Title: Analysis of Biological Specimens for Cannabinoids by Liquid Chromatography/Mass Spectrometry (LC/MS)

1.0 Scope

Marijuana, obtained from the Cannabis sativa plant, is a commonly abused illicit drug. It is typically dried and smoked. The chemical Δ^9-tetrahydrocannabinol (THC) primary psychoactive component of marijuana. Another chemical, 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) is a major active metabolite of THC and 11-nor-9-carboxy- Δ^9-tetrahydrocannabinol (THC-COOH) is a major inactive metabolite of THC.

This procedure is used to confirm and quantitate THC and it's primary metabolites in blood specimens. When appropriately validated, it can also be used to qualitatively identify these compounds within other matrices (e.g., vitreous humor, plasma, serum).

2.0 Principle

Biological specimens are assayed for the presence of THC, THC-OH, and THC-COOH. A deuterated internal standard solution is added to specimens which contain deuterated analogs to the analytes of interest. Extraction of analytes occurs through the use of polymeric solid phase extraction (SPE) technology. Eluted solutions are analyzed using high performance liquid chromatography/mass spectrometry (HPLC/MS or LC/MS) including multiple reaction mode (MRM) detection.

3.0 Specimens

This procedure uses a biological fluid (e.g., blood, serum, plasma). Typically, 0.5mL of sample is used during the analysis, but differing volumes may be necessary. Dilution of samples due to limited specimen or due to suspicion of high drug or metabolite concentrations (as possibly indicated by immunoassay results) is acceptable.

4.0 Equipment/Materials/Reagents

a. General laboratory glassware

b. Disposable borosilicate test tubes (e.g., 16 x 100 mm, round bottom, borosilicate glass with Teflon caps)

c. Vortex mixer

d. Automatic pipettes (with disposable tips)

e. Positive pressure solid phase extraction device (e.g., Cerex, or equivalent)

f. Sample concentrator with nitrogen (e.g., Cerex 48 heated, or equivalent)

g. Centrifuge

h. Liquid Chromatograph/Mass Spectrometer (Shimadzu LCMS-8030, or equivalent)

i. HPLC column (Kinetex 2.6µ, C18, 100Å, 50 mm x 2.1 mm (Phenomenex), or equivalent)

j. Pre-Column – SecurityGuard ULTRA Cartridge UHPLC Kinetex for 4.6mm ID Columns (Phenomenex or equivalent)

k. Autosample vials with inserts (LC/MS grade 1.8mL or equivalent)

l. Acetic acid, glacial (Reagent grade or equivalent)

m. Acetonitrile (CH_3CN or ACN, Optima grade or equivalent)

n. Ammonium acetate (NH_4OAc ; Reagent Grade or equivalent)

o. Ammonium hydroxide (NH_4OH, Reagent grade or equivalent)

p. Ethyl acetate (HPLC grade or equivalent)

q. Hexane (HPLC grade or equivalent)

r. Hydrochloric acid – concentrated (HCl ; Reagent grade or equivalent)

s. Methanol (MeOH, Reagent grade or equivalent)

t. Water (H_2O, Millipore, Deionized (DIW) or equivalent)

u. $NH_4OAc_{(aq)}$ (5M or 38.5% (w/v): Can be prepared by dissolving 3.85 g of NH_4OAc in 10mL of water. Stable for at least two (2) months in refrigerator.

v. SPE Conditioning-Solution – $HCl_{(aq)}$ (0.1M or 0.83% (v/v)) – Can be prepared by slowly adding 8.3 mL of $HCl_{(conc)}$ to water within a 1 L volumetric flask. Dilute to volume with water. Stable for one (1) year at room temperature. This solution can be stored in a re-pipetter bottle.

w. SPE Sample Wash Mixture – H_2O:CH_3CN:NH_4OH (85:15:1): Can be prepared by combining 85mL of deionized H_2O, 15 mL of CH_3CN, 1 mL NH_4OH and mix well. Store in glass at room temperature. Should be prepared fresh.

x. Elution Solvent (THC, THC-OH) – Ethyl acetate – stable indefinitely.

y. Elution Mixture (THC-COOH) – Hexane:Ethyl Acetate:Acetic Acid (80:18:2): Can be prepared by combining 80 mL of hexane, 18 mL of ethyl actetate and 2 mL of acetic acid and mixing well. Store in glass at room temperature. Stable for at least one (1) month.

z. Mobile Phase A – $NH_4OAc_{(aq)}$ (5mM or 0.04% (w/v)): Can be prepared by diluting 1mL of the 5 mM (38.5% (w/v)) $NH_4OAc_{(aq)}$ in 1 L of water (1:1000 dilution) and mixing well. Store in glass at room temperature. Stable for one (1) week.

aa. Mobile Phase B – (ACN or CH_3CN). Store in glass at room temperature – stable indefinitely.

bb. Δ^9-tetrahydrocannabinol (THC) Reference Standard (1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

cc. Δ^9-tetrahydrocannabinol-D_3 (THC-D_3) Reference Standard (0.1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

dd. 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) Reference Standard (1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

ee. 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol-D_3 (THC-COOH-D_3) Reference Standard (0.1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

ff. 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) Reference Standard (1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

gg. 11-hydroxy-Δ^9-tetrahydrocannabinol-D_3 (THC-OH-D_3) Reference Standard (0.1 mg/mL or equivalent). Storage conditions per manufacturer's instructions.

5.0 Standards and Controls

Negative Control Solution: See below.

Positive Control Solution: Can be obtained from an outside source or prepared in-house. If prepared in-house, reference standards from different manufacturers or from different lot numbers should be used when making controls and calibrator solutions.

a. Stock Deuterated Internal Standard (IS) Mix Solution –
((THC-D_3 + THC-OH-D_3 + THC-COOH-D_3 (1 µg/mL each))):
Can be prepared by individually combining 100 µL each of 0.1 mg/mL solutions of THC-D_3, THC-OH-D_3, and THC-COOH-D_3 into a 10 mL volumetric flask and diluting to volume (10 mL) with MeOH.
Alternatively can be prepared, if using 1 mg/mL solutions of the reference standards, only add 10 µL each of the reference standards to a 10 mL volumetric flask and diluting to volume with MeOH.

b. Working Deuterated Internal Standard (IS) Mix Solution –
((THC-D_3 + THC-OH-D_3 + THC-COOH-D_3 (0.1 µg/mL each))):
Can be prepared by diluting the Stock Deuterated Internal Standard (IS) Mix Solution 1:10 with MeOH (e.g., 1 mL of Stock IS Mix Solution in a total volume of 10 mL of MeOH).

c. Working Standard Solution for Controls (High) –
((THC + THC-OH + THC-COOH (1 µg/mL each))):
Can be prepared by individually combining 25 µL each of the 1 mg/mL purchased referernce standard solutions of THC, THC-OH, and THC-COOH into a 25 mL volumetric flask and diluting to volume (25 mL) with MeOH.

d. Working Standard Solution for Controls (Low) –
((THC + THC-OH + THC-COOH (0.1 µg/mL each))):
Can be prepared by diluting the Working Standard Solution (High) 1:10 with MeOH.
This can be done by transferring 25 µL of the Working Standard Solution (High) into 25 mL volumetric flask and diluting to volume (25 mL) with MeOH.

e. Working Standard Solution for Calibrators (High) –
((THC + THC-OH + THC-COOH (1 µg/mL each))):
Can be prepared by individually combining 25 µL each of the 1 mg/mL purchased referernce standard solutions of THC, THC-OH, and THC-COOH into a 25 mL volumetric flask and diluting to volume (25 mL) with MeOH. Should be from different lot number, manufacturer, or prepared by a different analyst compared to the Working Standard Solution for Controls (High).

f. Working Standard Solution for Calibrators (Low) –
((THC + THC-OH + THC-COOH (0.1 µg/mL each))):
Can be prepared by diluting the Working Standard Solution (High) 1:10 with MeOH.
This can be done by transferring 25 µL of the Working Standard Solution (High) into 25 mL volumetric flask and diluting to volume (25 mL) with MeOH. Should be from different lot number, manufacturer, or prepared by a different analyst compared to the Working Standard Solution for Controls (Low).

g. High Positive Control (Hi-Ctrl) Blood
(THC + THC-OH + THC-COOH (50 ng/mL)):
Can be prepared by adding 25 µL of the Working Standard Solution (High ; 1 µg/mL)) to a screw-capped test tube containing 0.5 mL of blank blood. Store refrigerated in glass. Stable for at least one (1) month. [See table 1]

h. Low Positive Control (Lo-Ctrl) Blood
(THC + THC-OH + THC-COOH (5 ng/mL)):
Can be prepared by adding 25 µL of the Working Standard Solution (Low ; 0.1 µg/mL)) to a screw-capped test tube containing 0.5 mL of blank blood. Store refrigerated in glass. Stable for at least one (1) month. [See table 1]

i. Positive Control [Other Matrix]
(THC + THC-OH + THC-COOH (5 ng/mL)):
Can be prepared by adding 25 µL of the Working Standard Solution (Low ; 0.1 µg/mL)) to a

screw-capped test tube containing 0.5 mL of blank [other matrix (e.g., serum, plasma, vitreous fluid)]. Store refrigerated in glass. Stable for at least one (1) month.

j. Calibrator Solutions (THC + THC-OH + THC-COOH (1, 5, 50, 200 ng/mL))
Can be prepared by using either the Working Standard Solution (Low) or Working Standard Solution (High) according to Table 2.

k. Negative Control [Othr Matrix]:
Synthetic or in-house negative control [other matrix]. Store refrigerated or obtain fresh. If purchased, stability should be determined by manufacturer. A negative control [other matrix] should be extracted and analyzed with each appropriate assay or batch.

l. Negative Control Blood:
Purchased (e.g., Diagnostics Products Corporation, UTAK Labs), donated (e.g., American Red Cross), or in-house negative control blood (e.g., from a proven blank specimen). Store frozen, refrigerated, or obtain fresh. If purchased or donated, stability should be determined by manufacturer. A negative control blood should be extracted and analyzed with each blood assay or batch.

Table 1: Control (In-House) Solutions

Pipette into 0.5 mL Neg. Ctrl. Blood or [Other Matrix] [drug-free]				
Level	**THC THC-OH THC-COOH (ng/mL)**	**Volume of Working Std. Solution for Control (Low) (0.1 µg/mL) (µL)**	**Volume of Working Std. Solution for Control (High) (1 µg/mL) (µL)**	**Volume of Neg. Ctrl. Blood or [Other Matrix] [drug-free] (mL)**
Negative Ctrl.*	–	–	–	0.5 (Blood or [Other Matrix])
Positive Ctrl.*	5	25	–	0.5 [Other Matrix]
Low Blood	5	25	–	0.5 Blood
High Blood	50	–	25	0.5 Blood

*If [Other Matrix] samples are not being analyzed, controls will not be necessary.

6.0 Calibration

This procedure may be used quantitatively through the construction of a multipoint calibration graph for the analyte(s) of interest.

Table 2: Calibrator Solutions

Pipette into 0.5 mL Neg. Ctrl. Blood [drug-free]				
Level	**THC THC-OH THC-COOH (ng/mL)**	**Volume of Working Std. Solution for Calibrator (Low) (0.1 µg/mL) (µL)**	**Volume of Working Std. Solution for Calibrator (High) (1 µg/mL) (µL)**	**Volume of Neg. Ctrl. Blood [drug-free] (mL)**
1	1	5	–	0.5
2	2	10	–	0.5
3	5	25	–	0.5
4	20	100	–	0.5
5	50	–	25	0.5
6	100	–	50	0.5

7.0 Sampling

Not applicable.

8.0 Procedure

The Appendix contains an abbreviated version of this procedure. This form may be used at the bench by analysts who perform the procedure.

8.1. Prepare calibrator and control solutions according to tables 1 and 2.

8.2. Add 0.5mL of unknown specimens to properly labeled test tubes.

8.3. Add 100 µL of the Working Deuterated Internal Standard (IS) Mix Solution [10 ng of each component] to each test tube and mix.

8.4. Slowly add cold (e.g., -20 °C) ACN to each blood sample while vortex-mixing for at least 30 seconds. This should cause a protein precipitation to occur. This step can be skipped for [Other Matrix] specimens.

8.5. Cap and centrifuge each tube (e.g., ~6 minutes at ~5200 RPM).

8.6. Add 2 mL of 0.1M $HCl_{(aq)}$ into each SPE column.

8.7. Individually add supernatant from each sample into SPE columns that already contain the 0.1M $HCl_{(aq)}$ and mix with pipette. Be careful not to transfer protein sediments.

8.8. Allow solutions to migrate through SPE columns via gravity. If needed, apply a small amount of pressure to push solutions through SPE columns.

8.9. Wash SPE columns with 1 mL of the SPE Sample Wash Mixture (H_2O:ACN:NH_4OH (85:15:1)) via gravity. If needed, apply a small amount of pressure to push solutions through SPE columns.

8.10. Dry the SPE columns for ~5-8 minutes using high pressure (60-80 psi). Do not over-dry (not longer than 8 minutes).

8.11. Elute SPE columns with 2 mL of ethyl acetate (Elution Solvent for THC and THC-OH) at the rate of ~0.5 mL/min. and into properly labeled test tubes. Increase the pressure briefly and expel remaining elution solvent from the SPE sorbent beds and from the Luer tips.

8.12. Dry the SPE columns for 2 minutes using high pressure (60-80 psi).

8.13. Elute SPE columns with 2 mL of Elution Mixture for THC-COOH (Hexane:Ethyl Acetate:Acetic Acid (80:18:2)) at the rate of ~0.5 mL/min. into the same test tubes as were used for the THC and THC-OH extracts. Increase the pressure briefly and expel remaining elution solvent from the SPE sorbent beds and from the Luer tips.

8.14. Evaporate the combined sample extracts to dryness (e.g., $N_{2(g)}$ at <40°C).

8.15. Dissolve the dried residue in 50 µL ACN and vortex-mix the solutions to solubilize the residues from the surfaces of the collection tubes.

8.16. Add 50 µL of water to each test tube and vortex-mix. Total volume of each solution should be ~100 µL.

8.17. Individually transfer solutions to properly labeled autosample vials containing volume inserts.

8.18. Analyze solutions by LC/MS instrumentation.

9.0 Instrumental Conditions

The following are the typical operating parameters for the instrument used in this procedure. The instrument conditions may be modified to adjust or improve the procedure. The appendix contains an abbreviated version of the procedure and this checklist can be used by analysts.

HPLC Parameters:

HPLC Column: Phenomenex, Kinetex, 2.6µ, C18, 100 Å, 50 mm x 2.1 mm

Mobile Phase A: $NH_4OAc_{(aq)}$ (5mM or 0.04% (w/v))
Mobile Phase B: Acetonitrile (neat)
Column Temperature: 40 °C
Constant Flow: 0.5 mL/min.
Autosampler Temperature: 15 °C

Gradient Program: Initial 20% B Flow 0.5 mL/min

Time (min.)	**Mobile Phase A $NH_4OAc_{(aq)}$ – (5mM ; 0.04% (w/v)) (%)**	**Mobile Phase B Acetonitrile (100%) (%)**
Initial	80	20
0.25	80	20
1.75	10	90
2.40	10	90
2.41	80	20
4.00	80	20

Mass Spectrometer Parameters:

Ionization Source	Electrospray	Polarity	Negative Ion
Ion Spray Potential	-3.5 kV	Heat Block	500 °C
Nebulizer Gas	Nitrogen	Nebulizer	2.5 L/min
Drying Gas	Nitrogen	Drying Gas	10 L/min.
Scan Type	MRM (&/or Full Scan)	Resolution	Unit
Desolvation Line	300 °C		

Multiple Reaction Montioring (MRM) Transition Ions:

Compound	Presursor (m/z)	Product (m/z)	Pause Time (msec)	Dwell Time (msec)	Q1 Pre-Bias (V)	Coll. Energy (V)	Q3 Pre-Bias (V)
THC	313.20	245.05	3	10	10	28	27
THC	313.20	191.20	3	10	10	30	21
THC	313.20	203.10	3	10	14	31	13
THC-OH	329.20	268.15	3	15	13	28	19
THC-OH	329.20	172.90	3	15	16	31	18
THC-COOH	342.20	325.25	3	15	11	20	23
THC-COOH	342.20	191.15	3	15	15	33	22
THC-COOH	342.20	299.20	3	15	19	23	20
THC-D_3	316.10	194.20	3	15	15	35	15
THC-OH-D_3	332.30	271.20	3	15	15	35	15
THC-COOH-D_3	346.20	248.30	3	15	15	35	15

10.0 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. Retention time (chromatrographic characteristic), fragmentation pattern and qualified ion ratios (mass spectrometric characteristics), and other characteristics are used as the basis for detection, identification, and quantitiation. In most cases, all of the criteria below should be met in order to identify the appropriate cannabinoids within biological specimens.

10.1. Chromatography

Analyze solutions by LC/MS instrumentation.

All chromatographic peaks for the analytes of interest should show good chromatographic characteristics, with reasonable peak shape, width, and resolution. For low concentrations of an analyte (<5 ng/mL), there may be transitions that are not optimal. In order to be determined as acceptable, a chromatographic peak in an unknown sample should compare

favorably to the same analyte's chromatographic peak in a known sample which has been analyzed on the same system and in the same, or subsequent, analytical timeframe. Additionally, the following two criteria should be met:

10.1.1. Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a reference standard or positive control.

10.1.2. Signal-to-Noise

To justify the existence of a peak, it's baseline signal to noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a negative control or blank sample injected prior to that sample.

10.2. Mass Spectrometry

Independent MS/MS experiments are conducted for each analyte. Ion ratios should compare favorably to ion ratios from reference standards or an extracted positive control at a comparable concentration. Generally ion ratios are within the following limits:

Expected [Set] Ion Ratio	Allowance
>50 %	20%
20% - 50%	25%
<20%	30%

11.0 Calibration

Calibration is accomplished by the addition of known amounts of analytes (in addition to the internal standard) into matrix-matched blank samples. A run-specific calibration graph is generated from the resulting data and used as the basis for the quantitation of unknown samples. A calibration graph for each drug and metabolite is analyzed with each batch and a correlation coefficient should be ≥ 0.990 when using deuterated internal standards. If the correlation coefficient is lower than 0.990 then approval from the appropriate lead analyst or Deputy Director must occur and, if deemed acceptable, justification must accompany the applicable data. Following linear regression, reprocessed calibrators generally should be within 20% of their target value in order to be included in the calibration graph used to quantitate case samples. Calibration for each analysis batch is done independently.

12.0 Uncertainty of Measurment

The critical sources of measurement uncertainty in this procedure include:

- Historical random uncertainty of repeated measurements
- Accuracy of the pipette used to deliver the sample
- Accuracy of the pipette used to deliver the calibrators
- Uncertainty in the concentration of the calibration standards
- Precision of the delivery of the internal standard

When quantitative results that are determined from this procedure are included in a report, applicable measurment uncertainties will be estimated. Information used to derive uncertainty measurements should be tracked electronically.

13.0 Limitations

Limit of Detection (LOD), Limit of Quantitation (LOQ), Upper Limit of Quantitiation (ULOQ)

Cannabinoids	LOD – **Urine (ng/mL)	LOD – Blood (ng/mL)	LOQ – Blood (ng/mL)	ULOQ – Blood (ng/mL)
THC	**5***	**1**	**2**	**100**
THC-COOH	**5***	**1**	**1**	**100**
THC-OH	**5***	**1**	**2**	**100**

*Administratively set – values may actually be lower
**Unconjugated – analytes simply spiked in urine

	THC (Blood)	THC-COOH (Blood)	THC-OH (Blood)
Linear Range (ng/mL)	2-100**	2-100**	2-100**
Bias(Low Conc. – 5 ng/mL)	Average Range: -4.7% to 12.4%		
Bias(Medium Conc. – 50 ng/mL))	Average Range: -10% to 0.6%		
Bias(High Conc. – 100 ng/mL)	Average Range: 4.7% to 11.8%		
Precision(Low Conc. – 5 ng/mL)	Average Range: 8.4% to 19.3%		
Precision(Medium Conc. – 50 ng/mL))	Average Range: 7.6% to 18.6%		
Precision(High Conc. – 100 ng/mL)	Average Range: 9.5% to 11.8%		

**Administratively set – values may actually be higher

Bias was evaluated by finding the average quantitative values from three (3) batches of data. Bias was expressed as % difference.

Precision was evaluated by finding the mean and standard deviation of the quantitative values from three (3) batches of data and dividing the standard deviation by the mean. Precision was expressed as the coefficient of variation (%CV) for the three (3) batches.

14.0 Safety

This procedure is carried out in a laboratory environment and standard safety procedures appropriate for such an environment should be utilized, including gloves, safety glasses, and protective clothing (e.g., lab coat). Biological specimens will be handled using universal precautions and will be treated as biohazardous. Potentially contaminated items and surfaces will be cleaned prior to use, especially. When casework samples are being processed/analyzed brown paper (or other similar barrior) should be placed in between the surface and the specimens.

15.0 References

FBI Laboratory, Analysis of Cannabinoids from Biological Specimens by LC/MS/MS

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).

Monroe County MEO, 11-Nor-9-Carboxy-Δ^9-Tetrahydrocannabinol,11-Hydroxy Tetrahydocannabinol and Δ^9-Tetrahydrocannabinol Quantitation In Blood.

SPEware Corporation, Extraction of Δ^9-Tetrahydrocannabinol (THC) and its Metabolties from Whole Blood for LC-MS Confirmations using Cerex® THC Extraction Columns, 3mL, 35mg

Appendix:
Bench Method: Analysis of Cannabinoids from Biological Specimens by LC/MS/MS

The following is an abbreviated version of this procedure.
This form may be used at the bench level by the analyst performing the procedure.

Pipette into 0.5 mL drug-free blood/[other matrix]		**Lot:**	
Standard Lot:	**Standards**		
Level	**THC THC-OH THC-COOH (ng/mL)**	**Volume of Working Std. Solution for Calibratos (Low) (0.1 µg/mL) (µL)**	**Volume of Working Std. Solution for Calibrators (High) (1 µg/mL) (µL)**
1	1	5	–
2	2	10	–
3	5	25	–
4	20	100	–
5	50	–	25
6	100	–	50

Control Lot:		**In House Controls**		
Level	**THC THC-OH THC-COOH (ng/mL)**	**Volume of Working Std. Solution for Controls (Low) (0.1 µg/mL) (µL)**	**Volume of Working Std. Solution for Controls (High) (1 µg/mL) (µL)**	**Volume of Neg. Ctrl. Blood or [Other Matrix] [drug-free] (mL)**
Negative Ctrl.	–	–	–	0.5 (Blood or [Other Matrix])
Positive Ctrl.	5	25	–	0.5 [Other Matrix]
Low Blood	5	25	–	0.5 Blood
High Blood	50	–	25	0.5 Blood

*If [Other Matrix] samples are not being analyzed, certain controls will not be necessary.

Appendix (Cont'd):

Specimen Procedure	
Add 0.5 mL standard/control/sample into properly labeled 16 x 100 mm test tubes	
Add 100 µL of working internal standard solution (1ug/mL)	**Lot:**
Slowly add 1 mL cold acetonitrile (ACN) while vortexing. Mix/vortex ~1 min	
Cap, centrifuge ~6min at ~5200 rpm	
Add 2 mL of 0.1 M HCl to the SPE column.	**Lot:**
Load sample onto SPE column, mixing into 0.1 M HCl (e.g., Polycrom THC 682-0353). Apply pressure as needed.	**Lot:**
Wash with 1.0 mL (85/15/1) DIW, ACN, NH_4OH Prepare fresh	
NH_4OH	**Lot:**
Acetonitrile	**Lot:**
Dry column~ 5 to 8 min. at full pressure. Do not over dry	
Elute with 2.0 mL Ethyl Acetate (THC, THC-OH fraction)	
Ethyl Acetate	**Lot:**
Dry column for 2 min at full pressure (Do not over dry)	
Elute with 2 mL (80:18:2) Hexane:Ethyl Acetate:Acetic Acid) (THC-COOH fraction)	
Hexane	**Lot:**
Ethyl Acetate	**Lot:**
Acetic Acid	**Lot:**
Evaporate combined fractions to dryness at 40 °C under nitrogen (Do not overdry)	
Dissolve the dried residue in 50 µL ACN. Mix/vortex tube rinsing sides of the tube to solubilize the residue from the surface of the collection tube.	
Acetonitrile	**Lot:**
Add 50 µL DIW to tube. Mix/vortex and ensure sides of tubes are rinsed.	
Transfer to appropriately labeled LC autosample vials.	

Appendix A (Cont'd):

Specimen Procedure (Cont'd)

Analyze by LC/MS/MS:

Instrument: Shimadzu 8030	Instrument Identifier:
HPLC Column: Phenomenex Kinetex C18 ; 100Å ; 2.6µm ; 50mm x 2.1 mm):	Lot:
Mobile Phase A: 5mM (0.04% NH_4OAc)	Lot:
Mobile Phase B: ACN (CH_3CN) – 100%	Lot:

Gradient Program: Initial 20% B Flow 0.5 mL/min

Time (min.)	%B
0.25	20
1.75	90
2.40	90
2.41	20
4.00	Stop

MRM Transitions:

Analyte	Precursor (m/z)	Product (m/z)	Pause time (msec)	Dwell time (msec)	Q1 Pre-Bias (V)	Coll. Energy	Q3 Pre Bias (V)
THC	313.20	245.05	3	10	10	28	27
THC	313.20	191.20	3	10	10	30	21
THC	313.20	203.10	3	10	14	31	13
THC-OH	329.20	268.15	3	15	13	28	19
THC-OH	329.20	172.90	3	15	16	31	18
THC-COOH	342.20	325.25	3	15	11	20	23
THC-COOH	342.20	191.15	3	15	15	33	22
THC-COOH	342.20	299.20	3	15	19	23	20
THC-D_3	316.10	194.2	3	15	15	35	15
THC—OH-D_3	332.30	271.20	3	15	15	35	15
THC-COOH-D_3	346.20	248.30	3	15	15	35	15

Rev. #	History
1	New procedure for blood or other appropriate matrices.