3.2.6 GC/MS Instrument Setup

- An unextracted, derivatized control containing THC, THC-COOH, D3-THC and D9-THC-COOH should be run to verify instrument function prior to the start of the batch analysis. It is further used to qualitatively verify acceptable chromatographic and mass spectral data is being generated by the instrument.
- Carryover may be seen at high concentrations. Each high calibrator (Std 4), control and unknown sample will be followed by a solvent blank in order to minimize the likelihood of carryover between samples. Additional blanks may be run between samples if a sample is suspected of having exceptionally high concentration as may be present in overdose situations.
- Any sample run directly after a sample which has an analyte concentration greater than the highest calibrator will be assessed for carryover. Reinjection will be necessary if carryover is detected.
- Samples shall be run in SIM mode for quantification of THC and THC-COOH.

4.3.2.7 Data Interpretation

• Calibration models, quantification ions, internal standards and peak detection algorithms have been established for each quantified analyte as follows:

4.3.2.7.1 Calibration Curve

Analyte	D3-THC	THC	D9-THC-COOH	THC-COOH
Curve Type		Linear		Linear
Weighting		Inverse (1/x)		Inverse (1/x)
Origin		Ignore		Ignore
Quantification Ion	380	377	649	640
Additional Ions	395,420,463	392,417,460	486,498,631	477,489,625
Internal Standard		D3-THC		D9-THC-COOH
Peak Detection Algorithm		ICIS		ICIS

- Calibration model information in the processing method or Quan Browser shall not be changed unless validated.
- A standard curve should be generated with $r^2 \geq 0.95$ for each analyte. If the r^2 value is not within this range for THC or THC-COOH, the analytical run shall be repeated.
- Evaluate the curve by examining the value of the calibrators. Values of ±30% from the target calibrator concentration are acceptable. Verification of calibrator lot number and expiration date shall occur at the time calibrator/control packs are reviewed.
- At least 5 calibrators must be included in the calibration curve. No more than 1 calibrator between the lowest calibrator and highest calibrator may be removed from the calibration curve.

- If the lowest or highest calibrators are removed from the curve, this change in calibrators may require a repeat analysis of case specimens that lie outside the new calibrator range. Alternately, the cases may be reported out as "< (new lowest calibrator)" or "> (new highest calibrator)" at the discretion of the unit supervisor.

4.3.2.7.2 Controls

- Each batch analysis should contain 10% controls. Control samples may be re-injected, it is not necessary to extract multiple sets of controls. When multiple positive controls are run within a batch, 2 out of 3 positive controls (or 67%) must fall within 20% of target concentration for an acceptable quantitative analysis. Verification of control lot number and expiration date shall occur at the time calibrator/control packs are reviewed. If controls do not meet this acceptance criteria all positive case samples should be repeated. Qualitative results may be reported when reanalysis is not possible or practical.
- The negative control should not contain enough of the analyte of interest to be confirmed.

4.3.2.7.3 Limits of Detection, Limits of Quantification and Upper Limits of Quantification

In SIM mode limits of detection, limits of quantification and upper limits of quantification are shown in the below table.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	ULOQ (ng/mL)
THC	1	1	50
THC-COOH	2	5	100

4.3.2.7.4 Chromatographic and Mass Spectral Quality Control

- Chromatographic Quality
  - Chromatographic quality for Toxicology SIM data is defined as a reasonably symmetrical shaped peak consistent with those observed in calibrators and positive controls and is able to be differentiated from a negative control.
    - This document is intended to aid analysts in the evaluation of chromatographic quality. The most common evaluation will occur with THC. Quantitative values < 1ng/mL do not require chromatographic quality evaluation as they will be reported negative per protocol.
- Retention Time
  - Whenever possible, the retention time of positive analytes shall match a known reference standard run with each batch of unknowns. If a known reference standard is unavailable, a relative retention time based upon deuterated internal standards should be used. It is

recognized that retention times may "shift", slightly, from that of the known reference standard. Flexibility is given to the experienced analyst to prevent misidentification and under-identification.

- Mass spectrum and ion ratios
  - Whenever possible, the mass spectrum and ion ratios of an identified analyte shall match a known reference standard. If a known reference standard is unavailable, a library match using an approved library is acceptable. It is recognized that ion ratios may change, slightly, from that of the known reference standard based upon factors such as analyte concentration, co-eluting substances and background noise. Flexibility is given to the experienced analyst to prevent misidentification and under-identification.
- Library Matches
  - If a known reference standard is unavailable, a library match from an accepted library may be used to aid in identification of an analyte. Approved libraries are:
    - DD2010
    - SWGDRG
    - nistdemo
    - mainlib
    - caymanspectrallibrary
    - Any MSP in-house library in which the name of the analyte, lot number and manufacturer have been recorded.

Note: *Chromatographic Quality and Mass Spectral Quality shall also be evaluated for all calibrators and controls. Calibrators and controls are not required to meet the criteria of showing chromatographic peaks for three m/zs (three m/zs should be observed in the mass spectrum however). All other criteria, a reasonably symmetrical peak, able to be differentiated from a negative control, retention time and mass spectral data shall be assessed. By completing the calibrate/control request within Forensic Advantage, the reviewer is stating that all applicable requirements have been met.*

4.3.2.8 Reporting Results

- Quantitative results will be reported in ng/mL.
- Quantitative results will be truncated (not rounded) and reported to the nearest whole number.
- THC values will be reported from 1-50 ng/mL.
- THC values less than 1 ng/mL will be reported as “not detected”.
- THC values ≥ 1 ng/mL that do not meet chromatographic quality guidelines will be reported as:
  - "not detected"
- THC values greater than 50 ng/mL will be reported as “> 50 ng/mL”.
- THC-COOH will be reported from 5-100 ng/mL.
- THC-COOH values between 2 ng/mL and 4 ng/mL will be reported as < 5 ng/mL.
- THC-COOH values less than 2 ng/mL will be reported as “not detected”.
- THC-COOH values greater than 100 ng/mL will be reported as “> 100 ng/mL”.
- If the initial sample volume is less than 2 mL the results should be reported qualitatively.
- For low volume samples, the quantitative value required for reporting as "present" shall be ≥ LOQ with no volume correction calculations.

4.3.2.9 Preparation of Calibrators, Controls and Internal Standards

Prepared standards, controls and internal standards shall be stored refrigerated.

Expiration is one year from date made, or that of the earliest expiring component, whichever is earlier.

4.3.2.9.1 Calibrators and Controls

Standard reference material stock solutions required to make 5 mL volume of each:

- THC-COOH, 100 ug/ml; 1975 µLtotal volume of stock solution required (2 ampules)
- THC, 1 mg/mL; 100 µL total volume of stock solution required (1 ampule)
- Make a 1:10 dilution of the THC 1 mg/mL stock solution by adding 100 µL of the stock solution to 900 µL of methanol in a glass culture tube, or equivalent.
- Mix/vortex.
- Prepare calibrators and controls according to the below table in 5 mL screw top vials, or equivalent:

Calibrator/ Control	Final THC Concentration (ng/µL)	Final THC-COOH Concentration (ng/µL)	µL 1 mg/mL THC stock 1:10 dilution	µL 100 mg/mL THC-COOH stock	Methanol (µL)
1	0.1	0.5	5	25	4970
2	0.25	1	12.5	50	4937.5
3	0.5	2.5	25	125	4850
4	1	5	50	250	4700
5	2.5	7.5	125	375	4500
6	5	10	250	500	4250
low	0.3	1	15	50	4935
medium	2	4	100	200	4700
high	4	8	200	400	4400

4.3.2.9.2 Internal Standard

Standard reference material stock solutions required to make 5 mL volume:

- D9-THC-COOH, 100 µg/mL; 500 µL (1 ampule)

- D3-THC, 100 µg/mL; 50 µL (1 ampule)

Prepare internal standard in 5 mL screw top vial, or equivalent according to the table below:

Internal Standard	Final Concentration (ng/mL)	µL of 100 µg/mL stock	µL of Methanol
D9-THC-COOH D3-THC	100 10	500 50	4450