
**Technical Procedure for Blood Cannabinoid Liquid-Liquid Extraction (BCLLE)
for Analysis by LC-MS/MS**

1.0 Purpose - This procedure specifies the required elements for the extraction and quantitation of THC, 11-OH-THC and THCA using liquid-liquid extraction (LLE) for LC-MS/MS analysis.

2.0 Scope – This procedure applies to the blood cannabinoid extractions performed in the Toxicology Units of the State Crime Laboratory.

3.0 Definitions

- **Quality control (QC) check** – Periodic confirmation of the reliability of equipment, instrumentation, and/or reagents.
- **Limit of Quantitation (LOQ)** – The LOQ is the lowest calibrator concentration included in the calibration curve.
- **Upper Limit of Quantitation (ULOQ)** – The ULOQ is the highest calibrator concentration included in the calibration curve.

4.0 Equipment, Materials and Reagents

4.1 Equipment

- Centrifuge
- Mechanical Pipettes
- Class A Volumetric flasks
- TurboVap or equivalent evaporator
- Test tube rocker

4.2 Materials

- Test tubes (16 x 125, 16 x 150 mm) with caps
- Glass Conical test tubes
- Vortexer
- Pipette tips
- LC vials with pre-slit septa caps

4.3 Reagents

- Deionized water
- Water, HPLC grade or higher
- Hexane, HPLC grade or higher
- Ethyl acetate, HPLC grade or higher
- Acetonitrile, HPLC grade or higher
- Glacial acetic acid, ACS grade or higher
- Formic Acid, 99 % or higher
- Ammonium Formate
- Negative Blood

4.4 Primary Reference Standards

- Δ -9-tetrahydrocannabinol (Δ -9-THC)
- 11-nor- Δ -9-tetrahydrocannabinol-9-carboxylic acid (THCA)
- 11-hydroxy- Δ -9-tetrahydrocannabinol (11-OH-THC)
- Δ -9-tetrahydrocannabinol (Δ -9-THC)-D₃
- 11-nor- Δ -9-tetrahydrocannabinol-9-carboxylic acid(THCA)-D₃
- 11-hydroxy- Δ -9-tetrahydrocannabinol (11-OH-THC)-D₃

4.5 Prepared Reagents/Standards – Reagents/Standards may be prepared by the Forensic Scientist in any amount provided that the component ratios are kept constant.

4.5.1 Cannabinoid Stock Internal Standard Solution (CSIS)

4.5.1.1 Prepare a stock solution containing 1.0 $\mu\text{g/mL}$ of the deuterated reference standards.

4.5.1.1.1 Pipette 100 μL of each of the 100 $\mu\text{g/mL}$ (or 10 μL of 1.0 mg/mL) deuterated reference standards to a 10 mL volumetric flask. Add methanol in a quantity sufficient (qs) to bring to volume.

4.5.1.2 Lot number: Eight digit format year/month/day + CSIS

4.5.1.2.1 Example: 20101231CSIS

4.5.1.3 Expiration: One year

4.5.1.4 Storage: Freezer

4.5.1.5 QC check: Successful control check

4.5.2 Cannabinoid Internal Standard Working Solution (CISW)

4.5.2.1 Prepare a working solution containing 100 ng/mL of the deuterated reference standards.

4.5.2.1.1 Pipette 1.0 mL of the 1.0 $\mu\text{g/mL}$ stock Internal Standard solution into a 10 mL volumetric flask and qs to volume with methanol.

4.5.2.2 Expiration: Prepare Daily

4.5.2.3 QC check: n/a

4.5.3 Cannabinoid Stock Calibration Solution (CSC)

4.5.3.1 Prepare a solution containing 1.0/5.0 $\mu\text{g/mL}$ of Δ -9-THC, 11-OH-THC/THCA primary reference standards in methanol.

4.5.6 Mobile Phase A- 5mM Ammonium Formate with 0.1 % Formic Acid

4.5.6.1 Combine 315 mg of ammonium formate and 1 mL of formic acid in HPLC grade water in a 1 L volumetric flask and qs to volume with HPLC grade water.

4.5.6.2 Lot number: "MP-A1-" + Eight digit format year/month/day

4.5.6.2.1 Example: MP-A1-20140123

4.5.6.3 Expiration: One month

4.5.6.4 Storage: Room Temperature

4.5.6.5 QC check: n/a

4.5.7 Mobile Phase B- Acetonitrile with 0.1 % Formic Acid

4.5.7.1 Combine acetonitrile and 1 mL of formic acid in a 1 L volumetric flask and qs to volume with acetonitrile.

4.5.7.2 Lot number: "MP-B1-" + Eight digit format year/month/day

4.5.7.2.1 Example: MP-B1-20140123

4.5.7.3 Expiration: One month

4.5.7.4 Storage: Room Temperature

4.5.7.5 QC check: n/a

4.5.8 10 % Acetic Acid

4.5.8.1 Add 50 mL of glacial acetic acid to 450 mL of deionized water.

4.5.8.2 Lot number: Eight digit format year/month/day

4.5.8.2.1 Example: 20101231

4.5.8.3 Expiration: One year

4.5.8.4 Storage: Room Temperature

4.5.8.5 QC check: Tests acidic to pH or litmus paper

4.5.9 9:1 Hexane:Ethyl Acetate

4.5.9.1 Combine 900 mL of hexane with 100 mL of ethyl acetate.

4.5.9.2 Lot number: Eight digit format year/month/day

4.5.9.2.1 Example: 20101231

4.5.9.3 Expiration: One year

4.5.9.4 Storage: Room Temperature

4.5.9.5 QC check: n/a

5.0 Procedure

5.1 Allow all solutions and samples to be analyzed to equilibrate to room temperature

5.2 Calibration and Control Sample Preparation

5.2.1 Calibrator Preparation

5.2.1.1 Pipette the following volumes of the **CSC** and the **CCW** into the labeled screw cap test tubes.

Amount of CSC (μL)	Amount of CCW (μL)	Final Concentration of cannabinoids (ng/mL)
100		100/500
50		50/250
25		25/125
	100	10/50
	50	5/25
	25	2.5/12.5
	10	1.0/5.0

5.2.1.2 Add 1 mL of negative blood to each calibrator to obtain the appropriate concentrations.

5.2.2 Positive Control Preparation

5.2.2.1 Pipette the following volumes of the **CSV** into the labeled screw cap test tubes.

Amount of CSV (μL)	Final Concentration of cannabinoids (ng/mL)
100	50/250
10	5/25

5.2.2.2 Add 1 mL of negative blood to each control to obtain the appropriate concentrations.

5.2.3 Negative Control Preparation

5.2.3.1 Add 1 mL of negative blood to the labeled screw cap tubes.

5.2.4 An extraction batch will include at least two negative and two positive controls. Case specimens shall be bracketed by one of each.

5.2.5 Control samples shall make up at least 10 % of an extraction batch.

5.3 Maintenance

5.3.1 Add water to the TurboVap if needed.

5.4 Sampling

5.4.1 Pipette 1 mL of case specimens to be analyzed into a labeled screw cap tube.

5.4.1.1 Ensure that all body fluids are homogenous.

5.4.1.2 If a homogenous sample cannot be obtained, a notation shall be made in the worksheet detailing the condition of the sample and its handling.

5.5 Extraction Procedure

5.5.1 Add 100 µL of the internal standard working solution to each calibrator, control, and case specimen to be analyzed.

5.5.2 Add 2.0 mL of water and vortex.

5.5.3 Add 800 µL of 10 % acetic acid and vortex.

5.5.4 Add 8.0 mL of 9:1 hexane:ethyl acetate solution, cap and rock tubes for 30 minutes.

5.5.5 Centrifuge tubes for 20 minutes. Transfer the organic (upper) layer to a clean labeled glass conical tube.

5.5.6 Evaporate to dryness using a TurboVap at 50 °C.

5.5.7 Add 50 µL of 50:50 acetonitrile:water to each tube and centrifuge for 10 minutes.

5.5.8 Transfer reconstituted specimens to labeled LC vials and cap.

5.5.9 Analyze samples on a LC-MS/MS as specified in the [Toxicology Liquid Chromatography-Tandem Mass Spectrometry \(LC-MS/MS\) procedure](#).

5.6 Data Processing and Calibration/Control Acceptance Criteria

5.6.1 Process the run using the Cannabinoid method.

5.6.1.1 Ensure that the boxes are checked to update the retention times and ion ratios of the analytes and their internal standards.

5.6.2 Save the processed data with the name of the procedure and the extraction date added to the end.

5.6.3 Calibration Curve Acceptance Criteria

- 5.6.3.1** Evaluate the curve by back-calculating the calibrator concentrations against the curve. Values of +/- 30 % from the target concentration are acceptable for the lowest calibrator. All other calibrators shall be within 20 % of the target concentration.
- 5.6.3.2** A maximum of two calibration points may be dropped from the curve with cause (e.g., statistical outlier, laboratory accident, fails **5.6.3.1**, etc.).
- 5.6.3.2.1** If the low calibration point is dropped, this will change the LOQ and may require a repeat analysis for case specimens whose quantitation is between the lowest extracted calibrator and the new LOQ.
- 5.6.3.2.2** If the high calibration point is dropped, this will change the ULOQ and may require a repeat analysis for case specimens whose quantitation is between the highest extracted calibrator and the new ULOQ.
- 5.6.3.3** The calibration curves for each analyte shall show a correlation of determination (r^2) of 0.985 or greater.
- 5.6.3.4** The x-intercept shall be less than the lowest extracted calibrator.
- 5.6.3.5** It is acknowledged that some assays are inherently non-linear (e.g., LC-MS/MS) and the use of quadratic calibration curves may be necessary and appropriate.

5.6.4 Quality Control Acceptance Criteria

- 5.6.4.1** Each analyte in a positive control shall give a quantitation within +/- 25 % of the expected concentration.
- 5.6.4.2** The qualifier ion ratios for an analyte/internal standard in the controls shall be within +/- 20 % of the analyte ion ratios determined by the average of the calibration sample ion ratios.
- 5.6.4.3** The negative control fails for an analyte if there is an integrated peak for both transitions at the expected retention time that is greater than 20 % of the lowest calibrator area of that analyte and meets the requirement in **5.6.4.2**.
- 5.6.5** If manual integration is performed, the chromatogram showing the integration prior to manual integration shall be printed and attached to the chromatogram showing the result of the manual integration. The reason for the manual integration shall be documented on the chromatogram.
- 5.6.6** Create a data packet for the run, including the following quality control data:
- Summary page with FA workstation reference
 - Completed extraction worksheet

-
- LC-MS/MS sequence list
 - Calibration Verification Report
 - Experiment and Calibration Report
 - Quantitation reports of all calibrators and controls

5.6.7 The Quality Control data packet shall be reviewed by a Forensic Scientist qualified to perform the Technical Procedure for Blood Cannabinoid Liquid-Liquid Extraction for Analysis by LC-MS/MS and, if acceptable, approved in the Toxicology Unit section object repository of FA with a file name beginning with “THCQC” (capitalization optional), followed by eight digit format year/month/day.

5.6.7.1 Example: THCQC20121004

5.6.8 Control Charting

5.6.8.1 Complete the Toxicology Control Chart Form and submit to the Toxicology Technical Leader.

5.7 Identification of THC, 11-OH-THC, and THCA

5.7.1 If an analyte that failed to meet the acceptance criteria listed in **5.6.3** or **5.6.4** is present in a case sample, the case shall be re-analyzed, sample volume permitting.

5.7.2 The internal standard area must be within 50 % -200 % of the average internal standard area of the controls.

5.7.2.1 If the internal standard area is less than 50 %, the quantitation may be used if the signal to noise (S/N) calculated by the instrument software is greater than 10:1. If not, the case shall be re-analyzed to confirm the quantitation, sample volume permitting.

5.7.2.2 If the internal standard area is greater than 200 %, the case shall be re-analyzed to confirm the quantitation, sample volume permitting.

5.7.3 If there is insufficient sample volume remaining, the data may be reported qualitatively only if the acceptance criteria in **5.6.4.2**, **5.6.4.3**, **5.7.4**, **5.7.5**, and **5.7.6** are met.

5.7.4 The qualifier ion ratios of each component to be identified shall be within +/- 20 % of the target value.

5.7.5 The retention time of each component to be identified shall not differ from the target value by more than 4.0 %.

5.7.6 The quantitation result shall be greater than the LOQ of each analyte to be reported.

5.8 Calculations

5.8.1 Percent Difference Calculation:
$$\frac{|(\text{standard retention time} - \text{analyte retention time})|}{(\text{standard retention time})} * 100$$

5.9 Uncertainty of Measurement

- 5.9.1** The process uncertainty for all analytes quantitated by this procedure is 18 %.
- 5.9.2** The uncertainty of measurement shall be reported as a percentage only.
- 5.9.3** In accordance with the [Drug Chemistry Procedure for Measurement Assurance](#) these values shall be updated annually.

5.10 Reporting

5.10.1 THC, 11-OH-THC and THCA identified by LC-MS/MS analysis shall have a positive indication for Cannabinoids from an Immunoassay Drug Screen analysis to be reported. Refer to the Drug Chemistry Section Toxicology Unit [Toxicology Analysis](#) procedure for reporting of THC, 11-OH-THC, or THCA.

5.10.2 All quantitative results will be rounded to two significant figures for reporting.

5.10.2.1 If the number to the right of the second significant figure is five or higher, round up. If the number to the right of the second significant figure is four or lower, round down.

5.10.3 Example Reporting Statement:

- Tetrahydrocannabinol (THC) – 10 ng/ml +/- 18 %
- 11-hydroxy- Δ -9-tetrahydrocannabinol (11-OH-THC) – 5.0 ng/ml +/- 18 %
- 11-nor- Δ -9-tetrahydrocannabinol-9-carboxylic acid (THCA) – 45 ng/ml +/- 18 %

5.11 Record the following in the case record:

- Approved THCQC data packet for the run
- Quantitation report of the sample

6.0 Limitations

6.1 Samples with THC, 11-OH-THC or THCA whose concentrations exceed the upper level of the calibration curve may be reanalyzed after dilution with the proper matrix to bring them within the calibration range.

7.0 Safety

7.1 Refer to the [Laboratory Safety Manual](#).

8.0 References

Alabama DFS Cannabinoids method

Virginia DFS Cannabinoids method

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).

C. Coulter, E. Miller, K. Crompton, C. Moore. "Tetrahydrocannabinol and Two of its Metabolites in Whole Blood Using Liquid Chromatography-Tandem Mass Spectrometry." *Journal of Analytical Toxicology*. 32: 653-658 (2008).

C. Jamey, E/ Swarc, A. Tracqui, and B. Ludes. "Determination of Cannabinoids in Whole Blood by UPLC-MS-MS." *Journal of Analytical Toxicology*. 32: 349-354 (2008).

9.0 Records

- Quality Control data packet
- Case record
- Toxicology Control Chart Form

10.0 Attachments- N/A

Revision History		
Effective Date	Version Number	Reason
03/14/2014	1	Original Document
08/29/2014	2	4.5 – Modified language 5.2.2.1 – Removed reference to CVW 5.6.5 – Added requirement