Section Three
Blood Toxicology

3.10 SPE Methods for GC/MSD Confirmation

3.10.1 Extraction of 11-nor-Δ^9-THC-9-COOH (Carboxy-THC) from Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® THC Column

3.10.1.1 BACKGROUND

Δ^9-THC (Figure 1) is the chief psychoactive cannabinoid resulting from exposure to marijuana. Δ^9-THC has a peak blood concentration within 5 to 15 minutes following smoking of a marijuana cigarette.[3,4,5] This blood concentration drops rapidly after cessation of smoking.[3,4] The level may fall to less than 5µL within 30 to 60 minutes, although longer detection times have been reported.[3,4] Detection of low dose (1.75%) post-smoking Δ^9-THC has been reported to vary from 3 to 12 hours.[4] This detection window was based on a limit of quantitation of 0.5ng/mL. The number, duration, and spacing of puffs, hold time, and inhalation volume all impact the degree of drug exposure and thus bioavailability.[4] Longer detection times have been observed for frequent users. The Δ^9-THC metabolite, 11-nor-Δ^9-THC-9-COOH (Carboxy-THC), concentration gradually increases and may plateau for several hours.[4] There is poor correlation between blood Δ^9-THC and psychoactive effects since the Δ^9-THC concentrations begin to decline prior to the time of peak effects.[3,4,5] Research continues on models using the relative amounts of Δ^9-THC and Carboxy-THC to assist with establishing recent drug use.

Negative behavioral effects reported from exposure to marijuana include altered time perception, lack of concentration, impaired learning and memory which can lead to impairment of cognitive and performance tasks.[4] Establishing impairment in an individual is based on evaluation of all available information in conjunction with quantitative blood levels.

For additional background, refer to analytical method 2.4.4 and provided references.

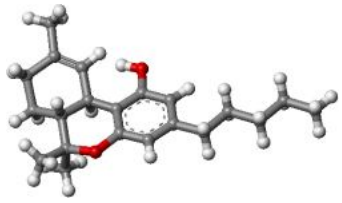

Figure 1.

3.10.1.2 PRINCIPLE & SCOPE

This procedure outlines the use of the 200mg United Chemical Technologies (UCT) CLEAN SCREEN® THC Column for the extraction of carboxy-THC from blood. The CLEAN SCREEN® THC column utilizes a copolymeric sorbent which combines a cationic exchanger and a hydrophobic functionality (reverse phase) to interact effectively, physically and chemically, with analytes of interest while interacting minimally with interfering substances in the blood sample. The cation exchanger utilizes an anionic sorbent to bind to cations. Additional retention mechanisms include hydrophobic interactions and polar adsorption.

For the extraction of carboxy-THC, a deuterated internal standard is added to the sample. Blood proteins are precipitated with 10% Methanol-Acetonitrile solvent mix and are removed via centrifugation. The supernatant is transferred to a clean tube; nitrogen is used to reduce the volume by one-half. The sample pH is then adjusted with an acetate buffer (pH 4.5) and loaded onto a pre-conditioned SPE column. The conditioning of the SPE column creates an environment which allows for optimal interaction between the sorbent and the analyte of interest. The column is subsequently washed to selectively remove matrix components and interfering substances from the column. Next, the column is dried to remove traces of aqueous and organic solvents. After drying, the analyte of interest is eluted from the SPE column with an organic solvent mixture. Following elution and evaporation of the solvent, the extract is derivatized for compound confirmation using a gas chromatograph equipped with a mass selective detector (GC-MSD).

3.10.1.3 EQUIPMENT AND SUPPLIES

3.10.1.3.1 200mg CLEAN SCREEN® THC Extraction Column
3.10.1.3.2 Disposable inserts for SPE manifold ports
3.10.1.3.3 Drybath or laboratory oven capable of 70°C
3.10.1.3.4 Evaporative concentrator equipped with nitrogen tank.
3.10.1.3.5 Tube rocker
3.10.1.3.6 Vortex mixer
3.10.1.3.7 Laboratory centrifuge capable of 3400- 3500rpm
3.10.1.3.8 Vacuum Manifold/ Vacuum pump
3.10.1.3.9 Fixed and adjustable volume single channel air displacement pipetters, and appropriate tips, capable of accurate and precise dispensing of volumes indicated.
3.10.1.3.10 16 x 100mm glass tubes (silanized recommended)
3.10.1.3.11 Screw Cap for 16mm O.D. tubes
3.10.1.3.12 GC/MS Automated Liquid Sample (ALS) vials
3.10.1.3.13 GC/MS Vial Microinsert (silanized recommended)
3.10.1.3.14 Gas Chromatograph equipped with a Mass Selective Detector and a nonpolar capillary column with a phase composition

comparable to 100%-dimethylpolysiloxane or 95%-dimethylpolysiloxane with 5%-diphenyl.

3.10.1.4 REAGENTS

Refer to manual section 5.12 for solution preparation instructions.

3.10.1.4.1 Deionized/distilled (DI) water
3.10.1.4.2 Methanol (Certified ACS Grade)
3.10.1.4.3 Hexane (Certified ACS Grade)
3.10.1.4.4 Ethyl Acetate (Certified ACS Grade)
3.10.1.4.5 Acetonitrile (Certified ACS Grade)
3.10.1.4.6 10% Methanol in Acetonitrile
3.10.1.4.7 100mM Acetate Buffer (pH 4.5)
3.10.1.4.8 100mM HCl
3.10.1.4.9 70:30 Hexane:Ethyl Acetate
3.10.1.4.10 70:30 100mM HCl:Acetonitrile
3.10.1.4.11 BSTFA + 1% TMCS

3.10.1.5 QUALITY ASSURANCE MATERIAL

3.10.1.5.1 Calibrator and Control Solutions

Corresponding calibrator and control reference material must be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available.

3.10.1.5.1.1 **Reference Material Stock Solutions**

Concentration: 100µg/mL or 1mg /mL
Carboxy-THC
Store remaining stock solution as recommended by vendor.

3.10.1.5.1.2 **Reference Material Working Solutions**

Working solutions are stable for 6 months when stored under refrigeration.

Concentration: 1.0ng/µL

As appropriate, add 100µL of 100µg/mL or 10µL of 1mg/mL Stock Solution to approximately 9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

Concentration: 0.1ng/µL

Add 1000µL of 1ng/µL working calibration solution to approximately 8mL Methanol in 10mL volumetric class A flask. QS to 10mL.

3.10.1.5.2 Internal Standard Solutions

3.10.1.5.2.1 **Stock Solution**

Concentration: 100µg/mL or 1mg/mL Carboxy-THC-D_9

Store remaining stock solution as recommended by vendor.

3.10.1.5.2.2 **Working Internal Standard Solution**

Working internal standard solution is stable for 6 months when stored under refrigeration.

Concentration: 1.0ng/µL

Add 100µL of 100µg/mL or 10µL of 1mg/mL stock solution to approximately 9mL Methanol in a 10mL volumetric class A flask. QS to 10mL.

3.10.1.5.3 Whole Blood Negative Control

Negative Whole Blood

3.10.1.6 PROCEDURE

3.10.1.6.1 Initial set-up

Label extraction tubes (x3), 200mg CLEAN SCREEN® extraction columns (x1), and GC/MSD vials (x1) with microinserts for calibrators, controls and case samples.

3.10.1.6.2 Calibrator Preparation

Use the same lot of negative blood for all controls and calibrators.

3.10.1.6.2.1 Add 1mL of negative whole blood to six extraction tubes.

3.10.1.6.2.2 Add the volume of 0.1ng/µL carboxy-THC calibrator working solution as indicated in the following table:

Level	Desired ng/mL	µL Working RM
1	2.5	25
2	5	50
3	10	100

3.10.1.6.2.3 Add the volume of 1.0ng/µL carboxy-THC calibrator working solution as indicated in the following table:

Level	Desired ng/mL	µL Working Reference Material
4	25	25
5	50	50
6	100	100

3.10.1.6.3 Positive Control Sample Preparation

Use the same lot of negative blood for all controls and calibrators.

3.10.1.6.3.1 Add 1mL of negative whole blood to two extraction tubes.

3.10.1.6.3.2 Add indicated amount of 0.1ng/µL working control solution.

Desired ng/mL	µL Working Control
6.0	60.0

3.10.1.6.3.3 Add indicated amount of 1.0ng/µL working control solution.

Desired ng/mL	µL Working Control
60.0	60.0

3.10.1.6.4 Negative Control Sample Preparation

Add 1mL of negative whole blood into an extraction tube.

3.10.1.6.5 Case Sample Preparation

Place sample container on tube rocker for a minimum of five minutes. If sample is clotted, homogenize as necessary. Transfer 1mL of blood into a labeled extraction tube.

3.10.1.6.6 Internal Standard Addition

3.10.1.6.6.1 To calibrators, controls and case samples, add 25µL of internal standard.

3.10.1.6.6.2 Vortex tube briefly and allow to stand 15 to 30 minutes for sample equilibration.

3.10.1.6.7 Protein Precipitation

3.10.1.6.7.1 While vortexing, add 2mL cold 10% methanol in acetonitrile dropwise to case, calibrator and control samples.

3.10.1.6.7.2 Cap tube and continue vortexing tube for approximately 30 seconds.

3.10.1.6.7.3 Allow tube to stand for approximately five minutes.

3.10.1.6.7.4 Centrifuge at approximately 3500 rpm for 10 minutes.

3.10.1.6.7.5 Decant organic supernatant into second labeled glass tube.

3.10.1.6.7.6 Transfer tube to Evaporative Concentrator and evaporate under nitrogen at ≤40°C to approximately 1mL. ***Do not allow extract to go to dryness.***

3.10.1.6.7.7 To the evaporated extract add 2mL **100mM acetate buffer (pH 4.5)**. Vortex briefly to mix.

3.10.1.6.7.8 If necessary, centrifuge buffered solution for an additional 5 minutes at approximately 3500 rpm to remove blood fragments or foam.

3.10.1.6.8 SPE Column Preparation

3.10.1.6.8.1 Insert valve liners and labeled SPE columns into appropriate location on vacuum manifold. For each following SPE step, allow to gravity flow or aspirate at ≤ 3 in. Hg to prevent sorbent drying.

3.10.1.6.8.2 To each SPE column, add 3mL **70:30 Hexane:Ethyl acetate**.

3.10.1.6.8.3 To each SPE column, add 3mL **methanol** to the column.

3.10.1.6.8.4 To each SPE column, add 3mL **deionized or distilled water** to the column.

3.10.1.6.8.5 To each SPE column, add 1mL **100mM HCl**.

3.10.1.6.9 Blood Extract Loading
Decant buffered blood extract onto the SPE column.

3.10.1.6.10 Column Wash

3.10.1.6.10.1 To each SPE column, add 2mL of **deionized or distilled water**.

3.10.1.6.10.2 To each SPE column, add 2mL **70:30 100mM HCl:Acetonitrile**.

3.10.1.6.11 Dry Disc
Turn on/increase vacuum to ≥10 in. Hg (≥34 kPa) for ≥ 5 minutes.

3.10.1.6.12 Compound Elution

3.10.1.6.12.1 Open vacuum manifold, wipe collection tips, and insert the collection rack containing the labeled glass tubes.

3.10.1.6.12.2 To each SPE column, add 200uL **hexane** (important for elution solvent reception).
*Gravity flow **only**. Do not allow column to dry.*

3.10.1.6.12.3 To each SPE column, add 3mL **70:30 Hexane:Ethyl Acetate** elution solvent. *Collect eluate with gravity flow or apply minimal vacuum.*

3.10.1.6.13 Eluate Evaporation
Transfer centrifuge tube to Evaporative Concentrator. Evaporate eluates to dryness, under a gentle stream of nitrogen at ≤ 40°C.

3.10.1.6.14 Derivatization

3.10.1.6.14.1 Add 40µL ethyl acetate and vortex.

3.10.1.6.14.2 Add 40µL BSTFA (1% TMCS) to each extract. Cap tubes and vortex briefly.

3.10.1.6.14.3 Heat tubes at 70°C for 15 minutes.

3.10.1.6.14.4 Remove tubes from heat and allow to cool to room temperature.

3.10.1.6.14.5 Transfer derivative to labeled GC/MSD ALS vial with microinsert.

3.10.1.6.15 Preparation for Analysis Run

3.10.1.6.15.1 Into Sequence log table, enter the sample case numbers, blanks and controls.

3.10.1.6.15.2 Load samples, standards, blank and controls into the quadrant rack as noted in the sequence table.

3.10.1.6.16 GC-MS Calibration Curve

3.10.1.6.16.1 The calibration curve must be established with a minimum of four data points.

3.10.1.6.16.2 Calibrators should be analyzed in order of increasing concentration.

3.10.1.6.16.3 The least squares line resulting from the analysis of the calibrators must have a coefficient of correlation of ≥0.98.

3.10.1.6.16.4 If calibrators are run in duplicate, it is not required that duplicate calibration points be included as long as the linearity requirement is met.

3.10.1.7 GC and MSD ACQUISITION PARAMETERS

Critical parameters are specified below. Parameters not specified are at the discretion of the analyst and should be optimized for the particular GC-MSD instrument. Each laboratory should maintain a centrally stored printed or electronic copy of current and past GC-MSD methods. The data supporting the GC-MSD method should be stored centrally.

3.10.1.7.1 GC Temperature Parameter

Injection Port: 250°C or 260°C

3.10.1.7.2 MSD Instrument Parameters

Detector/Transfer Line: 280°C

3.10.1.7.3 ALS Parameters

Injection Volume: 1µL (1 stop)
Viscosity Delay: A minimum of 1 second

Solvent Washes (A & B): A minimum of 3 pre- and post-wash rinses.

3.10.1.7.4 MS SIM Parameters

Analyte	Target Ion	Qualifier Ion 1	Qualifier Ion 2
Carboxy-THC	371	473	488
Carboxy-THC-D9	380	482	497

3.10.1.8 REPORTING CRITERIA

3.10.1.8.1 Qualitative Chromatographic Criteria

Acceptable retention time window established by calibrators is ± 0.1 minutes.

3.10.1.8.2 Qualitative Mass Spectral SIM Criteria

Ion ratios for the analyte and its corresponding internal standard, established by calibrators for target and qualifier ions, must not differ by more than ±20% (relative). Refer to section 3.10.1.8.4.5for cutoff criteria.

Cut-off for Carboxy-THC is the lowest calibrator (2.5ng/mL), or the lowest calibrator that meets quality assurance requirements. Any analyte with a quantitative value below this cut-off will be reported as "none detected." If the concentration exceeds the calibration range, the sample can either be appropriately diluted with negative whole blood for reanalysis or qualitatively confirmed in full scan mode (refer to section 3.10.1.8.3 for confirmation criteria).

3.10.1.8.3 Qualitative Mass Spectral Full Scan Criteria

Analytes may be confirmed from full scan data if the retention time for the sample versus applicable reference material does not differ by more than ±0.1 minutes and there are no significant differences in the mass spectral data.

3.10.1.8.4 Quantitative Mass Spectral Criteria

3.10.1.8.4.1 Refer to Section 3.10.1.9.2 for determination of when this method may be used for quantitative purposes.

3.10.1.8.4.2 Quantitative results can be accepted if the calculated concentrations of all calibrator and control samples are within ±20% of their respective concentrations (relative).

3.10.1.8.4.3 Quantitation is achieved through the plotting of the target ion response ratio versus the concentration for each calibrator.

3.10.1.8.4.4 Quantitative values for case samples, calibrators and controls will be truncated for reporting purposes.

3.10.1.8.4.5 Administrative limit of detection (LOD) for carboxy-THC is 2.5ng/mL, the lowest calibrator level. Results < this LOD should be reported as negative unless there are extenuating circumstances. The Toxicology Discipline Leader must be consulted to evaluate exceptions.

3.10.1.8.4.6 If the concentration exceeds the calibration range, the sample must be appropriately diluted with negative whole blood for reanalysis. Alternatively, the analyte may be reported using full scan data; refer to section 3.10.1.8.3 for criteria.

3.10.1.9 REPORTING OF RESULTS

3.10.1.9.1 Qualitative Confirmation

If carboxy-THC meets confirmation criteria, it may be reported. The administrative cut-off of 2.5ng/mL, or the lowest calibrator meeting quality assurance requirements, will be used to determine if the analyte is detected.

3.10.1.9.2 Quantitative Value

This method is currently only approved for the qualitative identification of drugs. Quantitative values are not to be reported or expressed. They are currently being used to establish an administrative cut off. Once the uncertainty of measurement is established for this method, it will be evaluated for quantitative reporting.

3.10.1.10 QUALITY ASSURANCE REQUIREMENTS

3.10.1.10.1 General

3.10.1.10.1.1 Blood samples are to be stored under refrigeration after aliquots are removed for analysis.

3.10.1.10.1.2 Refer to toxicology manual section 5.1, 5.2, 5.8, and 5.10 for quality assurance and reference material authentication requirements.

3.10.1.10.2 Per Analysis Run Control Requirements

3.10.1.10.2.1 Minimally, a solvent blank must follow the highest calibrator, as well as precede each case sample.

3.10.1.10.2.2 A minimum of the spiked blood controls described in section 3.10.1.6.3 must be run per batch of samples. Controls should not be grouped at the beginning of the acquisition sequence. Rather, controls should be interspersed throughout the sequence.

3.10.1.10.2.3 If the number of case samples exceeds 10, in addition to the two controls described in 3.10.1.6.3, one spiked or commercially obtained blood control must be run with each additional 10 case samples. Additional concentrations may be used.

3.10.1.10.2.4 Analysts may combine their samples into a single run to conserve supplies. However, each analyst with samples in the run must independently comply with the control requirements in section 3.10.1.10.2. A third-party reviewer must independently review the central file packet for compliance to method requirements.

3.10.1.10.3 Monitoring of Control Values

Once the method has been approved for quantitative purposes, the following is required: upon the completion of analysis, input blood control values on a spreadsheet used to assess uncertainty for this method.

3.10.1.11 ANALYSIS DOCUMENTATION

3.10.1.11.1 Case results are to be recorded in the LIMS system.

3.10.1.11.2 Original data for controls and standards will be prepared for each analysis run and stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

3.10.1.11.3 A copy of data for controls and standards may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of the control and standard printouts can be prepared from the centrally stored document.

3.10.1.12 REFERENCES AND RECOMMENDED READING

3.10.1.12.1 UCT CLEAN SCREEN® Extraction Columns Application Manual.

3.10.1.12.2 Standard Operating Procedure for Blood SPE Cannabinoids, Montana Department of Justice Forensic Sciences Division.

3.10.1.12.3 Standard Operating Procedure for Blood SPE THC and Carboxy-THC GC/MSD Assay, Edmonton, Canada Office of the Chief Medical Examiners, 2003.

3.10.1.12.4 Huestis, M.A., *Cannabis (Marijuana) - Effects on Human Behavior and Performance*, Forensic Science Rev. 14(1/2): 16-60, 2002.

3.10.1.12.5 Drummer, O.H., *Cannabis*, pp. 178-212. *in:* The Forensic Pharmacology of Drugs of Abuse, Arnold: London, 2001.

3.10.1.12.6 Bock, Peter, *Getting it right - R&D methods for science and engineering*, Academic Press, San Diego, 2001.

Revision History

Section Three

Blood Toxicology

3.10 Manual Solid Phase Extraction (SPE) Methods

3.10.1 Extraction of Carboxy-THC from Blood Employing the United Chemical Technologies (UCT) 200 mg CLEAN SCREEN® THC Extraction Column

Revision No.	Issue Date	History/Comments
0	11-22-2006	Original Issue Method is approved for qualitative purposes only. Upon review of uncertainty determinations for quantitative analysis this method will be applied for intended use.
1	07-28-2008	Clarified that negative blood used to prepare calibrators and positive controls is the same lot as used for negative control.
2	03-07-2011	Replaced 4°C storage with “under refrigeration”, emphasized need for sample homogeneity. Reformatted for clarity.
3	05-17-2013	Removed Δ^9-THC from method. Clarified that stock solutions are to be stored per vendor recommendations. Added provision for qualitative confirmation of analytes from full scan data. Removed quantitative references – method not for quantitation. Clarified that analytes will be deemed “none detected” if values achieved for that analyte is below the lowest calibrator meeting quality assurance requirements. Added provision for shared runs by multiple analysts, and clarified per-analyst control requirements. Made silanized extraction tubes a recommendation, due to supply availability limitations. Formatting for continuity.
4	03-13-2015	Clarified that cold 10% Methanol in Acetonitrile

		be added to blood for the protein precipitation step. Deleted repetitions of aspiration from the SPE method section; made single instruction at beginning of SPE section to replace repetitions. Clarified vortexing procedure in the derivatization step. Formatting for continuity. Consolidated quality assurance paragraphs. Added LIMS reporting requirement. Re-added quantitative criteria, but clarified that method not for quantitative use at this time. Removed tune information from 3.10.1.6.15, and reworded this section for method consistency. Made retention time requirement more rigorous.