Section Six
Urine and Blood Toxicology

6.1 Extraction Methods for LCMS-QQQ Confirmation

6.1.2 Confirmation of Cannabinoids in Blood and Urine

6.1.2.1 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.[4,5,6] This blood concentration drops rapidly after cessation of smoking.[4,5] The level may fall to less than 5µL within 30 to 60 minutes, although longer detection times have been reported.[4,5] Detection of low dose (1.75%) post-smoking Δ^9-THC has been reported to vary from 3 to 12 hours.[5] 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.[5] Longer detection times have been observed for frequent users. Δ^9-THC is metabolized to an active metabolite, 11-hydroxy-tetrahydrocannabinol, and an inactive metabolite, 11-nor-9-carboxy-tetrahydrocannabinol (carboxy-THC). The concentration of the Δ^9-THC inactive metabolite, carboxy-THC, gradually increases and may plateau for several hours.[5] 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.[4,5,6] The detection window for the active analytes is much shorter than that of carboxy-THC.[7] Cannabinol and cannabidiol are minor cannabinoids also found in marijuana.[8,9] Due to their low abundance, rapid clearance from blood, and similar pharmacokinetic profiles in chronic versus occasional smokers, the presence of cannabidiol and cannabinol in blood has been suggested to be a sufficient but unnecessary marker for recent marijuana use.[8]

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.[5] 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 methods 2.4.4 and 3.10.1, as well as provided references.

Figure 1.

6.1.2.2 SCOPE

This method is used for the confirmation of Δ^9-THC, 11-nor-Δ^9-THC-9-COOH (Carboxy-THC), 11-hydroxy-Δ^9-THC (Hydroxy-THC), cannbinol, and cannabidiol in blood and urine. The words *calibrator* and *calibration* are used to coincide with the terminology in instrument software and manufacturer manuals. The manufacturer's term *calibrator* refers to what is considered by ISP-FS as reference material that has a certified concentration of drug present.

6.1.2.3 EQUIPMENT AND SUPPLIES

6.1.2.3.1 Agilent 6410B LC/MS/MS system and MassHunter software
6.1.2.3.2 16x100mm silanized extraction tubes & caps
6.1.2.3.3 Tapered glass tubes for evaporation and reconstitution
6.1.2.3.4 Glass transfer pipettes
6.1.2.3.5 Calibrated pipettes for accurate dispensing of volumes 10 µL to 4 mL
6.1.2.3.6 Auto-sampler vials with snap-caps for Agilent 1200 and/or 1260 ALS
6.1.2.3.7 Flat-bottomed inserts compatible with the ALS vials in 6.1.2.3.6
6.1.2.3.8 Test tube rocker or rotator
6.1.2.3.9 Centrifuge
6.1.2.3.10 Oven or Waterbath
6.1.2.3.11 Evaporative concentrator
6.1.2.3.12 pH indicator strips
6.1.2.3.13 Calibrated thermometer

6.1.1.4 REAGENTS

Refer to manual section 5.12 for preparation instructions.

6.1.2.4.1 Glacial Acetic Acid
6.1.2.4.2 10N NaOH
6.1.2.4.3 10% Acetic Acid in Water
6.1.2.4.4 Hexane
6.1.2.4.5 Ethyl Acetate
6.1.2.4.6 LC/MS grade Water
6.1.2.4.7 Deionized Water
6.1.2.4.8 LC/MS grade Acetonitrile
6.1.2.4.9 Extract reconstitution solvent: 1:1 Water:Acetonitrile (LC/MS grade only)
6.1.2.4.10 LC/MS grade formic acid (optional)
6.1.2.4.11 0.1% formic acid in water (mobile phase A)
6.1.2.4.12 0.1% formic acid in acetonitrile (mobile phase B)

6.1.2.5 QUALITATIVE ASSURANCE: REFERENCE MATERIALS AND CONTROLS

6.1.2.5.1 **Calibrator and Control Solutions**

Corresponding calibrator and control reference material shall be obtained from different vendors, or be from different lot numbers if suitable second vendors are not available. *NOTE: Stock solution concentrations other than those listed here may be obtained, but appropriate addition volume adjustments must be made when direct spiking or preparing working solutions. Stock solutions should be stored as recommended by vendor.* ***Lipomed reference materials shall not be used for controls (except the***

carboxy-THC-glucuronide control) described in this method, as the ion ratios consistently failed during validation experiments.

6.1.2.5.1.1 **Reference Material Stock Solutions**

6.1.2.5.1.1.1 Single component cannabinoid-class reference solutions
Δ^9-THC
11-nor-Δ^9-THC-9-COOH
11-hydroxy-Δ^9-THC
Cannabinol
Cannabidiol

6.1.2.5.1.1.2 **Reference Material Working Solutions**
Refer to Appendix 1 for the preparation instructions and stability of the working solutions.

6.1.2.5.1.2 **Internal Standard Solutions**

6.1.2.5.1.2.1 **Stock Solution (100 µg/mL)**
Δ^9-THC-D3
11-nor-Δ^9-THC-9-COOH-D3
11-hydroxy-Δ^9-THC –D3
Cannabinol-D3
Cannabidiol-D3

6.1.2.5.1.2.2 **Working Solution**
Refer to Appendix 1 for the preparation instructions and stability of the working solution.

Required Extracted Controls for all options contained in this method:

6.1.2.5.2 **Extracted Negative Control**
An extracted negative control will be run for each matrix that is included in the run. The controls may be commercially obtained or in-house urine or blood verified to be negative for drugs of interest. The same lot of negative urine or blood should be used for extracted calibrators and all controls made in house.

6.1.2.5.3 **Extracted Positive Control**
Extracted positive controls will be run for each matrix that is included in a run. Positive Controls can be prepared with single or multi-component working solutions and/or obtained commercially.

6.1.2.5.3.1 URINE
The positive control must have at least two compounds in it that are included in the scope of the method, at an approximate concentration between 5 and 25 ng/mL for all

compounds except carboxy-THC. The response for carboxy-THC should be between 25 ng/mL and 125 ng/mL. (Note: spike with 5-25 µl of the low control working solution). *The compounds in the controls* ***cannot*** *be the same lots as were used for the calibrators*. For the control to be considered passing, it should give a response greater than the LOD for each intended analyte. *NOTE: carboxy-THC-glucuronide may be one of the compounds included in this control. If included in the positive control, a separate glucuronide control is not required. The concentration of the glucuronide compound shall meet criteria specified in section 6.1.2.5.4*

6.1.2.5.3.2 BLOOD

In blood, two positive controls should be run. One control shall be a low control, with a concentration between 5 ng/mL and 10 ng/mL for all compounds except carboxy-THC. The response for carboxy-THC should be between 25 ng/mL and 50 ng/mL. The second control shall be either a mid- or high-concentration control at an approximate concentration between 25 ng/mL and 100 ng/mL for all compounds except carboxy-THC. The response for the carboxy-THC should be between 125 ng/mL and 500 ng/mL. (Note: for the low control, spike with 5-10µl of the low control working solution and for the mid to high control, spike with 25-100 µl of the high control working solution). Both positive controls must minimally contain two compounds included in the scope of the method as follows: any analytes being quantitated, and at least one other analyte. For the control to be considered passing, it should give a response within 20% of the target concentration for the analyte being reported quantitatively. For analytes being reported qualitatively, a response greater than the LOD is considered passing.

6.1.2.5.4 **Extracted Glucuronide Controls (URINE ONLY).**

A positive glucuronide control is required for any run that includes urine samples. This control may be obtained commercially or prepared in-house by spiking negative urine. Carboxy-THC-glucuronide should be used, and the approximate concentration should be between 10 and 200 ng/mL.

6.1.2.5.4.1 Stock Solution

100 µg/mL Carboxy-THC-Glucuronide

6.1.2.5.4.2 Working Glucuronide Solution (10 ng/µL)

Add 10 µL of 100 µg/mL Stock Solution to 990 µL of MeOH or Acetonitrile. *Solution is stable for one week when stored under refrigeration.*

6.1.2.6 PROCEDURE

6.1.2.6.1 Calibrator preparation (calibrators shall be prepared and extracted with each run; calibrators must be prepared and extracted for each matrix included in the analysis run).

6.1.2.6.1.1 Label an extraction tube for each calibrator. Add 1 mL of matrix (blood or urine), then spike each with 10 µL of 1.0 µg/mL ISTD mix, as well as the following volumes of reference material. Extract as described in section 6.1.2.6.2. *NOTE: A minimum of 4 calibration points are required to establish a valid calibration curve. Analysts may determine which concentration levels are appropriate and elect to run the minimum number of calibrators; all quality assurance requirements must be successfully met.*

Sample Type	0.1/0.5 µg/mL Target Mix	1.0 µg/mL Target Mix	100 µg/mL e-THC stock	OR 1.0/5.0 µg/mL Target Mix
1/5 ng/mL Cal 1	10 µL	-	-	-
2/10 ng/mL Cal 2	20 µL	-	-	-
5/25 ng/mL Cal 3	50 µL	-	-	-
10/50 ng/mL Cal 4	100 µL	-	-	-
25/125 ng/mL Cal 5	250 µL	-	-	25 µL
50/250 ng/mL Cal 6	-	50 µL	2.5 µL	50 µL
100/500 ng/mL Cal 7	-	100 µL	5 µL	100 µL

6.1.2.6.2 Blanks

A non-extracted blank or negative control will be run directly preceding each case sample to rule out carryover. The area response of the blank preceding a sample must be at least 10 times less than any compound confirmed in the case sample, and must be below the limit of confirmation for any analyte confirmed in the case sample. If confirmation criteria (e.g. ion ratios) are not met, the analyte is not considered present.

6.1.2.6.2.1 Multiple non-extracted blanks may be prepared and used.. To prepare, spike a tapered bottomed tube with the appropriate amount of internal standard *(ie 10* µL *if reconstituted in 100* µL *reconstitution solvent or 20* µL *internal standard if reconstituted in 200* µL *reconstitution solvent,* etc.)

6.1.2.6.2.2 Evaporate the spiked internal standard to dryness under nitrogen at ~ 40 degrees C. **It is critical that the extracts**

are evaporated completely to dryness, but *DO NOT* over-dry.

6.1.2.6.2.3 Reconstitute the dry extract in the appropriate volume of 1:1 Acetonitrile:Water. (*NOTE: The reagents for this step shall be LC/MS grade.*). Transfer the reconstituted sample from the evaporation tube into to the corresponding autosampler vial and cap.

6.1.2.6.3 Casework sample and control preparation

6.1.2.6.3.1 Casework and Control Samples (Blood or Urine)

6.1.2.6.3.1.1 Transfer 1 mL casework and controls to extraction tubes.

6.1.2.6.3.2 Internal Standard Addition

6.1.2.6.3.2.1 Add 10 µL of 1.0 µg/mL ISTD mix to each blank, QC and case sample. Vortex to mix.

6.1.2.6.2.3 Sample Hydrolysis (***Urine ONLY***)

6.1.2.6.3.3.1 Enzyme hydrolysis: using a calibrated pipette, add 40 µL 10N NaOH to each calibrator, control and case sample. Vortex and verify that pH >10.

6.1.2.6.3.3.2 Verify water-bath or oven temperature. Cap and incubate at 60°C for 20 minutes. Remove from heat and allow to cool.

6.1.2.6.3.3.3 Using a calibrated pipette, add 25 µL of Glacial Acetic Acid to each calibrator, control and case sample. Vortex and verify that pH is ~5.5 (pH 5-6 is acceptable). Proceed with extraction at section 6.1.2.6.2.4.3.

6.1.2.6.3.4 Extraction

6.1.2.6.3.4.1 Add 2 mL of deionized water to each tube containing blood. Vortex to mix.

6.1.2.6.3.4.2 Using a calibrated pipette, add 0.8 mL of 10% Acetic Acid in Water to each tube containing blood. Vortex to mix.

6.1.2.6.3.4.3 Add 8 mL of 10% Ethyl Acetate in Hexane to each tube (blood) **or** 3 mL of 10% Ethyl Acetate in Hexane to each tube (urine).

6.1.2.6.3.4.4 Rock or rotate tubes gently for no less than 10 minutes.

6.1.2.6.3.4.5 Centrifuge the tubes at approximately 2500-3500 rpm for 5 minutes.

6.1.2.6.3.4.6 Using a **glass** transfer pipette, transfer most of the upper organic layer from each tube to the corresponding labeled conical evaporation tube. **Avoid transferring any solids.**

6.1.2.6.3.4.7 Evaporate the organic phase to dryness under nitrogen at ~ 40 degrees C. *Start the airflow slowly (~5-10 psi) to avoid splashing tube contents.* **It is critical that the extracts are evaporated completely to dryness, but *DO NOT* over-dry.**

6.1.2.6.3.5 Reconstitution

6.1.2.6.3.5.1 Reconstitute the dry extract in 100 µL 1:1 Acetonitrile:Water. (*NOTE: The reagents for this step shall be LC/MS grade.*)

6.1.2.6.3.5.2 With a **glass** pipette, transfer most of the reconstituted sample from the evaporation tube into to the corresponding auto-sampler vial with flat-bottom insert, and cap. *Do not transfer any solids. If the reconstituted extract is cloudy or viscous, centrifuge at ~2000 rpm for ~5 minutes, then transfer only the supernatant to the ALS insert.*

6.1.2.6.4 Instrument and run set up

See analytical method 5.13 for instrument maintenance, operation and data analysis procedures

6.1.2.6.4.1 Begin the Worklist by clicking on the Multiple Vial icon on the top center of the MassHunter Acquisition screen. The cycle time for each injection is ~15 minutes.

6.1.2.6.5 **Batch Review**

6.1.2.6.5.1 The lab criterion for acceptable calibration curve R^2 is $\geq$0.98.

6.1.2.6.5.2 The limit of detection (LOD) is the same as the limit of quantitation (LOQ) for blood quantitative analysis results. Once uncertainty of measurement has been established, Δ^9-THC may be reported quantitatively. For all other analytes in blood and all analytes in urine, only qualitative confirmation of results may be made.

The established LODs for each compound are as follows:

Analyte	LOD
Δ^9-THC	3 ng/mL
11-nor-Δ^9-THC-9-COOH	10 ng/mL
11-hydroxy-Δ^9-THC	5 ng/mL
Cannabinol	5 ng/mL
Cannabidiol	5 ng/mL

6.1.2.6.5.3 The default criteria for a positive result are:

6.1.2.6.5.3.1 The sample must have a concentration no less than the established LOD for each analyte. Samples with concentrations of qualitatively-reported analytes exceeding the highest calibrator may be reported without dilution/re-extraction provided that retention time and ion ratio requirements are met. For compounds that are quantitatively reported, the concentration shall fall within the range established by the calibrators for each analyte, or reported as >100 ng/mL provided all other reporting criteria are met.

6.1.2.7 QUALITY ASSURANCE REQUIREMENTS

6.1.2.7.1 Refer to toxicology analytical methods 5.8 and 5.10 for additional quality assurance and reference material authentication requirements.

6.1.2.8 ANALYSIS DOCUMENTATION

6.1.2.8.1 The printed results for each case sample and accompanying blank will be included with the analysts' notes. Case results are to be recorded in the LIMS system.

6.1.2.8.2 The reports for the batch and controls will be stored centrally by the lab in which the analysis was performed. When necessary, a copy of control printouts can be prepared from the centrally stored document.

6.1.2.8.3 The data from the run will be stored electronically, and if it is on a computer, will be backed up at least every two months.

6.1.2.9 LIMITATIONS OF METHOD

6.1.2.9.1 The hydrolysis process for glucuronides in urine has limited efficiency; based on the validation study, the estimated conversion is about 30-50 percent.

6.1.2.9.2 This method has only been evaluated for qualitative identification of the listed compounds in urine and blood. The uncertainty associated with the quantitative values has not been established; therefore, **quantitative values are not to be reported or expressed.** Once the uncertainty of measurement is established for this method, it will be evaluated for quantitative reporting of Δ^9-THC in blood samples only.

6.1.2.10 REFERENCES

6.1.2.10.1 This method was obtained independently from Agilent and Washington State Patrol (WSP) Toxicology Laboratory. Patrick Friel from Agilent came to the Idaho State Police Forensic lab located in Coeur d'Alene and provided application training July 23-26, 2012. Amanda Black (Quality Manager at WSP) provided copies of their validation documents to assist with the validation of this method in the ISP Forensic Services laboratory system.

6.1.2.10.2 Dr. Ryan van Wagoner with Sports Medicine Research and Testing Laboratory (SMRTL), provided consultation services for the validation of this project. This was funded by the National Institute of Justice under the 2013 Paul Coverdell Forensic Science Improvement Grants Program. The grant number is 70048-13NFSI00.

6.1.2.10.3 Williamson S.C, ISP Toxicology Analytical Methods 2.4.4 and 3.10.1.

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

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

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

6.1.2.10.7 Huestis, M. *Marijuana*. pp. 229-244. *in:* Principles of Forensic Toxicology, Second Edition. Levine, B. ed., AACC, 2003.

6.1.2.10.8 Desrosiers, N.A.; Himes, S.K.; Scheidweiler, K.B.; Concheiro-Guisan, M.; Huestis, M.A. *Phase I and II Cannbinoid Disposition in Blood and Plasma of Occasional and Frequent Smokers Following Controlled Smoked Cannabis.* Clinical Chemistry, 60:4, pp. 631-643, 2014.

6.1.2.10.9 Nadulski, T., et al. *Simultaneous and Sensitive Analysis of THC, 11-OH0THC, THC-COOH, CBD, and CBN by GC-MS in Plasma after Oral Application of Small Doses of THC and Cannabis Extract*. Journal of Analytical Toxicology, Vol 29, pp. 782-789, November/December 2005.

Appendix 1:

1.0 µg/mL ISTD mix in methanol
(*Document on a prep sheet with an expiration of one year, store frozen)*
Fill a 10 mL volumetric flask about half full with methanol, add 100 µl of 100 µg/mL stock solution of the following compounds. (If the stock solution is a different concentration you will need to adjust addition volumes.)

Δ^9-THC-D3, 11-nor-Δ^9-THC-9-COOH-D3, 11-hydroxy-Δ^9-THC –D3, Cannabinol-D3, Cannabidiol-D3

QS with methanol and ensure it is thoroughly mixed.

CALIBRATOR/CONTROL Working Solution Preparation Options:

High Working Solution OPTION 1: 1 µg/mL Target mix in methanol (Note: *11-nor-Δ^9-THC-9-COOH is NOT included in this working solution.)*
(Document on a prep sheet with an expiration of one year, store frozen)

In a 10 mL volumetric flask fill the flask about half full with methanol, add 10 µL of 1 mg/mL (or 100 µL of 100 µg/mL) stock solution of the following compounds (If the stock solution is a different concentration, you will need to adjust addition volumes.):

Δ^9-THC, 11-hydroxy-Δ^9-THC, Cannabinol, Cannabidiol

QS with methanol and ensure it is thoroughly mixed.

High Working Solution OPTION 2: 1/5 µg/mL Target mix in methanol
(Document on a prep sheet with an expiration of one year, store frozen)

NOTE: the 1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 5 µg/mL.

In a 10 mL volumetric flask fill the flask about half full with methanol, add 500 µL of 11-nor-Δ^9-THC-9-COOH stock solution (100 µg/mL) *AND* 10 µL of 1mg/mL (or 100 µL of 100 µg/mL) stock solution of the following compounds (or adjusted volume based on stock concentration):

Δ^9-THC, 11-hydroxy-Δ^9-THC, Cannabinol, Cannabidiol

If the analyst makes this working solution with carboxy-THC, no carboxy-THC stock should be spiked into the calibrators as described in Section 6.1.2.6.1.1.

QS with methanol and ensure it is thoroughly mixed.

Low Working Solution OPTION 1: 0.1/0.5 µg/mL Target mix in methanol
(Document on a prep sheet with an expiration of one year, store frozen)

NOTE: the 0.1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 0.5 µg/mL.

In a 10 mL volumetric flask fill the flask about half full with methanol. Add 1 mL of 1 µg/mL Target Mix working solution (*See OPTION 1 for Target Mix preparation*) *AND* 50 µL (100 µg/mL) carboxy-THC stock solution. QS with methanol and ensure it is thoroughly mixed.

Low Working Solution OPTION 2: 0.1/0.5 µg/mL Target mix in methanol
(Document on a prep sheet with an expiration of one year, store frozen)

NOTE: the 0.1 µg/mL concentration refers to all analytes except carboxy-THC; the concentration of carboxy-THC in the working solution is 0.5 µg/mL.

In a 10 mL volumetric flask fill the flask about half full with methanol. Add 1 mL of 1/5 µg/mL Target Mix working solution (*See OPTION 2 for Target Mix preparation*). QS with methanol and ensure it is thoroughly mixed.

Revision History

Section Six
Urine and Blood Toxicology

6.1 Extraction Methods for LCMS-QQQ Confirmation
6.1.2 Confirmation of Benzodiazepines and Z drugs in blood and urine

Revision No.	Issue Date	Revision/Comments
0	08/31/2015	Original Issue.
1	07/26/2016	Correction to section 6.1.2.6.3 referencing new method for instrument operation, minor formatting changes, addition of non-extracted blank to be used preceding case samples, changed concentration requirements for controls, changed acceptable blank to sample response ratio.