8.11 CANNABINOIDS PROCEDURE (VIA LC/MS/MS, SPE)

8.11.1 Purpose

To quantitatively and/or qualitatively identify tetrahydrocannabinol (THC), hydroxy-tetrahydrocannabinol (THC-OH), and carboxy- tetrahydrocannabinol (THC-COOH) compounds in submitted evidence using a solid phase extraction column followed by instrumental analysis with liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

8.11.2 Specimen Requirements

Samples for this analysis shall be presumptively positive or be suspected to contain a relevant drug per case circumstances. Acceptable samples for analysis include blood and/or urine. Matrix match calibrators and controls will be analyzed with similar matrix samples for quantitative purposes. Urine samples may be analyzed with blood calibrators and controls for qualitative determinations. For additional samples see Alternative Matrices (section 6.6).

8.11.3 Apparatus and Equipment

Disposable 10 mL culture tubes
UCT Styre Screen SSTHC 100mg/10mL SPE Column SSTHC11Z
Disposable transfer pipettes
Volumetric pipettes with disposable tips
Assorted volumetric glassware
Sample mixer
Centrifuge
Vacuum or Positive pressure manifold
Evaporation station
11 mm auto sampler vials, inserts, and caps
11 mm crimper/snap
LC/MS/MS Analyst compatible components

8.11.4 Reagents and Standards

Negative blood/urine or other matrix as needed
Reference standards
Deionized Water (DIH_2O)
HPLC DI Water (H_2O)
HPLC Methanol (MEOH)
Acetonitrile (ACN)
Glacial Acetic Acid (GAA)
Ammonium Hydroxide (NH_4OH)
Ethyl Acetate ($CH_3COOC_2H_5$)
Hexane (C_6H_{12})
1 M Ammonium Formate (NH_4HCO_2)
Formic Acid (CHO_2H)

Acetonitrile/Acetone 9:1
Wash solution (84% DIH_2O, 15% ACN, and 1% NH_4OH) or 84:15:1
Elution Solution 1: (98% Ethyl Acetate and 2% Glacial Acetic Acid) or 98:2
Elution Solution 2: (49% Hexane, 49% Ethyl Acetate, and 2% Glacial Acetic Acid) or 49:49:2
Mobile Phase A (99.6% HPLC suitable DI Water, 0.2% 1M Ammonium Formate, 0.2% Formic acid)
Mobile Phase B (97.6% HPLC suitable Methanol, 2% HPLC suitable DI Water, 0.2% 1M Ammonium Formate, 0.2% Formic acid)
Reconstitution Solution Mobile Phase A: Mobile Phase B 40:60

8.11.5 Standards and Reagents Preparation

The following are examples of how to prepare the standards and reagents used in this procedure.

8.11.5.1 Standards

Stock Reference Standard Solution 1:

[2500 ng/mL (Δ^9**-THC-COOH) 500 ng/mL (**Δ^9**-THC,** Δ^9**-THC-OH)]** Pipette 250 µL of Δ^9-THC-COOH [1 mg/mL] certified reference and 50 µL of each Δ^9-THC and delta Δ^9-THC-OH [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Stock Reference Standard Solution 2:

[250 ng/mL (Δ^9**-THC-COOH) 50 ng/mL (**Δ^9**-THC,** Δ^9**-THC-OH)]** Pipette 10 mL of Stock Reference Standard Solution 1 and dilute to 100 mL with acetonitrile.

Stock Reference Standard Control Solution:

[2500 ng/mL (Δ^9**-THC-COOH) 500 ng/mL (**Δ^9**-THC,** Δ^9**-THC-OH)]** Pipette 250 µL of Δ^9-THC-COOH [1 mg/mL] certified reference and 50 µL of each Δ^9-THC and Δ^9-THC-OH [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Internal Standard Reference Solution:

[200 ng/mL (Δ9-THC-D_3**, Δ9-THC-OH-**D_3**, and 1,000 ng/mL Δ9-THC-COOH-**D_3**)]** Pipette 20 µL of each Δ^9-THC-D_3 and Δ^9-THC-OH-D_3 [1 mg/mL] certified reference, and 100 µL of Δ^9-THC-COOH-D_3 [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Δ^8 Stock Reference Isomer Standard Solution:

[2500 ng/mL (Δ^8**-THC-COOH) 500 ng/mL (**Δ^8**-THC,** Δ^8**-THC-OH)]** Pipette 250 µL of Δ^8-THC-COOH [1 mg/mL] certified reference and 50 µL of each Δ^8-THC

and Δ^8-THC-OH [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Note: Other isomers/analogs of Tetrahydrocannabinol (THC), Hydroxy-tetrahydrocannabinol (THC-OH), and Carboxy-tetrahydrocannabinol (THC-COOH) may be used for additional isomer standard solutions using the above Stock Reference Isomer Standard Solution make up.

Working Reference Calibrator/ Control Standards Solution (for example)
To make the working reference calibrator/control standard solutions, add the following amounts to a final volume of 1 mL with blood or urine.

CONCENTRATIONS	AMOUNT USED	STANDARD SOLUTION
1&5 ng/mL	20 µL	Stock Reference Solution 2
2 &10 ng/mL	40 µL	Stock Reference Solution 2
4 &20 ng/mL	80 µL	Stock Reference Solution 2
10 &50 ng/mL	20 µL	Stock Reference Solution 1 **OR** Stock Reference Control Solution
20 &100 ng/mL	40 µL	Stock Reference Solution 1
50 & 250 ng/mL	100 µL	Stock Reference Solution 1
80 & 400 ng/mL	160 µL	Stock Reference Solution 1

Working Reference Calibrator/ Control Standards Solution (for example)

CONCENTRATION	AMOUNT USED	STANDARD SOLUTION
10 & 50 ng/mL	20 uL	Δ8 Stock Reference Control Solution

8.11.5.2 Expiration Date

All stock reference standard solutions expire 6 months from the preparation date.

Note: All stock reference standard solutions shall be stored in the freezer when not in use.

8.11.5.3 Prepared Chemicals

Methanol/Water 1:1 (needle rinse)
Add 500mL of methanol to a volumetric flask and dilute to 1000 mL with H_2O.

1M Ammonium Formate
Dissolve 63g of ammonium formate and dilute to 1000 mL with H_2O.

Mobile Phase A
Add 2 mL 1 M ammonium formate and 2 mL formic acid to H_2O and dilute to 1000mL with H_2O.

Mobile Phase B
Add 2 mL 1 M ammonium formate and 2 mL formic acid to 20 mL H_2O and dilute to 1000mL with Methanol.

Acetonitrile/Acetone 9:1 (protein crash solution)
Combine 900 mL of Acetonitrile, and 100 mL of Acetone.

Wash solution
Combine 84 mL of DI H_2O, 15 mL of Acetonitrile, and 1 mL Ammonium Hydroxide. Prepare daily. (84:15:1)

Elution Solution 1
Combine 98 mL of Ethyl Acetate and 2 mL Glacial Acetic Acid. (98:2)

Elution Solution 2
Combine 49 mL of Hexane, 49 mL Ethyl Acetate, and 2 mL Glacial Acetic Acid. (49:49:2)

Reconstitution Solution Mobile Phase A: Mobile Phase B 40:60
Mix 40 mL Mobile Phase A and 60 mL Mobile Phase B.

8.11.6 Procedure

1. Allow all stock reference standards and case samples to equilibrate to room temperature before beginning procedure.

2. Label, check, and load/unload all samples in accordance with the “Sample Pipetting Check List” (see Appendix section).

3. Prepare working reference calibrator and/or control standards from reference standard solutions. See the example above.

4. Pipette 1 mL of corresponding case sample, calibrator, positive control, or negative control into the appropriately labeled 10 mL culture tubes. Note: Smaller sample volumes may be analyzed on a case-by-case basis.

5. Pipette 100 µL of internal standard reference solution into each sample to make a final concentration of 20 ng/mL (THC-D_3 and THC-OH-D_3) and 100 ng/mL (THC-COOH-D_3).

6. Add 2 mL of acetonitrile/acetone 9:1 to each sample, slowly while mixing.

7. After completion of step 6, again mix each sample for approximately 30 seconds and let stand 5 to 10 minutes.

8. Briefly vortex and centrifuge the samples at the maximum RPM level possible not to exceed 4100 RPM for 10 minutes or more.

9. Decant the supernatant into a clean 10 mL test tube and add 5 mL of DIH_2O.

10. Decant the supernatant/DIH_2O into labeled UCT Styre Screen SSTHC SPE Columns.

11. Using the positive pressure manifold, pull the samples through the sorbent bed of the columns at a rate of 1-2 mL per minute (2-4 psi).

12. Wash the SPE column with 1 mL of DIH_2O:ACN:NH_4OH (84:15:1) Wash Solution. The wash solution needs to be made fresh daily and mixed well. Set the flow rate between 1 and 15 mL per minute, but not full positive pressure.

13. Dry the SPE column thoroughly under full positive pressure for 15 minutes or longer until the column is dry.

14. Switch the solid phase extraction apparatus to clean labeled 10 mL culture tubes.

15. Add 2 mL of the Elution solution (1) Ethyl Acetate:Glacial Acetic Acid (98:2) to each SPE column and allow the samples to elute slowly with a flow rate set on the manifold to approximately 1-2 mL/minute rate (2-4psi) pressure.

16. Add 3 mL of the Elution solution (2) Hexane:Ethyl Acetate:Glacial Acetic Acid (49:49:2) to each SPE column and allow the samples to elute slowly with a flow rate set on the manifold to approximately 1-2 mL/minute rate (2-4psi) pressure.

17. Gently evaporate the samples to dryness with heat approximately 40^{o}C or less and a dry gas (e.g. nitrogen) in an evaporation station (approximately 20 minutes).

18. Reconstitute the residue with 100 µL of 40:60 Mobile Phase A to Mobile Phase B, mix/vortex approximately 30 seconds, and centrifuge the samples at the maximum RPM level possible not to exceed 4100 RPM for 5 minutes or more.

19. Transfer to an 11 mm autosampler vial with insert, attempting to avoid transfer any of the particulate matter in the bottom of the tube, and seal with cap.

20. Analyze the samples by LC/MS/MS.

Note: Samples are not always analyzed immediately after extraction due to large batches or unforeseen delays. For example, the instrument may lose communication with its controller, inadvertently shutting down a batch run. The targeted drugs will be considered stable for up to 48 hours for testing purposes; after that time the samples need to be re-extracted. If the samples cannot be tested that day, refrigerate the samples until the next day. Prior to analyzing

on the LC/MS/MS, vortex the samples in the 11 mm autosampler vials for approximately 20 seconds before continuing with analysis.

8.11.7 Reporting

8.11.7.1 Qualitative

8.11.7.1.1 Retention times of drugs identified, and internal standards must fall within ± 2% of calibrator or control standards.
Note: Some drug retention times are concentration dependent and a comparison of ± 2% to a calibrator used in the calibration curve or control standard of similar concentration shall be acceptable.

8.11.7.1.2 Multiple reactions monitoring (MRM) ion ratios must fall within ± 20% of the calibrators or control standard. If a calibration point is removed, then the ion ratio range shall be recalculated from the calibrators used to establish the curve.
Note: Some drug MRM ion ratios are concentration dependent and a comparison of ± 20% to a calibrator used in the curve or control standard of similar concentration shall be acceptable.

8.11.7.1.3 If the control standard concentration is outside the expected value, the drug may be reported as "positive" if the retention time criteria and ion ratio criteria are met.
If the control standard concentration is within the expected value range, the drug shall not be reported if the retention time criteria and/or ion ratio criteria are not met.

8.11.7.1.4 Drug concentrations in casework may be reported as "positive" if the drug response ratio (i.e., area of drug/area of internal standard) is equal to or greater than the drug response ratio of the lowest calibrator used to establish the calibration curve.

8.11.7.1.5 The Stock Reference Isomer Standard Solution chromatography will be evaluated and reviewed for peak shape, peak shoulders and/or double peaks. An additional evaluation and review of the stock reference isomer standard solutions will be performed documenting ion ratio(s) and retention time(s).

8.11.7.2 Quantitative

8.11.7.2.1 All of the qualitative result criteria above must be met.

8.11.7.2.2 Sample drug concentrations greater than the highest calibrator level where results are not necessary for interpretation in the case may be reported as "greater than …." the highest calibrator level.
Note: Drug concentrations of 400 ng/mL for Δ^9-THC/ Δ^9-THC-OH or 2000 ng/mL for Δ^9-THC-COOH produced no carryover using this procedure.

8.11.7.2.3 Sample drug concentrations greater than the highest calibration level where the results are necessary for interpretation in the case shall be reanalyzed with a smaller sample amount (including dilution factor). When diluting a sample for reanalysis, the same matrix shall be used as the sample matrix for the diluent. If the lowest calibrator levels are removed from the calibration curve, this change in calibration levels may require a repeat analysis of case specimens with calibration levels at the method LOQ when necessary for interpretation in a case.

8.11.7.3 Results

8.11.7.3.1 Any qualitative or quantitative report data not used in a case shall either be lined through and initialed or all the data used shall be highlighted.

8.11.7.3.2 Sample drug concentrations below:

- 1 ng/mL for Δ^9-THC (the lowest calibration point) shall be reported as "No - tetrahydrocannabinol detected".
- 1 ng/mL Δ^9-THC-OH (the lowest calibration point) shall be reported as "No hydroxy- tetrahydrocannabinol detected".
- 5 ng/mL for Δ^9-THC-COOH (the lowest calibration point) shall be reported as "No carboxy- tetrahydrocannabinol detected".
- In cases where sample drug concentrations for Δ^9-THC, Δ^9THC-OH, Δ^9-THC-COOH are all below the lowest calibration point, results shall be reported as "No cannabinoids detected".

8.11.7.3.3 Qualitative results shall be expressed as "positive" and include any clarifying remarks, if applicable.

8.11.7.3.4 Quantitative results shall be reported in ng/mL and truncated to the whole number.

8.11.7.3.5 When a definitive conclusion cannot be made, the reason shall be documented on the report (e.g., insufficient sample for analysis"," sample unsuitable for analysis"," results are inconclusive due to sample condition", etc.).

8.11.7.3.6 If Δ^9-tetrahydrocannabinol (Δ^9-THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-OH), or 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH) are unable to be reported after the first analysis due to acceptance criteria being out of range, the sample does not need to be re-analyzed if it is not necessary for interpretation of the case.

8.11.7.3.7 This method was validated using the delta-9-tetrahydrocannabinol (Δ^9-THC), 11-hydroxy-delta-9-tetrahydrocannabinol (Δ^9-THC-OH), and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (Δ^9-THC-COOH) for reporting tetrahydrocannabinol (THC). However, known isomers of tetrahydrocannabinol have been indicated to co-elute with Δ^9-THC and its metabolites and may cause interference with the identification of THC if present in mixtures with Δ^9-THC and its metabolites. The potential impact of these isomers has been evaluated and addressed in the approach to cannabinoid data evaluation to avoid potential

misidentification. Due to the inability, at this time, to distinguish between Δ^9 and Δ^8 parent compounds and their metabolites, all usable data shall be reported as:

- tetrahydrocannabinol (THC) for Δ^9-tetrahydrocannabinol (Δ^9-THC)
- hydroxy-tetrahydrocannabinol (THC-OH) for 11-hydroxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-OH)
- carboxy-tetrahydrocannabinol (THC-COOH) for 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH).

8.11.7.3.8 Data review and reporting of cannabinoids should follow the below guidelines and reporting criteria. See appendix for cannabinoid reporting flow chart.

8.11.7.3.8.1 Perform a visual analysis of the chromatography noticing if there are peak shoulders and/or double peaks. Perform a check of acceptance criteria such as ion ratios out of range and/or retention time shifts. If the evaluated data does not meet chromatographic and qualitative acceptance criteria, then report as “inconclusive.”

8.11.7.3.8.2 Any analyte tetrahydrocannabinol (THC), hydroxy-tetrahydrocannabinol (THC-OH), or carboxy-tetrahydrocannabinol (THC-COOH) that does not meet acceptance criteria such as ion ratios and retention times shall be reported as inconclusive.

8.11.7.3.8.3 If the only reportable analyte is tetrahydrocannabinol (THC) and it meets all acceptance criteria, then tetrahydrocannabinol (THC) will be reported qualitatively.

8.11.7.3.8.4 If any of the analytes tetrahydrocannabinol (THC), hydroxy-tetrahydrocannabinol (THC-OH), and carboxy-tetrahydrocannabinol (THC-COOH) meet all acceptance criteria such as ion ratios and retention times, then report quantitative results, unless the circumstances outlined in 8.11.7.3.8.3 apply.

8.11.7.3.9 All cases where cannabinoids are reported as either qualitative, inconclusive, and/or quantitatively identified shall add the following statement to all reports “*The TBI Crime Laboratory does not currently have methodology to differentiate between isomers of THC and metabolites (for example, delta-8 and delta-9-THC) in biological samples. These variants of THC have similar psychoactive effects. This sample may contain a mixture of THC variants. Reported qualitative and quantitative results are based upon comparison to delta-9 reference materials. “*
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8.11.8 References:

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