
**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 Toxicology in the Raleigh, Triad, and Western locations of the State Crime Laboratory.
- 3.0 Definitions** – See **Toxicology Definitions List**
- 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
 - Methanol, HPLC grade or higher
 - Acetonitrile, HPLC grade or higher
 - Negative Blood
 - Acetic acid
 - Hexane
 - Ethyl acetate
- 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

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- 4.5 Prepared Reagents** – Refer to [Toxicology Solution Prep Guidelines](#) for instructions on how to prepare the reagents required by this procedure.
- 4.6 Prepared Standards** –Standards may be prepared by the Forensic Scientist in any amount provided that the component ratios are kept constant.

4.6.1 Cannabinoid Stock Internal Standard Solution (CSIS)

4.6.1.1 Prepare a stock solution containing 1.0 µg/mL of the deuterated reference standards.

4.6.1.1.1 Pipette 100 µL of each of the 100 µg/mL (or 10µL 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.

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

4.6.1.2.1 Example: 20101231CSIS

4.6.1.3 Expiration: One year.

4.6.1.4 Storage: Freezer.

4.6.1.5 QC check: Successful control check.

4.6.2 Cannabinoid Internal Standard Working Solution (CISW)

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

4.6.2.1.1 Pipette 1.0 mL of the 1.0 µg/mL stock Internal Standard solution into a 10 mL volumetric flask and QS to volume with methanol.

4.6.2.2 Expiration: Prepare Daily.

4.6.2.3 QC check: N/A

4.6.3 Cannabinoid Stock Calibration Solution (CSC)

4.6.3.1 Prepare a solution containing 1.0/5.0 µg/mL of Δ-9-THC, 11-OH-THC/THCA primary reference standards in methanol.

4.6.3.1.1 Pipette 25 µL of the 1.0 mg/mL Δ-9-THC and 11-OH-THC reference standard solutions and 125 µL of the 1.0 mg/mL THCA reference standard solution into a 25 mL volumetric flask and QS to volume with methanol. Note: Multiply volumes pipetted by 10 if using 100 µg/ml reference standard solutions.

4.6.3.2 Lot number: Eight-digit format year/month/day + CSC

4.6.3.2.1 Example: 20101231CSC

- 4.6.3.3 Expiration: One year.
- 4.6.3.4 Storage: Freezer.
- 4.6.3.5 QC check: Successful calibration (see 5.1).

4.6.4 Cannabinoid Calibration Working Solution (CCW)

- 4.6.4.1 Prepare a working solution containing 100/500 ng/mL of Δ -9-THC, 11-OH-THC/ THCA primary reference standards in methanol.
 - 4.6.4.1.1 Pipette 1.0 mL of the 1.0/5.0 μ g/mL Stock Calibration solution into a 10 mL volumetric flask and QS to volume with methanol.
- 4.6.4.2 Expiration: Prepare Daily.
- 4.6.4.3 QC check: N/A

4.6.5 Cannabinoid Stock Verification Solution (CSV)

- 4.6.5.1 Prepare a solution containing 0.5/2.5 μ g/mL of Δ -9-THC, 11-OH-THC/ THCA primary reference standards in methanol. The Verification Stock solution shall be prepared using standards from different manufacturers or different lot numbers from the ones used to prepare the calibration solution.
 - 4.6.5.1.1 Pipette 25 μ L of the 1.0 mg/mL Δ -9-THC and 11-OH-THC reference standard solutions and 125 μ L of the 1.0 mg/mL THCA reference standard solution into a 50 mL volumetric flask and QS to volume with methanol. Note: Multiply volumes pipetted by 10 if using 100 μ g/ml reference standard solutions.
- 4.6.5.2 Lot number: Eight-digit format year/month/day + CSV
 - 4.6.5.2.1 Example: 20101231CSV
- 4.6.5.3 Expiration: One year.
- 4.6.5.4 Storage: Freezer.
- 4.6.5.5 QC check: Successful control check.

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)
	10	1.0/5.0
	25	2.5/12.5
	50	5/25
	100	10/50
25		25/125
50		50/250
100		100/500

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)
10	5/25
100	50/250

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

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- 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 water:acetonitrile 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 Examine the calibration samples for outliers. See 5.6.5.1 and 5.6.5.2.
- 5.6.3 If manual integration of a compound is needed, the chromatogram showing the integration prior to manual integration shall be printed and included with the data as well. The reason for the manual integration shall be documented on the chromatogram.
- 5.6.4 Save the data with the name of the procedure and the extraction date added to the end.
 - 5.6.4.1 Example: BCLLE20150827
- 5.6.5 **Calibration Curve Acceptance Criteria**
 - 5.6.5.1 Evaluate the curve by back-calculating the calibrator concentrations against the curve. Values of +/- 25 % from the target concentration are acceptable for the lowest calibrator. All other calibrators shall be within 20 % of the target concentration.
 - 5.6.5.2 The qualifier ion ratios for an analyte/internal standard in the calibrators shall be within +/- 20 % of the analyte ion ratios determined by the average of the calibration sample ion ratios.
 - 5.6.5.3 A maximum of two calibration points may be dropped from the curve with cause (e.g., statistical outlier, laboratory accident, fails 5.6.5.1, 5.6.5.2, etc.) and documented in the QC packet.

5.6.5.3.1 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.

5.6.5.3.2 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.

5.6.5.3.3 If the two lowest or two highest calibration points for an analyte are dropped, the run fails for that analyte and the extraction must be repeated.

5.6.5.4 The calibration curves for each analyte shall show a coefficient of determination (r^2) of 0.985 or greater.

5.6.5.5 The absolute value of the x-intercept shall be less than the lowest extracted calibrator.

5.6.5.6 If a calibration curve for an analyte fails to meet the criteria in **5.6.5.4** or **5.6.5.5**, the extraction shall be repeated.

5.6.5.7 Some assays are inherently non-linear (e.g., THCA) and the use of quadratic calibration curves may be necessary and appropriate. All quadratic curves shall be approved by the Toxicology Technical Leader or designee.

5.6.6 Quality Control Acceptance Criteria

5.6.6.1 Each analyte in a positive control shall give a quantitation within +/- 20 % of the expected concentration.

5.6.6.2 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.

5.6.6.3 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 **5.6.6.2**.

5.6.7 The failure of an analyte to meet the criteria in **5.6.5** and **5.6.6** does not invalidate the acceptability of another analyte.

5.6.8 Create a data packet for the run, including the following quality control data:

- Summary page with FA workstation reference and calibration time stamp
- Completed extraction worksheet
- LC-MS/MS sequence list
- Approved LC-MS/MS system check
- Experiment, Method and Calibration Report
- Quantitation reports of all calibrators and controls

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- 5.6.9** The Quality Control data packet will be named beginning with “BCLLE” (capitalization optional), followed by eight digit format year/month/day ending with the instrument name. A suffix may be added to differentiate multiple runs.
- 5.6.9.1** Example: BCLLE20121004-QQQ-R-X
- 5.6.10** All quality control data packets shall be administratively and technically reviewed prior to use of the associated case data for reporting.
- 5.6.11** The reviewed data packet shall be uploaded to the Managed Files Section of the associated workstation and approved. The review and approval will be indicated by signing the summary page prior to uploading to FA.

5.7 Sample Acceptance Criteria

5.7.1 Analyte and Internal Standard Identification Criteria

- 5.7.1.1** The qualifier ion ratios shall be within +/- 20 % of the target value.
- 5.7.1.2** The retention time shall not differ from the target value by more than 4.0 %.

5.7.2 Quantitative Acceptance Criteria

- 5.7.2.1** The internal standard area must be within 50 % - 200 % of the average internal standard area of the controls.
- 5.7.2.1.1** If the internal standard area is less than 50 %, the quantitative results 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.
- 5.7.2.1.2** If the internal standard area is greater than 200 %, the case shall be re-analyzed to confirm the quantitation, sample volume permitting.
- 5.7.2.1.3** If the internal standard area fails to meet acceptance criteria and there is insufficient volume remaining, the data may be evaluated qualitatively with documented approval from the Toxicology Technical Leader or designee.
- 5.7.2.2** The signal to noise (S/N) of each analyte to be quantitated shall be greater than 10:1.
- 5.7.2.3** The quantitation result shall be equal to or greater than the limit of quantitation (LOQ) of each analyte to be reported.

5.7.3 Qualitative Acceptance Criteria

- 5.7.3.1** If there is insufficient sample volume remaining, the data may be reported qualitatively only if the following acceptance criteria are met:

- 5.7.3.1.1** Calibration curve is acceptable.
- 5.7.3.1.2** Quality control acceptance criteria in **5.6.6.2** and **5.6.6.3**.
- 5.7.3.1.3** Case sample acceptance criteria in **5.7.1** and **5.7.2**.

- 5.7.4** If an analyte meets the criteria in **5.7.1** and the corresponding internal standard fails to meet the same criteria, the case sample will be re-analyzed, sample volume permitting. If there is insufficient volume remaining, the data may be evaluated qualitatively with documented approval from the Toxicology Technical Leader or designee.
- 5.7.5** If an analyte meets the criteria listed in **5.7.2**, but fails to meet the criteria in **5.7.1**, the case sample will be re-analyzed. The data may not be used qualitatively for that analyte.
- 5.7.6** If an analyte meets the criteria in **5.7.1** and **5.7.2** that failed to meet the acceptance criteria listed in **5.6.6.1**, the case shall be re-analyzed, sample volume permitting.
 - 5.7.6.1** If there is insufficient volume remaining the data may be reported qualitatively, provided all acceptance criteria in **5.7.3** are met.

5.8 Calculations

- 5.8.1** Reported Measurement uncertainty calculation: see **5.10.6**.
- 5.8.2** Percent Difference Calculation:
$$|(\text{standard retention time} - \text{analyte retention time})| / (\text{standard retention time}) * 100$$

5.9 Uncertainty of Measurement

- 5.9.1** The current process uncertainty for each analyte quantitated by this procedure is located in the [Toxicology Reporting Index](#).
- 5.9.2** The uncertainty of measurement shall be reported in the same units as the compound quantitation.
- 5.9.3** 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 Toxicology Reporting](#) procedure for reporting of THC, 11-OH-THC, or THCA.
- 5.10.2** All quantitative results will be truncated and reported to two significant figures.
- 5.10.3** All calculated measurement uncertainties will be rounded and reported to the same level of significance as the quantitative results.
- 5.10.4** If a case sample is re-extracted, the average of all acceptable quantitations will be reported.

- 5.10.5** The full analyte result is used to calculate the associated measurement uncertainty (with coverage factor $k=2$, 95.45% coverage probability).
- 5.10.6** Example Reporting Statement and Uncertainty determination.
- 5.10.6.1** THC full quantitation = 5.45 ng/mL
- 5.10.6.2** Multiply the full quantitation by the current process uncertainty (see 5.9.1).
- 5.10.6.2.1** $5.45 \text{ ng/mL} * 0.21 (21\%) = 1.1445 \text{ ng/mL}$.
- 5.10.6.3** The THC result is truncated to 5.4 ng/ml, and the associated uncertainty is rounded to 1.1 ng/mL for reporting.
- 5.10.6.4** Report: Tetrahydrocannabinol (THC) – 5.4 +/- 1.1 ng/mL at a coverage probability of 95.45%
- 5.10.7** Case Samples with THC and 11-OH-THC concentrations that exceed the upper level of the calibration curve will be reanalyzed with a lower sample volume or after dilution with the proper matrix to bring within the calibration range.
- 5.10.8** For case samples where the THCA concentration exceeds the upper LOQ, the following reporting statement may be used.
- 5.10.8.1** 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THCA) –
Concentration is greater than the upper limit of calibration of 500 ng/ml

5.11 Record the following in the case record:

- Quantitation report of the sample

6.0 Limitations- n/a

7.0 Safety

- 7.1** It should be assumed that all body fluids contain bloodborne pathogens and should therefore be handled accordingly.
- 7.2** If the examination involves a biohazard, the Forensic Scientist shall use proper PPE, such as eye protection, a lab coat, and/or gloves.

8.0 References

Virginia DFS Cannabinoids method –Revision 5

Washington State Patrol Toxicology Laboratory Division – Revision 3

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
12/01/2023	7	Updated grammar and spacing throughout Removed "()" around QS throughout 5.6.5.3 – added "and documented in the QC packet." 5.9.3 - Removed reference to Toxicology Measurement Assurance procedure