HEALTH & ENVIRONMENTAL TESTING LABORATORY

Forensic Toxicology

BLOOD DRUG PROCEDURES

About This Document

The Forensic Lab Director / Quality Manager reviews this document at annually. If changes are made, analysts acknowledge the updated procedures. Obsolete procedures are archived and retained by the laboratory.

Contents

Maine HETL- Forensic Toxicology

1. Summary:

Forensic blood drug analysis is defined as the practical application of specialized devices, instruments, and methods by trained laboratory personnel to qualitatively detect drugs/drug metabolites in blood samples.

This document describes data acquisition protocols for the qualitative and quantitative detection of drugs/drug metabolites in blood as well as the toxicology quality guidelines for the analysis, evaluation, acceptance procedures, and reporting of blood toxicology results.

The methods described in this document are for the qualitative and quantitative detection of drugs and drug metabolites in blood. Recovery compounds and deuterated internal standards for the compound(s) of interest are added to the blood samples. Compounds of interest and corresponding recovery compound or deuterated internal standards are then efficiently partitioned from the blood sample via liquid/liquid or protein precipitation extraction technique and separated on a liquid chromatograph (LC) column and are analyzed using tandem mass spectrometers (MS/MS) utilizing multiple reaction monitoring (MRM) and select ion monitoring (SIM).

2. Definitions

Calibrators: Laboratory fortified whole blood matrixes that are prepared from a certified reference material that are used to create a calibration curve for an assay.

Calibration Range/Limit of Quantitation: The quantitative range between the Lower Limit of Quantitation (LLOQ) and the Upper Limit of Quantitation (ULOQ) used in the calibration curve.

Positive Quality Controls: Laboratory fortified matrix matched samples that are prepared from a certified reference material that are used to check the accuracy of a calibration curve.

Negative Quality Control: An extracted matrix matched sample containing internal standard or recovery compound used to confirm no compound of interest carry over from the batch calibrators and evaluate all reagents used in the analytical method for potential interference or contamination.

Internal Standard(s): Compound(s), most commonly compound of interest matched deuterated equivalents. Used to mitigate the effects of extraction and instrument variables. All calibrators, QC, and case samples are fortified with internal standard at a consistent concentration. Internal standard(s) are used to calculate quantitative values by measuring the area response of ion transition for each compound of interest as a ratio compared to the area of the compound of interest's associated internal standard.

Recovery Compound(s): A compound of interest that would be highly unlikely to be found in a case sample and not in the blood drug testing menu. A deuterated compound of interest may also be used as a recovery compound. All samples in an analytical batch are fortified at a consistent concentration. Recovery compound(s) are used in qualitative chromatographic assays to monitor overall batch and individual sample extraction recovery.

Reporting Limit (RL): The lowest concentration at which an analyte has been validated to be reported by the laboratory.

Lower Limit of Detection (LLOD)/Lower Limit of Quantitation (LLOQ): The lowest concentration at which an analyte has been validated to be accurately quantitated. The LLOD/LLOQ must exhibit the presence of the qualifier ion, have a signal to noise ratio of ≥3.3, and back calculate ±20% of expected concentration. If the LLOD/LLOQ does not meet all acceptability criteria, then the assay is repeated or a new LLOD/LLOQ is selected as the lowest calibration point that meets all of the acceptability criteria.

Upper Limit of Quantitation (ULOQ): The highest concentration calibration point included in the calibration curve, the ULOQ must exhibit the presence of the respective qualifier ion, have a signal to noise ratio of ≥3.3, and back calculate ±20% of expected concentration. If the ULOQ does not meet all acceptability criteria, then the assay is repeated or a new LLOQ is selected as the highest calibration point that meets all of the acceptability criteria. Case sample results above the ULOQ should be re-analyzed with a dilution, sample volume permitting else the sample result be qualified as above the calibration range.

Select Ion Monitoring (SIM): A type of mass spectrometry where the intensities of one or more specific ions are recorded rather than the entire mass spectrum.

Multiple Reaction Monitoring (MRM): A type of mass spectrometry were the intensities of one or more specific ion transitions (Parent>Fragments) are recorded.

Autotune: A tuning process that involves adjusting mass spectrometer parameters through the infusion of a tune solution.

Checktune: A tuning process that evaluates but does not adjust mass spectrometer parameters through the infusion of a tune solution.

3. <u>Evidence Handling and Preservation</u>

All laboratory personnel will handle submitted materials in a manner that assures the integrity of the evidence. Prior to initiating and during the processing of evidence, the analyst will employ the following practices:

- The work area will be clean and free of any excess debris

- Countertops will have adequate space for working with samples and shall be cleaned when dirty. Should any biological spill occur, work will be stopped, the area cleaned, and the counter wiped with an appropriate agent such as 10% bleach solution, or a "Lysol-like" product designed to clean and disinfect.
- All glassware and tools to be used will be clean
- Test tubes, capillary pipettes, Pasteur pipettes, and transfer pipets are used only once, then discarded. Repeat pipet syringes may be used multiple times as long as the syringe tip does not come into contact with a surface (countertop, extraction tube, etc)
- To prevent cross contamination of samples, only one case will be opened and aliquoted by the analyst at a time
- Reagents and solvents will be kept in closed containers when not being used in the analysis
- Prior to and following testing, the evidence will be properly secured. During analysis, the evidence will be under constant control in the custody of the analyst, as described by the Quality Manual.
- A worksheet(s) with the sample identification number will be used and will follow the sample throughout the analysis. Each unique case identification number will be placed onto all of the testing forms used.
- Samples and controls shall be fortified with internal standard(s) and stock(s) respectfully on the same day as the extraction.
- Samples will be stored in a secure location (i.e. evidence refrigerator) while thawing or if work stops for any reason. (i.e., lunch, end of workday, etc).

Opening & Storage of Submitted Blood Evidence

The following practices shall be employed in the opening and storage of submitted blood evidence:

- Evidence to be opened for analysis will be removed from evidence refrigerator and the reverse side of the pink Receipt/Request for Examination Contract will be filled out (i.e., internal chain of custody).
- The analyst will ensure a proper seal is in place prior to opening the evidence. If the item is found to not be properly sealed, or lacking initials across the seal, a note will be made on the Blood Kit Inventory form and communicated to the customer on the case report.
- The analyst opening the kit will initial stickers bearing the HETL Identification Number.
- If the subject's name is not available at the time of log-in, the analyst will write the subjects name on the labels at the time of kit opening (if known).
- The analyst will verify all identification numbers and names agree with the Receipt/Request for Examination Contract.
- If a non-HETL approved collection kit or materials were submitted, the WO# field in STARLIMS folder metadata shall be filled in with N/A.
- If the kit, upon opening, is found to contain non HETL approved collection materials this shall be noted on the inventory form and STARLIMS metadata shall be updated appropriately.
- The collection kit and all specimens will be labeled with the lab identification number, name of the subject (if known) and the initials of the analyst that opened the kit.
- All paperwork contained in the kit will be labeled with the laboratory identification number and initialed by the analyst that opened the kit.
- The analyst will verify and note in the case notes that the case information provided with the kit matches the HETL folder, sample information from the Laboratory Blood Drug Analysis Request form submitted with the sample and all Starlims labels. Any minor discrepancy shall be noted by the analyst within the case folder and communicated with the customer on the case report. Major discrepancies shall require customer communication and correction prior to testing. If a discrepancy is noted for type of testing requested between the Receipt/Contract for Examination and the Laboratory Blood Analysis Request form the following action shall be taken:

Receipt/Contract for Examination	Laboratory Blood Analysis Request	Action

None	BAC, TOX, or BAC/TOX	Perform testing requested on single form
BAC, TOX, or BAC/TOX	None	Perform testing requested on single form
BAC/TOX	BAC	Perform BAC & TOX testing
BAC/TOX	TOX	Perform BAC & TOX testing
BAC	TOX	Prior to any testing reach out to submitting agency to confirm testing requested
TOX	BAC	Prior to any testing reach out to submitting agency to confirm testing requested

- If a more detailed description is necessary, the analyst may document the item by making additional notations, or by taking a picture using a state controlled camera (not a personal camera or cell phone). If a picture is taken, a ruler shall be included in the photo. The photo shall be printed for the case file and have the following items documented on the printout: HETL case number, analyst initials, and date.
- The analyst will fill out the blood inventory worksheet, making any necessary notations. The analyst will document the HETL case number on the blood inventory worksheet. For non-HETL collection materials, refer to the manufacture's information or the below reference table:

Manufacturers cap/label	Additive(s)	Blood type
Light Blue	Sodium Citrate	Whole Blood (If centrifuged-Plasma)
Gold, Red/Black Mix	Clot activator & separation gel	Serum (*if centrifuged)
Red	None (glass) Clot activator (plastic)	Clotted whole blood (If centrifuged-Serum)
Green	Sodium Heparin Lithium Heparin	Whole Blood (If centrifuged-Plasma)
Light Green, Green/Gray Mix	Lithium Heparin & separation gel	Plasma (*if centrifuged)
Lavender/Pink	K2EDTA	Whole Blood
Gray	Potassium Oxalate & Sodium Fluoride	Whole Blood
*If centrifuged separation gel will travel from the bottom of the tube to the middle of the tube (to between the red blood cells and the serum/plasma).		

  - For continuity the volumes entered into the blood inventory worksheet and subsequent StarLIMS items received table shall adhere to the following format:

	Volume (mL)
Visibly empty blood tube, no label	0 (leave collection date & time blank)
Visibly empty blood tube, with label	0
Visible droplets of blood in tube (unusable testing volume)	<0.5
Low volume of blood in tube (unusable testing volume)	<0.5

Usable testing volume of blood in tube	Approximate volume

- If a blood sample is received by the laboratory with volume not sufficient to perform standard OUI blood drug testing screening (<2mL), analysts will refer to any submitted case documentation for indicated or suspected drugs. Alternatively, if there is no documentation referring to any indicated or suspected drugs, the analyst may reach out to the investigating officer notifying them that the sample is QNS to perform the standard OUI blood drug panel and discuss course of testing. If the investigating officer does not respond the analyst may use their discretion to determine the best course of testing.

- The blood kit inventory sheet will be labeled with the date the kit was opened and the analyst's initials who completed the kit inventory. The blood kit box (everything other than blood tubes) will be stored in an appropriately labeled storage box which is given a unique identification number. This storage box identification number will be recorded on the Chain of Custody Receipt. The box will be retained until filled in the laboratory. All filled storage boxes will be placed in long term evidence storage, for a period of at least six months, until being returned to the submitter or destroyed.
- The analyst that opened the kit shall ensure the correct test codes are applied to the sample in StarLIMS. See above table for guidance on requested test discrepancies.
- At the time of analysis an instrument sequence table with the specimen identification number will be created.
- After analysis, the blood tubes will be sealed in plastic tube containers, the seals initialed by the analyst, and stored in a tube storage rack in the locked evidence refrigerator. The tube rack and position will be recorded on the Chain of Custody Receipt. All blood tubes will be held in locked storage for a period of at least six months upon completion of analysis, until being returned to the submitter or destroyed.

4. Quality Assurance

Equipment & Supplies:

Volumetric Flasks various sizes
Volumetric cylinders various sizes
Disposable glass tubes and caps 5-15 mL (silanized or non-silanized)
2mL microcentrifuge tubes
Autosampler, caps, vials with inserts (silanized & non-silanized)
Vortex mixer
Disposable transfer pipettes
Pipets and tips- various
Sample Evaporator

Reagents & Stocks

Refer to Quality Manual, SOP Manual and Blood Drug Testing Reagent Sheets.

Laboratory Consumables

Vendor supplied storage directions and expiration dates shall be followed and no supplies past suggested expiration shall be used for testing subject specimens for all Blood Drug Testing Program laboratory consumables. Items without expiration date shall be assigned an expiration date of no later than one year from the date of receipt. For supplies stored and used in the laboratory for specimen testing the following information shall be recorded on the container or packaging:

- Date Received
- Date Opened with Initials of Analyst
- Laboratory Expiration Date-unless documented on vendor's COA.

Safety Precautions:

The solvents used are considered toxic. Repeated or prolonged exposure can produce targeted organ damage. Proper PPE shall be used when handling solvents. Chemists should be familiar with Safety Data Sheets for all chemicals and drug standards used in the blood drug testing methods.

Blood from unknown case samples shall be handled following Universal Precaution guidelines. Face/benchtop splash shields in addition to PPE shall be utilized while pipetting blood.

Waste Management:

The most current approved version of the laboratory's RCRA plan located on Sharepoint shall be followed for the generating, labeling, and disposal of all hazardous waste generated by these methods. Maine Department of Environmental Protection rules Chapter 850: Identification of Hazardous Waste, Chapter: 851 Standards for Generators of Hazardous Waste, and Chapter 858: Universal Waste Rules shall be adhered to.

- Materials contaminated with blood that have chemical residue are to be placed into Biohazardous Waste. Examples: Pipet tips used to transfer material to extraction tubes, empty extraction tubes.
- The liquid generated from the Hexane: Ethyl Acetate liquid-liquid extraction (Qualitative C, THC, and Buprenorphine methods) and the chloroform extraction (Qualitative A and B supernatant of water, methanol, and blood) is classified as dual waste (chemically and biologically hazardous) and is to be disposed of in the liquid waste hazardous waste stream.
- Organic based solvent, stocks, and standards are to be disposed of in the liquid waste hazardous waste stream.
- Instrument waste is to be disposed of in the liquid waste hazardous waste stream.

Whole Blood Matrix Quality Assurance

Laboratory whole blood matrix shall be purchased from approved vendors with quality assurance testing being performed with each new lot number provided from the aforementioned vendors.

Upon receiving a new lot number of vendor's certified negative whole blood, an aliquot of the lot specific matrix shall be screened for all compounds of interest in the blood drug testing menu as a blank sample. A blank sample is a unfortified whole blood matrix carried through the entire extraction procedure, being treated the same as a subject specimen and analyzed. This process shall be performed prior to the use of the matrix with a new lot number in a subject specimen batch. Acceptable results exhibiting no interferences caused by manufacturing contaminants or interfering compounds are required prior to the lot number being used for casework. Records containing the results, pass/fail status, and lot number of the certified negative sample matrix shall be maintained.

Blood Collection Kits Quality Assurance

Upon receiving a new lot number of HETL-Forensic Chemistry Blood Collection Kits to be issued a random kit for testing with the follow procedure being followed:

1. Record the kit lot, blood tube lot, and PI pad lot numbers on the blood kit check worksheet.
2. Add 1 mL of DI water to one tube and vortex.
3. Label an autosampler vial with identification number.
4. Transfer a portion of the water/sodium fluoride/potassium oxalate mixture to the labeled autosampler vial with insert.

5. Inject on a LC-MS/MS using data acquisition screening methods: Qualitative A, Qualitative B and Qualitative C. If there is a presumptive positive result from one of the screening methods the mixture shall be injected and analyzed with the respective confirmation method.
6. Give the blood kit check worksheet to the Quality Manager.

This process shall be performed prior to the issuing of the HETL-Forensic Chemistry Blood Collection Kits containing the new lot number. Acceptable results exhibiting no interferences caused by manufacturing contaminants or interfering compounds are required prior to the lot number being distributed. Records containing the results, pass/fail status, and lot number of the blood collection kits shall be maintained.

Specimen Requirements:

- Only whole blood samples shall be analyzed using these methods.
- Whole blood samples are collected in tubes provided by HETL or by a qualified medical professional and upon receipt, stored under refrigeration at HETL (<10°C).

Estimation of the Uncertainty of Measurement:

An estimation of the uncertainty of measurement shall be determined using an uncertainty budget for all quantitative analytical procedures and reported on the Certificate of Analysis. Documentation, when applicable shall be retained by the Quality Manager.

Traceability is established by using NIST/Guide 34 or ISO 17034 traceable standards, obtained by an approved vendor, and utilized equipment calibrated to ISO17025 standards.

The uncertainty budget shall include Type A (random) uncertainties and Type B (systematic) uncertainties. As illustrated below these uncertainties include:

Uncertainty Component	Method of Evaluation
Staff	
Multiple Analysts	Covered in Type A Evaluation of Process reproductivity data-blood matrix QC sample.
Training	
Experience	
Bias	
% Bias of method	Taken from Type A Evaluation of Process reproductivity data-blood matrix QC sample.
Calibrators	
CRM-uncertainty in the stated reference value	Type B Evaluation
Matrix of calibrators and measurand	Initially evaluated during method validation. Quantified in Type A Evaluation of process reproducibility data-blood matrix QC sample.

Quality Control Samples	
CRM-second source; uncertainty in the stated reference value	Type B Evaluation
Matrix control-stability	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample.
Sampling of Measurand	
Homogenization	Initially evaluated during method validation. Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample.
Temperature-all calibrators, quality control samples, and the measurand are brought to room temperature. Variation in the time allowed to reach room temperature. Variation in room temperature at different times of year.	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample.
Internal Standard Preparation	
Components	No influence. The measurement result will only be impacted by the volume of the internal standard added to each sample.
Concentration of Internal Standard	No influence. Procedural requirement to use the same lot of internal standard for all samples in an analytical batch.
Preparation of aliquots of Calibrators, Quality Control Samples and Measurand	
Pipets. Volume of sample, volume of internal standard and calibration uncertainty or criteria for calibration and proper function check.	Type B Evaluation.
Variation in use by multiple staff	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample
Autosampler vials	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample
Time between replicate sampling of measurand	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample.
Calibration of measuring system	
Uncertainty in the calibrator values	Duplicate listing of Component-see calibrator section above

Matrix of calibrators and measurand	Duplicate listing of Component-see calibrator section above
Instrument precision	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample
Analysis	
Instrument parameter settings	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample
Interference from the matrix	Duplicate listing of component-see Sampling of Measurand section above
Interference from reagents	This component is not an uncertainty component but is a quality control concern. The laboratory analyzes a matrix blank (Negative Control) that contains no analyte (compounds of interest) but does evaluate all reagents used in the analytical method. The laboratory procedure specifies acceptable criteria for this quality control sample.
Interference from other compounds	This component is not an uncertainty component but is a quality control concern. The laboratory, as part of the validation process to ensure proper functioning of the measuring system analyzed a mixture of compounds to ensure no interference.
Stability of sample(s) from preparation through analysis	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample and through the procedure administrative requirement for agreement of replicates
Data Processing	
Calibration model	Covered in Type A Evaluation of process reproducibility data-blood matrix QC sample and through CRMs used as QC

Adapted from: ASCLD/LAB Executive Director, ASCLD/LAB Guidance on the Estimation of Measurement Uncertainty-ANNEX D the Uncertainty Component and Method of Evaluation table. ASCLD/LAB Document Control Number AL-PD-3065 Ver 1.0 2013.

Type A uncertainties shall be evaluated using historical control charts to establish standard deviations. For new methods that lack historical control data a minimum of 30 controls that meet all detection, identification, and concentration accuracy (as set forth in the Data Analysis Acceptance Criteria: Quality Controls section of this document) shall be analyzed to determine the pooled relative standard deviation of the mean. The pooled relative standard deviation equation is as follows:

$$RSDpooled = \sqrt{\{[(n1-1)*(RSD1)^2] + [(n2-1)*(RSD2)^2] + [(n3-1)*(RSD3)^2]\} \div [(n1+n2+n3)-3]}$$

Instrument used for analysis:

Shimadzu LCMSMS 8030: MS/MS 8030 SN: 010255250026, LC-20AD HPLC Pump SN: L20105356432, LC-20AD HPLC Pump SN: L20105356433, SIL-20AHT SN: L20345256156, CTO-20A SN: L20205352542, CBM-20A SN: L20235355674.

Agilent LCMSMS (QQQ): MS/MS 6470A SN: SG200G112, G7112B BinPump SN: DEAE901050, G7129A Vial Sampler SN: DEAEQ30663, G7116A Column Compartment SN: DEAEM05847.

Type B uncertainties resulting from inherent biases in measuring systems and analytical methods that are considered of significance include for all quantitative LC-MS/MS methods:

Preparation of calibrator or internal standard using 5mL or 10mL volumetric flask

Preparation of calibrator or internal standard using pipets

Using a pipet to prepare calibrators

Using a pipet to aliquot a sample

Using a repeat pipet to dispense internal standard in to all calibrators, controls, and case samples.

Uncertainty associate with Certificates of Analysis on certified reference materials

It is noted that if K=2 is provided by a vendor than we shall use that value. If no K=2 is provided then we shall determine uncertainty of measurement based off of the tolerance provided by the calibration vendor.

The use of internal standard for all quantitative analysis minimizes other sources of uncertainty.

Data from controls are tracked in a Microsoft Excel Spreadsheet. Calibration certificates of the pipets and volumetric flasks used as well as the Certificates of Analysis of respective calibrators and QC standards are retained by the Quality Manager. From these tracking documents it can be determined that the data is or is not of a normal distribution, skewed and the mean and standard deviation calculated. Additional graphs can also be created as warranted. All values of uncertainty from individual components deem significant (as listed above) are concerted to percent uncertainty (See ASCLD/LAB Annex D AL-PD-3065 Ver 1.0.)

All impacting uncertainties are combined using the Root Sum Squares technique:

$$U_{combined} = \sqrt{(U_1{}^2 + U_2{}^2 + U_3{}^2 \ldots.)}$$

The expanded uncertainty for a confidence interval of 95.45% (more commonly referred to as 95%) is determined using the equation:

$$Uexpanded = Ucombined * k$$

For methods lacking sufficient historical data, a corrected coverage factor (K_{corr}) shall be used based on the Student's t Table to compensate for the unreliable estimates derived from random uncertainties in the instances where few measurements are made. A K_{corr} shall be selected to meet a 95.45% confidence interval using the correct degrees of freedom, also known as n-1 selected to express this. Methods containing sufficient historical data shall utilize an appropriate k value based on the Student's t Table.

Each quantitatively detected compound shall have its own individually calculated uncertainty of measurement. The schedule to review the measurement of uncertainty shall be conducted annually or upon the addition or replacement of laboratory equipment or other factors considered of significance once enough data has been obtained to be evaluated. The Quality Manager will retain calculations, verifications of spreadsheets, graphs, and other relevant data.

The expanded uncertainty of measurement shall always be rounded up to two decimal places and be reported as such in the test report with the coverage probability. In addition, the analytical test result and the rounded expanded uncertainty shall be reported to the same level of decimal places.

5. Screening and/or Confirmation by LC-MS/MS:

LC-MS/MS Batch Sequences

Prior to running a batch, a mobile phase blank shall be run to prime and flush the system, this is performed for maintenance purposes and instrument priming, the data will not be analyzed. Mobile phase blanks that are run following a sample containing a compound of interest at concentrations greater than the ULOQ shall be reviewed by the analyst for potential carry over.

All instrument sequences shall be created using the pre-made templates for each individual method, these templates already have sample types and calibrator levels pre-programed into the sequence.

Qualitative batches shall consist of a maximum of 20 subject samples and shall utilize the following sequence template:

Mobile Blank
Mix (A, B, or C) (Positive Control)
Negative Control
Sample 1
Sample 2, etc.

Quantitative batches shall utilize the following sequence template:

Mobile Blank
Calibrator 1
Calibrator 2
Calibrator 3
Calibrator 4
Calibrator 5
Calibrator 6
Calibrator 7
Calibrator 8
Negative Control *(run after highest calibrator to serve as a concurrent carryover check)**

Positive Low Control (QCL)*
Positive Medium Control (QCM)*
Positive High Control (QCH)*
Sample 1
Sample 2, etc.

* A maximum of 20 samples shall be run following the first set of quality controls, if greater than 20 samples are run in a batch then a second set of quality controls shall be run following the 20th sample.

Sequence Check: Each auto sampler vial position, dilution factor and associated data acquisition method for each sample shall be verified by a secondary individual in comparison to the instrument sequence upon placing into the auto sampler rack. This shall be performed by the secondary individual physically checking each autosampler vial number to the instrument sequence and shall be documented by the following:

1. Instrument Sequence: The secondary individual performing the check will physically check off on each sample's position and data acquisition method on the instrument sequence. The secondary individual will initial and date the sample position column and the data acquisition method column.
2. Batch Review Form: The secondary individual performing the check shall initial the applicable pre-check sections on the batch review form.

Each auto sampler vial shall be checked in comparison to the instrument sequence upon removing from the auto sampler rack; this shall be documented on the instrument sequence table and the batch sequence check form.

Qualitative Data Analysis Acceptance Criteria (Qualitative A, B, & C)

Select Ion Monitoring (SIM) Identification: As part of the qualitative screening procedure (Qualitative A & C) when SIM is used for identification of a compound of interest the retention time match (<+/-0.2 minutes) and compound ion match is required. Please note SIM may be backed up with an MRM for select difficult to detect compounds or methods that are utilized for confirmation. For compounds of interest with a SIM and MRM the presence in the respective Mix (positive control) shall be deemed acceptable when the compound of interest consists of a peak present at the expected retention time and at least the MRM signal to noise ratio is >3.3.

Qualitative Quality Controls

A corresponding Mix (positive control) shall be run with each new sequence. Mixes shall consist of blank matrix samples fortified at respective positive concentrations, extracted, and analyzed with each batch. Mixes function as both a positive quality control to confirm the success of an extraction for the compounds of interest and as a calibration point in the creation of a one-point calibration curve to be used as a quantitative frame of reference for case samples. It is noted that the Qualitative methods A, B, & C produce semi-quantitative results that are derived from a one-point calibration forced through zero curve, to achieve approximate quantitative results that are only to be used by the analyst as a guide for approximating values. The approximate quantitative values shall never be reported out on any certificates of analysis.

A qualitative batch shall consist of respective mixes and a negative quality control for every twenty samples.

Qualitative Mix A, B, & C (positive control) acceptance criteria:

- Contains all compounds of interest and recovery compound

- Compounds of interest and recovery compound exhibit acceptable chromatography
- Compounds of interest S/N ≥3.3
- All retention times updated
- All qualifier ion ratios updated (when applicable)

Qualitative Negative Quality Control shall consist of a blank matrix fortified with only recovery compound and shall be extracted and analyzed with each batch after the Mix (positive control) in every batch to monitor for carryover. All Qualitative Negative Quality Controls must exhibit/meet the following:

- Recovery of recovery compound ≥50% of concurrently run Mix
- Recovery compound retention time +/-0.20 min of concurrently run Mix
- Recovery compound exhibits qualifier ion ratios within +/-30% of concurrently run Mix (Qualitative B method only)
- Recovery compound S/N ≥3.3

If the batch negative control does not meet the above parameters, any associated data generated shall be rejected and the batch shall be re-extracted.

- Each compound of interest must meet one or more of the following to be considered negative:
    - No peak at expected RT
    - Calculated concentration is less than LLOQ
    - Unacceptable qualifier ion ratios
    - S/N <3.3

If a negative control does not meet the above parameters with regards to compounds of interest, all samples in the batch shall be evaluated and re-extracted only if a sample meets positive acceptance criteria for the compound(s) seen in the negative control.

Qualitative Sample Acceptance Criteria

1. All concurrently run Mixes (positive control) and negative control shall be evaluated prior to data analysis being performed on a subject sample to confirm the controls meet acceptance criteria.

2. The recovery compound in a subject sample shall then be evaluated for the following to determine if the sample is suitable for comparison to concurrently run controls.
    - Presence of the recovery compound peak with acceptable chromatography
    - A recovery of greater than 50% of concurrently run Mix
    - Signal to noise ratio of > 3.3
    - Retention time + 0.2 minutes
    - Qualifier ion ratios within +/-30% of the concurrently run Mix (Qualitative B only)

3. Once the subject sample has been deemed suitable for comparison to concurrently run controls, the analyst can determine the presence of a target compound by making a comparison to the concurrently run certified reference materials. In order for a compound to be positive, the following acceptance criteria must be met:
    - Compound(s) present with acceptable chromatography
    - The retention time of the compound(s) of interest being confirmed are within 0.2 minutes of the expected value as compared to the concurrently run certified reference materials (mid-range calibrator).
    - The signal to noise ratio shall be evaluated for the compound(s) of interest being confirmed and will be greater than a 3.3 ratio for the primary ion.
    - Concentration: For all compounds of interest within the qualitative method screening methods, the lower limit of detection shall be considered approximate and that, if all other acceptance criteria is met

and a calculated approximated concentration is close to the respective lower limit of detection the compound of interest may be deemed a presumptive positive screen result based on analyst discretion. It is noted that PCP shall not be deemed as a positive result by the qualitative method unless a concentration of above 10ng/mL is exhibited.

- Qualitative A & C: For all compounds detected by both SIM and MRM: to be considered a positive screen result the MRM must meet all qualitative identification and detection criteria as the SIM alone may not exhibit a robust enough response and/or may be subject to interference.
- Qualitative B confirmation testing only: Ion ratios for the compound(s) of interest shall be within +/- 30% of a concurrently run Mix.

Quantitative Data Analysis Acceptance Criteria

Multiple Reaction Monitoring (MRM) Identification: As part of a quantitative procedure when MRM is used for identification of a compound of interest the retention time match and the identification of two transition ions (Parent>Fragment) and two internal standard transitions (Parent>Fragment or Parent/Parent) for the compound of interest is required.

Calibration Curve

A calibration curve shall be prepared from certified reference standards and run for each new sequence. Calibration curves shall consist of a non-forced linear weighted 1/A calibration curve. An acceptable calibration curve shall consist of:

- A minimum of 5 calibration points per calibration curve
- A coefficient of determination (r2) of ≥.990
- Calibration points used in a calibration curve shall exhibit:
    - Concentrations within range of quantitation must be ±20% from the expected concentration (Calibrators 2-8), concentrations outside the range of quantitation must be ±30% from the expected concentration (Calibrator 1)
    - Signal to noise: the compound of interest peak signal to noise ratio must be ≥3.3 to be deemed a quantifiable peak signal and not baseline noise
    - Qualifier ion ratios of <±30% ion ratio difference to the set ratio within the quantitative range (Calibrators 2-8)

All calibration curves shall be evaluated individually for each compound of interest, if a compound of interest's calibration curve fails that compound shall be rejected for the entire batch.

The calibration curve shall be evaluated by back-calculating calibrator concentrations against the curve. Qualifier ion ratios and retention times shall be updated for each batch using a mid-point calibrator.

If limit of quantitation (LOQ) calibrators (Calibrators 2 or 8) are removed from the curve then the LOQ shall be changed to reflect this for the particular batch and compound, this may require a repeat analysis of case samples whose results are below or above the new LOQ range.

Quantitative Quality Controls

The methanolic controls for the LC-MS/MS assays are prepared "in-house" from a different manufacturer or different lot of certifiable reference material than used in the preparation of the calibrators. Results from the quality controls are recorded in an excel QC tracking document or StarLIMS and evaluated for trends.

For every set of twenty case samples a set of quality controls shall be extracted and analyzed, these controls shall consist of a Negative Control, Quality Control Low, Quality Control Medium, and Quality Control High.

Quantitative Positive Quality Controls shall consist of blank matrix samples fortified respectfully at low, medium, and high levels of each test methods calibration range. All positive controls shall be evaluated individually for each compound of interest, if a compound of interest's positive control fails that compound shall be rejected for the entire batch. Positive quality controls must meet the following acceptance criteria for a compound of interest:

- Internal standards retention times +/-0.20 min of mid-point calibrator
- Internal standard qualifier ion ratios within +/-30% of mid-point calibrator
- Internal standard S/N ≥3.3
- Compounds of interest and internal standard exhibit acceptable chromatography
- Compounds of interest S/N ≥3.3
- Calculated concentrations must be within ±20% of the expected value.
- Compound of interest qualifier ion ratios within +/-30% of mid-point calibrator

If a positive control fails for a compound of interest any sample not being confirmed for the failed compound of interest may be reported out.

Quantitative Negative Quality Control shall consist of a blank matrix fortified with only internal standard and shall be extracted and analyzed with each batch after the highest concentration calibrator in every batch to monitor for carryover. All Quantitative Negative Quality Controls must exhibit/meet the following:

- Internal standards retention times +/-0.20 min of mid-point calibrator
- Internal standard qualifier ion ratios within +/-30% of mid-point calibrator
- Internal standard S/N ≥3.3

If the batch negative control does not meet the above parameters, any associated data generated shall be rejected and the batch shall be re-extracted.

- Each compound of interest must meet one or more of the following to be considered negative:
    - No peak at expected RT
    - Calculated concentration is less than LLOQ
    - Unacceptable qualifier ion ratios
    - S/N <3.3

If a negative control does not meet the above parameters with regards to compounds of interest, all samples in the batch shall be evaluated and re-extracted only if a sample meets positive acceptance criteria for the compound(s) seen in the negative control.

Internal Standards

Internal standard recovery as measured by peak area shall be averaged for all calibrators and quality controls in a batch to evaluate internal standard recovery in case samples by exporting the data from the instrument and importing it into the excel LCMSMS Area Calculators.

Quantitative Sample Acceptance Criteria

1. All concurrently run calibrators, positive controls, and negative control shall be evaluated prior to data analysis being performed on a subject sample to confirm the calibrator and controls meet acceptance criteria.

2. The internal standard in a subject sample shall then be evaluated for the following to determine if the sample is suitable for comparison to concurrently run controls.

- Presence of the internal standard peak with acceptable chromatography
- Signal to noise ratio of > 3.3
- Retention time + 0.2 minutes
- Qualifier ion ratios within +/-30% of the concurrently run mid-point calibrator

3. Once the subject sample has been deemed suitable for comparison to concurrently run controls, the analyst can determine the presence of a target compound by making a comparison to the concurrently run calibrators and controls. In order for a compound to be positive, the following acceptance criteria must be met:
    - Compound(s) present with acceptable chromatography
    - The retention time of the compound(s) of interest being confirmed are within 0.2 minutes of the expected value as compared to the concurrently run certified reference materials .
    - Ion ratios for the compound(s) of interest shall be within +/- 30% of a concurrently run mid-point calibrator.
        - Samples exhibiting positive results that have an ion ration difference of >±30% in comparison to the set ratio shall be evaluated for causes such as but not limited to: elevated baseline, low concentration levels of compound of interest, and extremely high levels of compounds of interest. Remedial action may be taken in the case of these exceptions such as but not limited to: manual integration, manual comparison of ion ratios to a similar concentration calibrator, re-analysis, or re-analysis with a dilution. If remedial action results in an ion ratio difference that is still >±30% in comparison to the set ratio then the compound of interest in question shall not be reported out for the sample.
    - The signal to noise ratio shall be ≥3.3 ratio for the primary ion
    - A internal standard recovery of greater than 50% the average of the response seen in the calibrators and quality controls. (As the software of the current instruments does not have the ability to evaluate the internal standard recovery of a sample in comparison to the average response seen in the calibrators and controls the analyst must manually export the data from the instruments and import it into a excel LCMSMS Area Calculator. This cannot be done prior to determining the presence of a target compound.)
        - If a subject sample exhibits an internal standard response of <-50% of the average internal standard response in the calibrators and quality controls remedial actions, if appropriate include, but are not limited to, the following in recommended action order:
            1. Reinjection of the sample after visual evaluation of the vial insert for breakage or air bubbles.
            2. Re-extraction and reanalysis of the sample with or without a dilution
            3. Re-extraction and reanalysis of the sample using a different internal standard (this would be considered a major method deviation and therefore authorization from the Forensic Laboratory Director is required)
            4. The sample may be reported out as “unable to perform standard OUI Testing Panel due to sample matrix”

Quantitative values are calculated by measuring the area of characteristic primary ion transition for each compound of interest as a ratio compared to the area of the compound of interest’s associated internal standard. This compound of interest/internal standard peak area ratio is then used in a linear regression analysis to determine quantitative concentration.

As the case samples are biological matrixes that may contain multiple drugs, or co-eluting compounds exceptions may be created to the following chromatography guidelines and acceptability criteria. Deviations and Exceptions shall be documented in case notes, on chromatograms, or on reports when required.

Guidelines for Confirming Positive Results

Prior to identification of an unknown compound in a subject sample, review and acceptance of the applicable calibration curve, quality controls, and internal standards/recovery compounds are required to ensure suitability of an unknown compound in a subject sample.

The detection and identification of drugs should be confirmed (when possible) by a second technique utilizing a different instrument methodology, extraction method, and/or chemical principle. In the event that a second technique is not available, identification must be confirmed by a separate second extraction, on a different aliquot of the same sample. or, if necessary, an aliquot from a second tube (in the event of multiple tubes being drawn at the same time from one individual and one tube quantity being insufficient for a second extraction).

The following illustrates acceptable drug and drug metabolite confirmation practices:

- Qualitative screen followed by a qualitative confirmation performed by separate extraction on a different aliquot of the same sample or, if necessary, an aliquot from a second sample
- Qualitative screen followed by a quantitative confirmation performed by separate extraction on a different aliquot of the same sample or, if necessary, an aliquot from a second sample
- Quantitative screen followed by a quantitative confirmation performed by separate extraction on a different aliquot of the same sample or, if necessary, an aliquot from a second sample. Acceptable results within the quantitative range must be within the methods uncertainty of measurement and the lowest concentration detected shall be reported.

For second sample testing as described above the following practices shall be followed to insure that “same samples” are used for testing:

- Same samples: Collection tubes contain same chemical additives and collection times are within 10 minutes of each other.
- Different samples: Collection tubes contain same chemical additives and collection times are greater than 10 minutes of each other.
- Different samples: Collection tubes contain different chemical additives and collection times are within 10 minutes of each other.
- Different samples: Collection tubes contain different chemical additives and collection times are greater than 10 minutes of each other.

Reporting Criteria

Quantitative results are always reported in nanograms per milliliter (ng/mL) of blood and truncated to two decimal places (example: 0.1375 is truncated to 0.13). Qualitative results are always reported as “Qualitative Result Confirmed: (Compound Detected).”

An uncertainty of measurement for each result is calculated based upon the most current expanded uncertainty value. The resulting value is always rounded up to two decimal places, regardless of what the 3th significant figure is (example: 0.0142 is rounded up to 0.015).

An inventory table will be included on the report to indicate the number and types of tubes received, the collection information, the approximate volume of each tube, and an indication as to which tubes were tested.

Expert witness letters will list all references used and contain, at minimum, the HETL case number and Subject's name, when applicable.

Low Volume Samples:
If a blood sample is received by the laboratory with volume not sufficient to perform standard OUI blood drug panel and only a portion of panel can be screened and confirmed for, then the following comment shall be included in the COA: "Unable to perform standard OUI blood drug panel due to low sample volume."

If a case sample is found to have insufficient quantity to extract and analyze as a neat sample, then the customer shall be notified either through a No Exam report or a comment on the Blood Alcohol report stating, "Unable to perform blood drug testing due to low sample volume."

If a No Exam report is being utilized the result field on the report must say "No Exam" and the comment field on the report must say "Unable to perform blood drug testing due to low sample volume."

High Concentration Samples:
If a case sample is found to be above the upper limit of quantitation then a re-extraction with dilution is necessary, testing method and sample volume permitting. If sample volume or testing method does not allow for a dilution or excessively high concentrations of the compound(s) of interest are still above the adjusted upper limit of quantitation, then the case sample shall be resulted out as "Compound detected >(Upper Limit of Quantitation)ng/mL".

Miscellaneous Comments:
Any method deviations will be communicated to the customer on the report in the form of the comment "Deviation occurred: See casefile".

Blood samples that were collected using non-DHHS certified blood collection tubes shall have the following comment included in the COA: "(Color) topped tube used for analysis."

All postmortem samples tested shall have the following comment included in the COA: "Postmortem Specimen: (collection site)." The collection site shall be indicated in the comment section of the COA, if a submitted sample was obtained from multiple sites, then the term "Pooled" shall be used for the collection site.

If a blood sample is received by the laboratory and the matrix does not allow the completion of the standard OUI blood drug panel and only a portion of panel can be screened and/or confirmed for, then the following comment shall be included in the COA: "Unable to perform standard OUI blood drug panel due to sample matrix."

<u>Reinjection</u>
Extracted sample stability studies have been performed for all compounds of interest in the blood drug testing panels, all compounds of interest, with the exception of Norfentanyl, are considered stable extracted in the reconstitution agent for 24 hours. Norfentanyl was found to be stable in the reconstitution agent for 16 hours. Reinjections of samples within this time range shall not require reinjection of the controls but the samples must meet all qualitative/quantitative acceptance criteria to be used to report out any positive results.

- Reinjections made outside of the extracted sample stability time range for quantitative methods must have all associated positive (QCL, QCM, & QCH) and negative controls reinjected prior to reinjecting the case sample. All

reinjected positive and negative controls must meet the respective acceptance criteria, if the controls do not meet the acceptance criteria the case sample must be rejected and re-extracted volume permitting.

- Reinjections made outside of the extracted sample stability time range for Qualitative methods must have the associated Mix (positive control) and negative control reinjected prior to reinjecting the case sample. All reinjected controls must meet the respective acceptance criteria. As the Mix serves as a positive control and a calibrator a new data analysis batch must be created otherwise the software will not properly quantitate the batch. If the controls do not meet the acceptance criteria the case sample must be rejected and re-extracted volume permitting.

Dilution

Each quantitative method has been validated for set dilutions, see Specific Extraction Procedure for each method's approved dilutions. If multiple dilutions or diluted and undiluted are analyzed, the least dilute compound that falls within the quantitation range of the method for that sample is reported. If a diluted and undiluted sample are analyzed any diluted results must be within ±20% of the undilute sample result for any concentrations that are within the quantitative range/adjusted quantitative range to be deemed acceptable. Diluted samples containing a concentration within the adjusted quantitative range that are not within ±20% of the undilute sample result shall be rejected for all compounds. If two diluted sample are analyzed the results within the respective adjusted quantitative ranges must be within 20% of each other for these two diluted samples to be deemed acceptable.

Dilutions shall be noted with the dilution factor in the sample name or written on the generated Masshunter data analysis report.

- Quantitative methods shall have the multiplication/dilution factor put into the sequence table to allow the software to properly quantitate the diluted sample.
- Qualitative methods shall not have the multiplication/dilution factor put into the sequence table as this will impact the surrogate recovery compound calculations in the data analysis methods.

If there is a need for a sample dilution:

- If an undiluted screen sample or confirmation sample indicates the need for a dilution, the sample shall be extracted/re-extracted with a dilution (or undiluted and diluted as needed).
    - Buprenorphine and Norbuprenorphine special consideration: As the screening method's lower limit of detection is greater than the quantitative method's lower limit of detected if the result from the qualitative screen indicates the need for a dilution the sample must be run undiluted and diluted (sample volume permitting).
- Undiluted sample and diluted sample shall be run in the following order in a batch sequence:
    1. Diluted sample
    2. Undiluted sample
    3. Mobile phase blank

Dilutions of case samples shall be performed as needed for confirmation testing by the analyst. Dilutions may need to be performed for, but are not limited to:

- Case samples with low sample volume: If a case sample does not contain enough volume to perform an extraction then a dilution may be performed to allow for extraction.

- Case samples with high levels of compounds of interest or analyst discretion/experience: If a case sample screens high or is found to contain high levels of a compound of interest, then a dilution may be performed.

- Matrix interference: Difference whole blood matrixes may have ion suppressing effects on the compounds of interest and associated internal standard. Ion suppression is indicated by low recovery response of the internal standard and the sample may need to be diluted.

Documentation

Each batch (example: QUALC082819EAF) folder shall contain: (*Each piece of paper within a batch shall contain the analyst initials.)*

- Raw/Summary data from the instrument for all calibrators, mixes, quality controls, and carryover mobile phase blanks
- Calibration Report from the instrument *(when applicable)*
- LC-MSMS Batch Review Form
- Qualifier Ion Ratio Report *(when applicable)*
- Internal Standard Area Report *(when applicable)*
- Extraction Batch Summary Sheet (*when applicable)*
- Instrument Sequence Table
- STARLIMs Blood Drug Screen Sequence
- Blood Bench Sheet Form

Each Sample case file/folder shall contain: (*Each piece of paper within a casefile shall contain the Laboratory Identification Number (Sample #) and analyst initials.)*

- Laboratory Blood Analysis Request Form
- Receipt/Contract for Examination Form/Chain of Custody
- Blood Kit Inventory Worksheet
- Blood Drug Screen Worksheet
- Raw/Summary data from the instrument including reinjections if applicable
- Blood Drug Results Worksheet
- Technical Review Compound Verification Form
- Case Review Form
- Any paperwork/cards included in the blood collection kit as well as any communications regarding the sample

If an entire batch is rejected the batch name shall be recorded each on each impacted sample's Blood Drug Screen/Result Worksheet, any sample data generated by a failed batch shall be included in the casefile with documentation rejecting the data and the reason for batch failure. The failed batch's bench sheet and sequence table as well as any raw batch data with documentation giving the reason for batch failure shall be stored in the batch folder for the failed batch.

Case notes and comments shall be documented in the case file by the analyst. Minor and major deviations shall be authorized by the Supervisor and documented in the case file with a "Deviation Request Form". Any deviations shall be documented within each case sample file in the affected batch and a comment shall be included on the report documenting a deviation from test method SOP.

Each case sample and each calibration and quality control batch shall have a technical and administrative review performed as described in the Quality Manual, these reviews shall be documented on the Case Review form (technical and administrative) and LCMSMS Batch Review form (technical).

Carry Over

Carryover may occur due to extremely high drug concentrations in biological samples and extreme caution is warranted when carryover is detected. When this occurs, the supervisor must be notified to provide guidance and review of the analytical results. All analytical test methods have been validated to establish that at extremely high concentrations of the compounds of interest exhibit no carryover into the following blank matrix samples. However, carryover must be evaluated and confirmed on all samples that exhibit results greater than the specific method/batch ULOQ.

Mobile phase blanks run after these undilute samples shall be analyzed and evaluated for carryover. Each of these mobile phase blanks shall have a comment included on them to indicate what compound(s) the previous sample was >ULOQ for. Examples: “Previous sample contained concentration > ULOQ: Benzoylecgonine.” OR “Previous sample contained no concentrations >ULOQ.”. All mobile phase blanks shall be stored in the batch folder.

- If the resulting mobile phase blank does not exhibit the compound of interest, associated qualifier ion, & area counts at ≤10% of the response from the methods LLOQ then no further action is required.
- If the resulting mobile phase blank does exhibit the compound of interest, associated qualifier ion, and >10% response from the methods LLOQ then the case samples following the >ULOQ concentration sample(s) may require re-extraction and analysis if the following case sample(s) exhibit the >ULOQ compound of interest.

If a case sample is run without a following mobile phase blank and exhibits a result >ULOQ:

- If the case sample immediately following the sample exhibiting the >ULOQ concentration does not exhibit the compound of interest no further action is required.
- If the case sample immediately following the sample exhibiting the >ULOQ concentration does exhibit the compound of interest further action is required. All impacted samples following the >ULOQ sample must be re-extracted, see follow example:

Example Sequence	
Sample 1: **Fentanyl >ULOQ**	Re-extract with dilution
Sample 2: Fentanyl present	Reject sample due to potential carryover contamination and re-extract.
Sample 3: Fentanyl present	Reject sample due to potential carryover contamination and re-extract.
Sample 4: Sample negative for Fentanyl	Sample acceptable, report results.
Sample 5: Fentanyl present	Sample acceptable, no risk of carryover contamination as preceding sample was negative for fentanyl, report results.

6. Specific Extraction Procedures

The following sample preparation setup is to be utilized for all extractions:

- Case samples shall be removed from refrigeration storage, allowed to warm to room temperature and placed on a rocker for a minimum of ten minutes.
- Homogenize the sample and break up any clots, where possible. If homogenized, sample must be placed on a rocker a second time for a minimum of ten minutes.
- When aliquoting working stock into calibrators or controls the tubes shall be segregated from the case samples.
- When aliquoting samples into extraction tube, the tube shall be segregated
- Repeat pipettors must be used when adding Recovery compound stock or Internal standard stock for all extractions.
- Segregation or capping extraction tubes shall be utilized when possible, during the extraction process.
- If a pipet tip/pipet syringe/transfer pipet touches a potentially dirty surface, (side of extraction tube, work counter, etc) it shall be discarded, and a new pipet tip/syringe shall be used.

Qualitative A & B Extraction Procedure:

Table: Mixes A & B Control Levels			
ID	Blood Volume (µL)	Mix A Working Stock (µL)	Mix B Working Stock (100 ng/mL) (µL)
Mix A	500	100	
Mix B	500		150
QC Negative	500		

1. Mixes are prepared as per Table 4: Mix A & B Control Levels in glass tubes.
2. Transfer 500µL of each case sample into a glass tube.
3. Pulse vortex
4. To each case sample and negative control add 200 µL of Methanol
5. Pulse vortex
6. Add 25µL Recovery compound stock (1000 ng/mL) to each tube (Note: the same lot of recovery compound shall be used for all samples in an analytical batch.)
7. Pulse vortex
8. In a fume hood add 2 mL Chloroform to each tube and cap
9. Pulse vortex ~1 minute
10. Centrifuge at high speed for ten minutes
11. In a fume hood remove the supernatant and discard, then remove the chloroform bottom layer and transfer into new labeled glass tubes
12. Dry down at room temp w/ Nitrogen
13. Reconstitute in 200 µL 80:20 mobile phase Water w/0.1% Formic Acid and 5mM Ammonium Formate: Methanol
14. Pulse vortex
15. Transfer to an autosampler vial with insert for analysis

Method Limitations:

- Qualitative A & B can only be analyzed on the Agilent 6470A at this time. The Shimadzu 8030+ has decreased in sensitivity and a method optimization and validation is required for this instrument.
- Qualitative A: The following compounds were found to exhibit statistically significant ionization suppression which could produce a false negative result for a sample.
    - 7-Aminoclonazepam
    - 7-Aminoclonazepam
    - Clonazepam
    - Morphine
    - 6-Acetylmorphine
- Qualitative A: The following compounds were found to exhibit statistically significant ionization enhancement which could produce a false positive preliminary screen result for a sample. These compounds are confirmed using a different method and would never be reported out as positive from only a preliminary screen result.
    - Oxazepam
    - Alpha-Hydroxyalprazolam

- Qualitative B: The following compounds were found to exhibit statistically significant ionization suppression which could produce a false negative result for a sample.
    - Buprenorphine
    - Norbuprenorphine
    - Olanzapine
    - Chlordiazepoxide
    - Carbamazepine
    - Imipramine
    - Desipramine
    - Nortriptyline
    - Fluoxetine
    - Sertraline
- Qualitative B: Buprenorphine and Norbuprenorphine: As the screening method's lower limit of detection is greater than the quantitative method's lower limit of detected if the result from the qualitative screen indicates the need for a dilution the sample shall be run with a dilution as well as undiluted (sample volume permitting)
- Qualitative A is a screening method that exhibits some compounds with irregular chromatograms and presents compounds with shared MRM ion transitions in the following retention time order:
    - Morphine and Hydromorphone have similar retention times and the chromatograms are not fully resolved. Morphine retention time is earlier than Hydromorphone. Since they are not resolved samples are recorded as being preliminary positive for Morphine/Hydromorphone.
    - 351: 3-Methylfentanyl before Butyrylfentanyl
    - 302: Oxymorphone before Dihydrocodeine then Noroxycodone
    - 300: Codeine before Hydrocodone

Qualitative C Extraction Procedure:

Table: Mix C and Negative Control Levels		
	Volume Blood (µL)	Mix C Working Stock (µL)
Mix C	500	25
QC Negative	500	

1. Mixes are prepared as per Table 5: Mix C and Control Levels in glass tubes.
2. Transfer 500µL of each case sample into a glass tube.
3. Pulse vortex
4. Add 25µL Recovery compound stock (1000 ng/mL) to each tube (Note: the same lot of recovery compound shall be used for all samples in an analytical batch.)
5. Pulse vortex
6. Add 500µL HPLC Grade Water to each tube
7. Pulse vortex
8. Add 100 µL 1N HCl to each tube
9. Pulse vortex
10. In a fume hood add 2.5 mL 80:20 Hexane: Ethyl acetate to each tube and cap.
11. Pulse vortex ~1 minute
12. Centrifuge at high speed for ten minutes
13. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
14. Dry down at room temp w/ Nitrogen
15. Reconstitute in 100 µL 50:50 mobile phase Water w/0.1% Formic Acid: Methanol
16. Pulse vortex
17. Transfer to an autosampler vial with insert for analysis

Method Limitations:

- Carboxy-delta-9-THC and delta-9-THC have a partially resolve isomer that has a retention time after the compound of interest, this results in two chromatograms that are partially merged. Carboxy-delta-9-THC and delta-9-THC retention times are earlier than the partially resolved isomer and is the first chromatograph peak.

Cannabinoids Extraction Procedure:

Dilutions: No dilutions shall be performed for cannabinoids testing.

Table:	**Calibration Levels**					
Calibration Level	Target Concentration THC, OH-THC	Target Concentration THC-COOH	Vol 1000/5000 ng/mL Stock	Vol 100/500 ng/mL Stock	Vol 10/50 ng/mL Stock	Blank Whole Blood
	ng/mL	ng/mL	(µL)	(µL)	(µL)	(µL)
1	0.5	2.5			25	500
2	1	5			50	500
3	2	10			100	500
4	5	25		25		500
5	20	100		100		500
6	50	250	25			500
7	80	400	40			500
8	100	500	50			500

Table:	**Quality Control Levels**				
Quality Control Level	Target Concentration THC, OH-THC	Target Concentration THC-COOH	Vol 1000 ng/mL Stock	Vol 100 ng/mL Stock	Blank Whole Blood
	ng/mL	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	5	15		25	500
Medium (QCM)	20	60		100	500
High (QCH)	80	240	40		500
Negative (Neg)	--	--			500

1. Calibrators are prepared as per Table: Calibration Levels in glass tubes.
2. Quality Controls are prepared as per Table: Quality Control Levels in glass tubes.
3. Transfer 500µL of each case sample into a glass tube.
4. Pulse vortex
5. Add 25µL Internal Standard to each tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
6. Pulse vortex
7. Add 500µL HPLC Grade Water to each tube
8. Pulse vortex
9. Add 100 µL 1N HCl to each tube
10. Pulse vortex
11. In a fume hood add 3mL 80:20 Hexane: Ethyl acetate to each tube and cap.
12. Pulse vortex ~1 minute
13. Centrifuge at high speed for ten minutes
14. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
15. Dry down at room temp w/ Nitrogen
16. Reconstitute in 100 µL 50:50 mobile phase Water w/0.1% Formic Acid: Methanol
17. Pulse vortex
18. Transfer to an autosampler vial with insert for analysis

Method Limitations:

- Delta-9-THC and Carboxy-delta-9-THC have a resolved isomer that has a retention time after the compound of interest, this results in two chromatogram peaks within the retention time window. Delta-9-THC and Carboxy-delta-9-THC retention times are earlier than the resolved isomer.
- This method is unable to distinguish between delta-9-THC and delta-9,11-THC.
- No dilutions shall be performed for cannabinoids testing.

Stimulants Extraction Procedure:

Dilutions:

Dilution Factor	Volume of Case Sample	Volume of Water
1:2	100µL	100µL
1:4	50µL	150µL
1:10	20µL	180µL
1:20*	10µL	190µL

**Please note: Methamphetamine cannot be diluted 1:20.*

Table: Calibration Levels					
Calibration Level	Target Concentrations	Blood	Vol Stock A (2000 ng/mL)	Vol Stock B (200 ng/mL)	Vol Stock C (20 ng/mL)
	(ng/mL)	(uL)	(uL)	(uL)	(uL)
1	5	200			50
2	10	200			100
3	20	200		20	
4	50	200		50	
5	100	200		100	
6	200	200	20		
7	400	200	40		
8	500	200	50		

Table: Quality Control Levels				
Quality Control Level	Target Concentration Group 1	Vol QC Stock A (2000ng/mL)	Vol QC Stock B (200ng/mL)	Blank Whole Blood
	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	25		25	200
Medium (QCM)	100		100	200
High (QCH)	300	30		200
Negative (Neg)	--			200

1. Calibrators are prepared as per Table: Calibration Levels using micro centrifuge tubes.
2. Quality Controls are prepared as per Table: Quality Control Levels using micro centrifuge tubes.
3. Transfer 200µL of each case sample into a micro centrifuge tube.
4. Pulse vortex
5. Add 25µL Internal Standard to each micro centrifuge tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
6. Pulse vortex
7. In a fume hood add 1mL of Acetonitrile to each micro centrifuge tube and cap.
8. Pulse vortex ~1 minute
9. Centrifuge at ~8000 rpm for ten minutes.
10. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
11. Dry down at room temperature with Nitrogen
12. Reconstitute in 200 µL 80:20 mobile phase Water w/0.1% Formic Acid 5mM Ammonium Formate: Methanol

13. Pulse vortex
14. Transfer to an autosampler vial with insert for analysis

Method Limitations:

- None

Narcotics Extraction Procedure:

Dilutions:

Dilution Factor	Volume of Case Sample	Volume of Water
1:2*	100µL	100µL
1:4*	50µL	150µL
1:10*	20µL	180µL
1:20*	10µL	190µL

**Please note: Sufentanil shall not be diluted if >ULOQ.*

Table: Calibration Levels

Calibration Level	Target Concentrations (ng/mL)		Blood	Vol Stock A (100/1000 ng/mL)	Vol Stock B (10/100 ng/mL)	Vol Stock C (1/10 ng/mL)
	Group 1	Group 2	(uL)	(uL)	(uL)	(uL)
1	0.2	2	200			40
2	0.5	5	200			100
3	1	10	200		20	
4	2.5	25	200		50	
5	5	50	200		100	
6	25	250	200	50		
7	40	400	200	80		
8	50	500	200	100		

Table:	**Quality Control Levels**				
Quality Control Level	Target Concentration (ng/mL)		Vol Stock A (100-1000 ng/mL)	Vol Stock B (10-100 ng/mL)	Blood
	Group 1	Group 2	(µL)	(µL)	(µL)
Low (QCL)	1	10		20	200
Medium (QCM)	5	50		100	200
High (QCH)	40	400	80		200
Negative (Neg)	--	--			200

1. Calibrators are prepared as per Table: Calibration Levels using micro centrifuge tubes.
2. Quality Controls are prepared as per Table: Quality Control Levels using micro centrifuge tubes.
3. Transfer 200µL of each case sample into a micro centrifuge tube.
4. Pulse vortex
5. Add 25µL Internal Standard to each micro centrifuge tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
6. Pulse vortex
7. In a fume hood add 1mL of Acetonitrile to each micro centrifuge tube and cap.
8. Pulse vortex ~1 minute
9. Centrifuge at ~8000 rpm for ten minutes.
10. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
11. Dry down at room temperature with Nitrogen
12. Reconstitute in 200 µL 80:20 mobile phase Water w/0.1% Formic Acid 5mM Ammonium Formate: Methanol

13. Pulse vortex
14. Transfer to an autosampler vial with insert for analysis

Method Limitations:

None.

Benzodiazepine Extraction Procedure:

Dilutions:

Dilution Factor	Volume of Case Sample	Volume of Water
1:2	100µL	100µL
1:4*	50µL	150µL

**Please note: 7-aminoflunitrazepam cannot be diluted 1:4.*

Table: Calibration Levels					
Calibration Level	Target Concentrations (ng/mL)	Blood	Vol Stock A (400 ng/mL)	Vol Stock B (40 ng/mL)	Vol Stock C (4 ng/mL)
		(uL)	(uL)	(uL)	(uL)
1	2	200			100
2	4	200		20	
3	20	200		100	
4	60	200	30		
5	100	200	50		
6	120	200	60		
7	160	200	80		
8	200	200	100		

Table: Quality Control Levels				
Quality Control Level	Target Concentration	Vol QC Stock A (400ng/mL)	Vol QC Stock B (40ng/mL)	Blank Whole Blood
	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	6		30	200
Medium (QCM)	60	30		200
High (QCH)	160	80		200
Negative (Neg)	--			200

1. Calibrators are prepared as per Table: Calibration Levels using micro centrifuge tubes.
2. Quality Controls are prepared as per Table: Quality Control Levels using micro centrifuge tubes.
3. Transfer 200µL of each case sample into a micro centrifuge tube.
4. Pulse vortex
5. Add 25µL Internal Standard to each micro centrifuge tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
6. Pulse vortex
7. In a fume hood add 1mL of Acetonitrile to each micro centrifuge tube and cap.
8. Pulse vortex ~1 minute
9. Centrifuge at ~8000 rpm for ten minutes.
10. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
11. Dry down at room temperature with Nitrogen
12. Reconstitute in 100 µL 70:30 mobile phase Water w/0.1% Formic Acid 5mM Ammonium Formate: Methanol
13. Pulse vortex

14. Transfer to an autosampler vial with insert for analysis

Method Limitations:

Lorazepam: There is a resolved chromatogram peak that has a retention time after the compound of interest, this results in two chromatogram peaks within the retention time window. Lorazepam retention time is earlier than the resolved isomer. Due to this the acceptance criteria for Lorazepam retention time is +/- 0.1 minutes in comparison to the concurrently run mid-range calibrator used to update the batch.

Buprenorphine Extraction Procedure:

Dilutions:

Dilution Factor	Volume of Case Sample	Volume of Water
1:2	500µL	500µL
1:4	250µL	750µL

Table: Calibration Levels					
Calibration Level	Target Concentrations (ng/mL)	Blood	Vol Stock A (1000 ng/mL)	Vol Stock B (100 ng/mL)	Vol Stock C (10 ng/mL)
		(uL)	(uL)	(uL)	(uL)
1	0.25	1000			25
2	0.5	1000			50
3	1	1000			100
4	5	1000		50	
5	20	1000	20		
6	50	1000	50		
7	80	1000	80		
8	100	1000	100		

Table: Quality Control Levels					
Quality Control Level	Target Concentration	Vol 2000 ng/mL QC Stock A	Vol 200 ng/mL QC Stock B	Vol 20 ng/mL QC Stock C	Blood
	ng/mL	(µL)	(µL)	(µL)	(µL)
Low (QCL)	2			100	1000
Medium (QCM)	20		100		1000
High (QCH)	80	40			1000
Negative (Neg)	--				1000

1. Calibrators are prepared as per Table: Calibration Levels in glass tubes.
2. Quality Controls are prepared as per Table: Quality Control Levels in glass tubes.
3. Transfer 1mL of each case sample into a glass tube.
4. Pulse vortex
5. Add 25µL Internal Standard to each tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
6. Pulse vortex
7. In a fume hood add 3 mL 80:20 Hexane: Ethyl acetate to each tube and cap.
8. Pulse vortex ~1 minute
9. Centrifuge at high speed for ten minutes
10. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
11. Dry down at room temp w/ Nitrogen
12. Reconstitute in 150 µL 80:20 mobile phase Water w/0.1% Formic Acid and 5mM Ammonium Formate: Methanol

13. Pulse vortex
14. Transfer to an autosampler vial with insert for analysis

Method Limitations:

- The Agilent 6470A sheath gas flow is set too fast and the instrument is no longer able to maintain the flow rate to run the buprenorphine method. The buprenorphine testing method shall only be run on the Shimadzu 8030+ until a method optimization and validation can be performed on the Agilent 6470A.
- Buprenorphine and Norbuprenorphine special consideration: As the screening method's lower limit of detection is greater than the quantitative method's lower limit of detected if the result from the qualitative screen indicates the need for a dilution the sample must be run undiluted and diluted (sample volume permitting).

7. Appendix

Chromatogram Integrations

Auto Integration is set up in the instrument data analysis method to have the software correctly integrate most of the peaks. As this auto integration is set to integrate all compounds uniformly and is not tailored to specific compounds there are conditions in which the analyst shall need to use manual integration. Sound scientific principals shall be followed for correct peak integration to ensure that there is uniformity in data analysis.

Each individual chromatogram shall be evaluated in regard to but not limited to poor baseline resolution, chromatogram splitting, rider peaks, co-eluting interferences, misidentified chromatograms, poor chromatogram shape and symmetry, retention time shifts. If improper auto-integration was performed by the computer software as deemed by analyst experience then manual integration shall be utilized.

Each individual chromatogram shall be evaluated in comparison to its corresponding primary or secondary ions. These corresponding ions, when possible, shall exhibit or be given comparable integration.

In the event that manual integration is required to be utilized then the following parameters shall be followed:

Manual integration shall be documented by chromatograms illustrating the integration as a variation of chromatogram shading or an asterisk/manual integration listed on the report.

Compounds within a sample or control shall not be manual integrated improperly to make the peak meet acceptance criteria.

A peak shall never be integrated unreasonably, generally speaking ~10%, below or above the baseline. (See examples of peak shaving or peak enhancing)

All samples and quality controls shall be integrated in the same manner.

All compound ions within a sample shall be, when possible, integrated in a comparable manner.

All un-integrated batches shall be available for review in the data analysis software program.

The following illustrates commonly seen chromatography, suggested integrations, and some possible causes of poor chromatography:

Figure 1: Properly integrated single peak.

The peak is symmetrically shaped and exhibits no indication of coelution, the baseline is flat and exhibits baseline to baseline integration that is normally integrated automatically by the software.

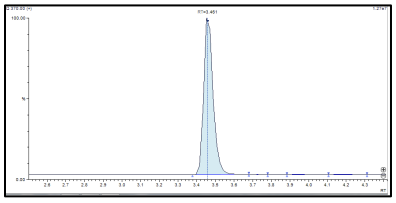


Figure 2: Properly integrated coeluting pea & Properly integrated co-eluting peak with a rising baseline.

Proper integration of two peaks that are not completely resolved, meaning that the response does not return to the baseline between the two peaks. The lowest point between two peaks is the appropriate integration end point.

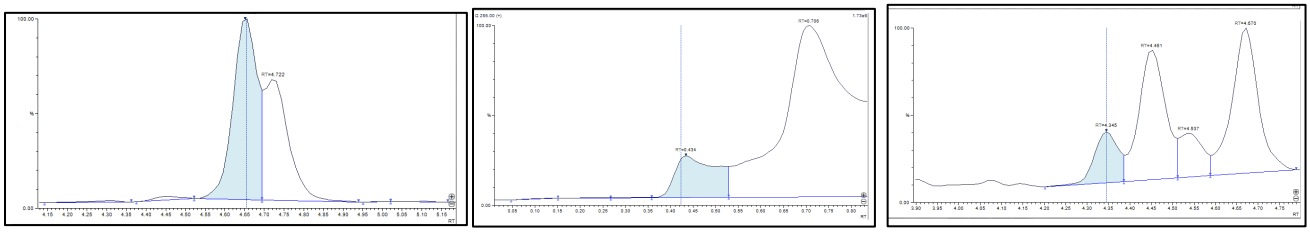


Figure 3: Uniform integrations.

These peaks exhibit slight interferences just prior to the target peak. These interfering peaks are not resolved and may be included in the automatic integration as shown in Figure 3. Overall this entire grouping would not be considered acceptable since the integration for this compound of interest was blatantly not uniform for all calibrators, quality controls, and case samples.

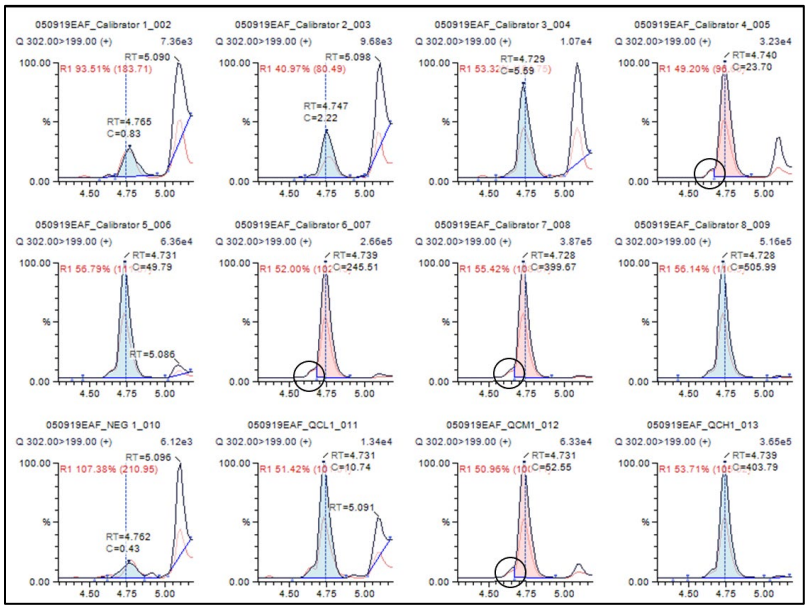


Figure 4: Baseline Noise Example.

This is an example of baseline noise as there are no definite peaks that distinguish themselves from the baseline and the 'peak' at the expected retention time has a signal to noise ratio of <3.

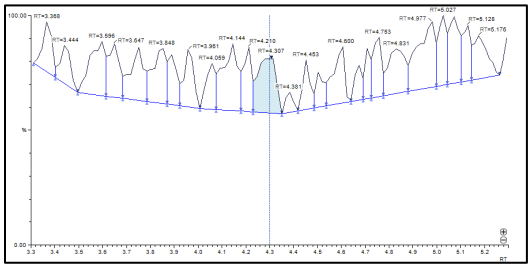


Figure 5: Peak Fronting

This is usually caused by an overloading of the column, HETL has also specifically seen this phenomenon when the reconstitution reagent concentration ratio has been swapped (please refer to durability studies within specific tests validations)

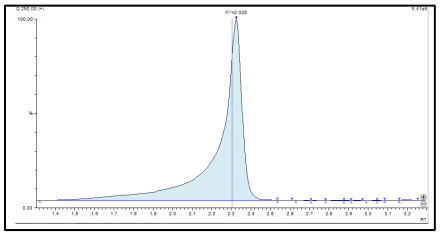


Figure 6: Peak Tailing

This is a limited example of peak tailing and could be caused by a number of factors including but not limited to: old mobile phases, old column guard cartridge, old column, overloading of the column, interfering coelutions. If the issue is gross and persistent troubleshooting of the instrument may be required.

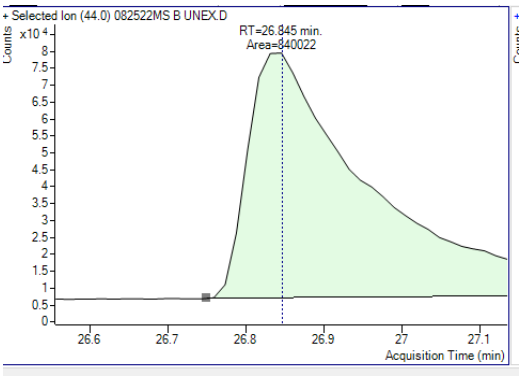


Figure 7: Improper Peak Shaving

Shaving is the exclusion of a large area of the peak, this includes: grossly elevating the baseline so that the integration runs from peak side to peak side as opposed to baseline to baseline or the eliminating the leading and tailing edges of the peak.

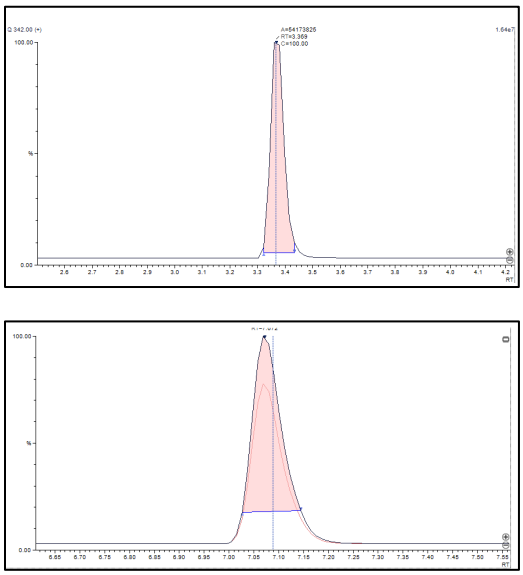
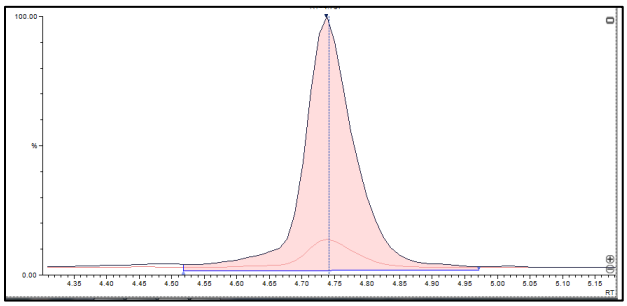


Figure 8: Improper Peak Enhancing

Enhancing is the integration of a large area that is not the target analyte peak, the following exhibits an improper peak enhancement by integration including a large amount below the baseline.



LCMSMS Data Acquisition Methods

The full data acquisition and data analysis method parameters will be printed out and placed in a method binder close to the instrument.

All Methods utilize the following parameters:

- Shimadzu 8030 Tandem Mass Spectrometer and LC system, Agilent 6470A Tandem Mass Spectrometer and LC system (or equivalent)
- LC oven 40ºC

Qualitative ABC: Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Phenomenex C18
- LC Column- Phenomenex Kinetex C-18 2.6 um 50 mm, 2.1 ID
- Qualitative A & B Flow rate 0.5 mL/Min
- Qualitative C Flow Rate Shimadzu 0.4mL/Min, Agilent 0.5mL/Min
- Screen A & B Mobile phase A: Water with 0.1% Formic Acid and 5mM Ammonium Formate
- Screen C Mobile Phase A: Water with 0.1% Formic Acid
- Mobile Phase B: Methanol
- Injection volume 20 uL

Table: Qualitative A LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid and 5mM Ammonium Formate	% B: Methanol
0.01	95	5
0.01	To MS	To MS
9.00	5	95
12.00	To Waste	To Waste
12.00	5	95
12.01	95	5
14.00	Stop	Stop

Table: Qualitative B LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid and 5mM Ammonium Formate	% B: Methanol
0.01	95	5
0.01	To MS	To MS
9.00	5	95
10.00	To Waste	To Waste
10.00	5	95
10.01	95	5
12.00	Stop	Stop

Table: Qualitative C LC Pump Gradient

Time	% A:Water with 0.1% Formic Acid	% B:Methanol
0.01	50	50
0.10	To MS	To MS
4.50	5	95
5.25	5	95
5.26	50	50
5.80	To Waste	To Waste
8.00	Stop	Stop

Table: Instrument Parameters for Target Analytes Qualitative A

Target Analyte	Precursor Ion (*m/z*)	Product Ion
Morphine	286	165
		152
Hydromorphone	286	185
		157
Oxymorphone	302	284
		227
Norfentanyl	233	84
Oxazepam	287	241
		104
Cocaine	304	
Benzoylecgonine	290	
Amphetamine	136	
Methamphetamine	150	
MDMA	194	
PCP	244	159
		91
Ketamine	238	
Norketamine	224	
Cocaethylene	318	
Methylphenidate	234	
Temazepam	301	
Nordiazepam	271	
Alprazolam	309	
Diazepam	285	
Etizolam	343	
Clonazepam	316	
Zolpidem	308	
7-amino flunitrazepam	284	
Flunitrazepam	314	
Alpha-Hydroxyalprazolam	325	
7-amino clonazepam	286	
lorazepam	321	
3-methylfentanyl	351	202
		105
6-Acetylcodeine	342	
6-acetylmorphine	328	

Butyrylfentanyl HCL	351	188
		105
Codeine	300	
EDDP	278	
Fentanyl	337	
Heroin	370	
Hydrocodone	300	
Methadone	310	
Norhydrocodone	286	
Noroxycodone	302	
O-Desmethyltramadol	250	
Oxycodone	316	
Sufentanil	387	
Tramadol	264	
Trazodone	372	
Meprobamate	219	
Zolpidem-d6	314	235

Table: Instrument Parameters for Target Analytes Qualitative B

Target Analyte	Precursor Ion (*m/z*)	Product Ion
Clonidine	230	213
		124
Norcodeine	286	165
		153
Olanzapine	313	256
		198
Zopiclone	389	245
		217
Lamotrigine	256	211
		58
Mirtazapine	266	195
		72
Meperidine	248	70
		91
LSD	324	223
		208
Risperidone	411	191
		110

Venlafaxine	278	58
		121
Norbuprenorphine	414	101
		187
Diphenhydramine	256	167
		152
Dextromethorphan	272	215
		171
Citalopram	325	109
		262
Doxepin	280	107
		115
Chlordiazepoxide-	300	227
		165
Buprenorphine	468	396
		101
Carbamazepine	237	194
		220
Imipramine	281	86
		208
Propoxyphene	340	58
		143
Cyclobenzaprine	276	215
		216
Desipramine	267	72
		193
Amitriptyline	278	91
		233
Nortriptyline	264	233
		117
Fluoxetine	310	44
		148
Carisoprodol	261	176
		97
Zolpidem-D6	314	235
		314
Triazolam	343	308
		239
Sertraline	306	159
		275

Table: Instrument Parameters for Target Analytes Qualitative C

Target Analyte	Precursor Ion (*m/z*)	Product Ion
Δ9-THC	315	193
		123
OH- Δ9-THC	331	313
		193
COOH-Δ9-THC	345	299
		327
Reserpine	609	195

Quantitative Cannabinoids Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Phenomenex C-18
- LC Column- Phenomenex Kinetex C-18 2.6 um 50 mm, 2.1 ID
- Flow rate 0.5 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid
- Mobile Phase B: Methanol
- Injection volume 25 uL

Table: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid	% B: Methanol
0	40	60
1	To MS	To MS
2	40	60
6	30	70
10	30	70
11	5	95
11.5	5	95
12	40	60
12.5	To Waste	To Waste

Table: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion		Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Δ9-THC	315	Quant	193	Δ9-THC-d3	318	Quant	196
		Qualifier	123			Qualifier	123
OH-Δ9-THC	331	Quant	193	OH- Δ9-THC -d3	334	Quant	316
		Qualifier	313			Qualifier	196
COOH-Δ9-THC	345	Quant	299	COOH-Δ9-THC-d3	348	Quant	302
		Qualifier	193			Qualifier	330

Stimulants Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Restek Raptor Biphenyl
- LC Column- Restek Raptor Biphenyl 2.7µm 100x2.1mm
- Flow rate 0.6 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 10 uL

Table: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid 5mM Ammonium Formate	% B: Methanol
0.01	90	10
0.25	To MS	To MS
9.00	5	95
9.01	To Waste	To Waste
12.00	5	95
12.01	90	10
13.00	Stop	Stop

Table: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion		Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Amphetamine	136	Quant	91	Amphetamine-d8	144	Quant	97
		Qualifier	119			Qualifier	127
Methamphetamine	150	Quant	91	Methamphetamine-d5	155	Quant	92
		Qualifier	119			Qualifier	121
MDMA	194	Quant	163	MDMA-d5	199	Quant	165
		Qualifier	105			Qualifier	135
Norketamine	224	Quant	207	Norketamine-d4	228	Quant	129
		Qualifier	125			Qualifier	211
Ketamine	238	Quant	125	Ketamine-d4	242	Quant	129
		Qualifier	207			Qualifier	224
Cocaethylene	318	Quant	82	Cocaine-d3	307	Quant	185
		Qualifier	196			Qualifier	85
Methylphenidate	234	Quant	84	Methylphenidate-d9	243	Quant	93
		Qualifier	56			Qualifier	243
Cocaine	304	Quant	182	Cocaine-d3	307	Quant	185
		Qualifier	82			Qualifier	85
Benzoylecgonine	290	Quant	168	Benzoylecgonine-d8	298	Quant	171
		Qualifier	105			Qualifier	298
PCP	244	Quant	159	PCP-d5	249	Quant	96
		Qualifier	91			Qualifier	164

Narcotics Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Restek Raptor Biphenyl
- LC Column- Restek Raptor Biphenyl 2.7µm 100x2.1mm
- Flow rate 0.6 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 20 uL

Table: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid 5mM Ammonium Formate	% B: Methanol
0.01	95	5
1.00	95	5
2.00	To MS	To MS
9.00	5	95
9.50	5	95
9.51	95	5
10.00	To Waste	To Waste
11.00	Stop	Stop

Table: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion		Internal Standard	Precursor Ion (*m/z*)	Product Ion	
6-Acetylcodeine	342	Quant	225	Codeine-d3	303	Quant	165
		Qualifier	165			Qualifier	215
6-acetylmorphine	328	Quant	165	6-acetylmorphine-d3	331	Quant	165
		Qualifier	211			Qualifier	331
Codeine	300	Quant	215	Codeine-d3	303	Quant	165
		Qualifier	165			Qualifier	215
EDDP	278	Quant	234	EDDP-d3	281	Quant	234
		Qualifier	249			Qualifier	281
Fentanyl	337	Quant	105	Fentanyl-d5	342	Quant	105
		Qualifier	188			Qualifier	188
Heroin	370	Quant	165	Heroin-d9	379	Quant	272
		Qualifier	268			Qualifier	335

Hydrocodone	300	Quant	199	Hydrocodone-d3	303	Quant	199
		Qualifier	171			Qualifier	171
Hydromorphone	286	Quant	185	Hydromorphone-d3	289	Quant	185
		Qualifier	157			Qualifier	157
Methadone	310	Quant	265	Methadone-d3	313	Quant	268
		Qualifier	105			Qualifier	105
Morphine	286	Quant	152	Morphine-d3	289	Quant	152
		Qualifier	165			Qualifier	289
Norfentanyl	233	Quant	84	Fentanyl-d5	342	Quant	105
		Qualifier	55			Qualifier	188
Norhydrocodone	286	Quant	199	Norhydrocodone-d3	289	Quant	202
		Qualifier	128			Qualifier	152
Noroxycodone	302	Quant	187	Noroxycodone-d3	305	Quant	190
		Qualifier	227			Qualifier	305
O-Desmethyltramadol	250	Quant	58	O-Desmethyltramadol-d6	256	Quant	64
		Qualifier	250			Qualifier	256
Oxycodone	316	Quant	298	Oxycodone-d3	319	Quant	301
		Qualifier	241			Qualifier	244
Oxymorphone	302	Quant	284	Oxymorphone-d3	305	Quant	230
		Qualifier	227			Qualifier	287
Sufentanil	387	Quant	238	Sufentanil-d5	392	Quant	238
		Qualifier	111			Qualifier	392
Tramadol	264	Quant	58	Tramadol-d3	268	Quant	58
		Qualifier	264			Qualifier	268
Trazodone	372	Quant	176	Trazodone-d6	378	Quant	150
		Qualifier	148			Qualifier	182
Meprobamate	219	Quant	158	Meprobamate-d3	222	Quant	161
		Qualifier	97			Qualifier	222

Benzodiazepine Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Restek Raptor Biphenyl
- LC Column- Restek Raptor Biphenyl 2.7µm 100x2.1mm
- Flow rate 0.6 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 20 uL
- LC oven 40ºC

Table: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid 5mM Ammonium Formate	% B: Methanol
0.01	70	30
1.00	70	30
1.00	To MS	To MS
4.00	35	65
5.00	35	65
9.00	5	95
9.01	To Waste	To Waste
9.50	5	95
9.51	70	30
11.00	Stop	Stop

Table: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion		Internal Standard	Precursor Ion (*m/z*)	Product Ion	
7-Aminoclonazepam	286	Quant	222	Clonazepam-d4	320	Quant	274
		Qualifier	121			Qualifier	320
Zolpidem	308	Quant	235	Zolpidem-d6	314	Quant	235
		Qualifier	263			Qualifier	314
7-Aminoflunitrazepam	284	Quant	135	7-Aminoflunitrazepam-d7	291	Quant	230
		Qualifier	227			Qualifier	291
Lorazepam	321	Quant	275	Lorazepam-d4	325	Quant	279
		Qualifier	321			Qualifier	325
α-hydroxyalprazolam	325	Quant	297	Alprazolam-d5	314	Quant	286
		Qualifier	216			Qualifier	314
Clonazepam	316	Quant	270	Clonazepam-d4	320	Quant	274
		Qualifier	214			Qualifier	320

Oxazepam	287	Quant	241	Oxazepam-d5	292	Quant	246
		Qualifier	104			Qualifier	292
Alprazolam	309	Quant	281	Alprazolam-d5	314	Quant	286
		Qualifier	205			Qualifier	314
Flunitrazepam	314	Quant	268	Flunitrazepam-d7	322	Quant	276
		Qualifier	239			Qualifier	322
Nordiazepam	271	Quant	140	Nordiazepam-d5	276	Quant	140
		Qualifier	165			Qualifier	276
Temazepam	301	Quant	255	Temazepam-d5	306	Quant	260
		Qualifier	177			Qualifier	306
Diazepam	285	Quant	154	Diazepam-d5	290	Quant	154
		Qualifier	193			Qualifier	290
Etizolam	343	Quant	314	Etizolam-d3	346	Quant	317
		Qualifier	259			Qualifier	346

Buprenorphine Instrumentation and Data Acquisition Parameters:

- LC Guard Cartridge – Raptor Biphenyl 2.7um 5x3.0mm
- LC Column- Raptor Biphenyl 2.7um 100x2.1mm
- Flow rate 0.5 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid with 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 50 uL

Table: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid and 5mM Ammonium Formate	% B: Methanol
0	60	40
1.0	To MS	To MS
1.9	60	40
2.0	45	55
5.0	45	55
9.0	5	95
9.5	5	95
9.75	60	40
10.0	To Waste	To Waste
11.0	Stop	Stop

Table: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion		Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Buprenorphine	468	Quant	101	Buprenorphine-d4	472	Quant	415
		Qualifier	396			Qualifier	240
Norbuprenorphine	414	Quant	187	Norbuprenorphine-d3	417	Quant	343
		Qualifier	101			Qualifier	83

Data Analysis Methods:

All Qualitative methods shall use the following data analysis parameters:

- Retention Time Setup: Criteria-Close RT
- Compound Calibration/Positive Control setup:

Mix	Level 1

- Compound Concentration Setup: CF-Linear, CF-Origin Force, CF Weight 1/x.
- Compound Qualifier and Mass Extraction Setup: Uncertainty-Relative, 30.
- Compound Noise Setup: Noise Alg. ASTM, Noise SD Multiplier. 1.0 (Agilent only)
- Compound Integration Parameters: Integrator-Agile2 (Agilent Only)
- Outlier Setup:
    - Retention Time: RT Window-0.4, RT Units-Minutes (Except for Lorazepam: RT Window-0.2)
    - Signal-to-Noise Ratio: Min S/N-3.3

All Quantitative methods shall use the following data analysis parameters:

- Retention Time Setup: Criteria-Close RT
- Compound Calibration/Positive Control setup:

Calibrator 1	Level 1
Calibrator 2	Level 2
Calibrator 3	Level 3
Calibrator 4	Level 4
Calibrator 5	Level 5
Calibrator 6	Level 6
Calibrator 7	Level 7
Calibrator 8	Level 8
QCL	Level 9
QCM	Level 10
QCH	Level 11

- Compound Concentration Setup: CF-Linear, CF-Origin Ignore, CF Weight 1/x.
- Compound Qualifier and Mass Extraction Setup: Uncertainty-Relative, 30.
- Compound Noise Setup: Noise Alg. ASTM, Noise SD Multiplier. 1.0 (Agilent only)
- Compound Integration Parameters: Integrator-Agile2 (Agilent Only)
- Outlier Setup:
    - Retention Time: RT Window-0.4, RT Units-Minutes (Except for Lorazepam: RT Window-0.2)
    - Signal-to-Noise Ratio: Min S/N-3.3

LCMSMS Instrument Maintenance & Use

This document describes instrument maintenance protocols for the daily prior to analyzing samples maintenance and the as needed maintenance for LC-MS/MS methodology. All daily and as needed maintenance shall be documented on the LCMSMS Shimadzu Maintenance Log or LCMSMS Agilent Maintenance Log, any additional maintenance shall be recorded in the maintenance notes section of the respective Maintenance Log with the date performed and analysts' initials.

Daily Maintenance:

Daily Maintenance shall be performed daily prior to sample analysis; if instrument is not used for sample analysis on a particular day then Daily Maintenance shall not be performed for that day. When Daily Maintenance is performed it shall be recorded on the Maintenance Log.

- **Mobile Phase Levels:** Mobile phase levels shall be checked to confirm that there is adequate volume to run the intended samples.
- **Check Rinse Line:** The Agilent autosampler rinse line is a small diameter line that draws from the Mobile Phase B, this line shall be checked to ensure that the end in submerged in the bottle.
- **Cleaning Electrospray Chamber& Shield-Daily:** The daily cleaning of the electrospray chamber and shield shall be performed prior to activating the MS to maintain optimal instrument performance.
    - Confirm that the MS is not activated.
    - Rinse interior of electrospray chamber with 50:50 HPLC grade 2-propanol: HPLC grade water.
    - Wipe interior of electrospray chamber with a clean Kimwipe taking care to avoid the nebulizing needle.
    - Rinse area around the spray shield with 50:50 HPLC grade 2-propanol: HPLC grade water. (Do not spray directly toward the end of the capillary. This can cause pressure surges in the vacuum system).
    - Wipe area around the spray shield with a clean Kimwipe taking care to avoid the nebulizing needle.
    - Rinse nebulizing chamber door and wipe with a clean Kimwipe.
    - Reattach door to chamber and close chamber.
- **Gas Level Check:** The Shimadzu Argon gas level shall be checked to ensure that a level of 70-110 psi is being maintained, if 70-110 psi is not being maintained then remedial action such as adjusting the regulator or changing the tank shall be performed. The Agilent Nitrogen gas level shall be checked to ensure that a level of ~20 psi is being maintained, if ~20 psi is not being maintained then remedial action such as adjusting the regulator or changing the tank shall be performed.
- **Nitrogen Generator Purity Check:** The Shimadzu Nitrogen generator purity shall be checked to ensure that a level of ≥99.7% is being maintained, if it is not being maintained then remedial action shall be taken such as Nitrogen generator maintenance. The Agilent Nitrogen generator purity shall be checked to ensure that a level of >95% is being maintained, if it is not being maintained then remedial action shall be taken such as Nitrogen generator maintenance.
- **Pump Level Check:** The pump pressure shall be recorded on the Maintenance Log to be monitored since pump pressure is an indicator of the condition of the LC system.
- **IG Vacuum Level & High Vacuum Level:** The Shimadzu IG Vacuum level shall be checked to confirm that it is maintaining a vacuum of ~3x10^-3 when the MS is on, if the MS is not maintaining a vacuum of ~3x10^-3 then a Shimadzu service engineer shall be called to troubleshoot the issue. The Agilent High Vacuum level shall be checked to confirm that it is maintaining a vacuum of 2.0-5.0x10^-5 when the MS is on, if the MS is not maintaining a vacuum of 2.0-5.0x10^-5 then a Agilent service engineer shall be called to troubleshoot the issue.
- **Check for Leaks:** Once the LC pump has been turned on the operator shall use a clean dry Kimwipe to check the jointures of the lines and capillary lines in the LC stack to ascertain that there are no slow leaks that could affect instrument performance.

- **Check Waste Level:** The instrument waste level bottle shall be checked and once the bottle is approximately half filled with waste then the bottle shall be emptied into the appropriate hazardous waste disposal container.
- **Checktune** Performed to check that optimal instrument detection performance is being maintained and shall be performed daily prior to sample analysis. If the Checktune does not meet parameters then action is required.
  Shimadzu: Shimazu does not have any pre-set/provided acceptance criteria for their LC-MS/MS instrument, historical review during the initial validations found that monitoring the Q1 and Q3 Scan positive ions allowed for optimal instrument monitoring. Acceptance criteria consist of Q1 and Q3 Scan positive ion 168.10, 256.15 and ion 300.20 having an intensity of >50% of the respective intensities from the most recent Autotune.
    - Confirm Nitrogen generator is on and activate MS.
    - Attach red end of tuning capillary to bottle of Tuning Solution, both located in tuning bottle chamber.
    - Remove capillary that attaches the LC to the MS and replace with the beige end of the tuning capillary.
    - In LabSolutions Data Acquisition Main Tab select Tuning to open the tuning menu.
    - Select Manual Tune and then the Standard Sample button this will start the flow of Tuning Solution, once peaks become visible and show acceptable chromatography select Tuning Results and select “No” to saving this file this will bring you back to the Autotune menu.
    - Once you are back in the Autotune menu select Autotuning Conditions and deselect all parameters and select OK, this ensures that none of the parameter will be changed only checked.
    - Select AutoTuning Start (and confirm that positive mode (+) is selected, this will start the Checktune. Upon completion print the Checktune file, labeling the document as a Checktune and return to the main data acquisition window in Labsolutions and select “No” to saving this file as the Checktune is automatically printed upon completion.
    - Remove Tune Solution bottle and replace with bottle of methanol.
    - Select Manual Tune and then the Standard Sample button this will start the flow of the methanol, once peaks disappear the tuning capillary has been flushed and is ready for storage. Select Tuning Results and select “No” to saving this file this will bring you back to the Autotune menu.
    - Disconnect the tuning capillary and place into storage in the Tune Solution bottle chamber.
    - Reconnect the LC flow capillary to the MS.

  Checktune Agilent: Acceptance criteria consist of passing of the following: Positive Results MS1, MS2 (unit, wide, & widest) Expected m/z: 118, 322, 622. It is noted that the tune does check larger m/z and negative results, these are not evaluated as they are not representative of the compounds that we test for.
    - Activate MS.
    - In MassHunter Data Acquisition Context select Tune. This shall change the window display to tuning.
    - Select Autotune tab and then select Checktune, this will start the Checktune. Upon completion print the Checktune file.
    - Once a successful Checktune has been performed in MassHunter Data Acquisition Context select Acquisition. This shall change the window display to Acquisition.

Bi-Weekly Maintenance: Bi-Weekly Maintenance shall be performed as defined as a minimum of once every two weeks. When Bi-Weekly Maintenance is performed it shall be recorded on the Maintenance Log.

- **Ballast Pump:** The Shimadzu ballast pump requires draining on a bi-weekly basis as defined as a minimum of every two weeks. If oil level is low post ballast pump drain or oil looks discolored pump maintenance shall be performed as per manufacturer’s recommendation. The Agilent pump is self-ballasting and does not need to be manually ballasted.

As Needed Maintenance: As Needed maintenance shall be performed on an as needed basis as indicated by instrument performance, frequency of instrument use, or by visual inspection by the operator.

- **Cleaning Electrospray Chamber/Shield-As Needed**: The As Needed cleaning of the Electrospray Chamber and Shield shall be performed as needed when indicated by visual inspection of the Electrospray Chamber and Shield by the operator. This maintenance is performed on the MS to maintain optimal instrument performance.
    - Confirm that the MS is not activated.
    - Rinse interior of electrospray chamber with 50:50 HPLC grade 2-propanol: HPLC grade water.
    - Wipe interior of electrospray chamber with a clean Kimwipe.
    - Remove spray shield.
    - Gently clean the spray shield with 6000 and 8000 abrasive paper moistened with 50:50 HPLC grade 2-propanol: HPLC grade water.
    - Rinse spray shield with 50:50 HPLC grade 2-propanol: HPLC grade water and wipe with a clean Kimwipe.
    - Replace spray shield.
    - Rinse area around the spray shield with 50:50 HPLC grade 2-propanol: HPLC grade water (Do not spray directly toward the end of the capillary. This can cause pressure surges in the vacuum system) and wipe area around the spray shield with a clean Kimwipe.
    - Rinse nebulizing chamber door with 50:50 HPLC grade 2-propanol: HPLC grade water and wipe with a clean Kimwipe.
    - Close chamber.
- **Change Column Guard Filter:** The column guard filter shall be changed on an as needed basis dependent upon instrument performance and frequency of instrument use at the discretion of the operator. This is performed to protect the integrity and longevity of the LC column.
    - Confirm that the LC pumps are off.
    - Unscrew the line connecting the column module to the column guard.
    - Unscrew the column guard from the column.
    - Using the appropriate wrenches unscrew the column guard and remove the filter.
    - Place a new filter in the column guard.
    - Using the appropriate wrenches screw the two pieces of the column guard back together.
    - Screw the column guard back into the column.
    - Screw the line connecting the column guard to the column module back in.
    - Turn the LC pumps on and using a clean dry Kimwipe confirm that there are no leaks at any of the jointures in the column module.
- **Autotune** Performed as needed such as if a Checktune shows marked decreased signal or following extensive instrument maintenance.

  Shimadzu:
    - Confirm Nitrogen generator is on and activate MS.
    - Attach red end of tuning capillary to bottle of Tuning Solution, both located in tuning bottle chamber.
    - Remove capillary that attaches the LC to the MS and replace with the beige end of the tuning capillary.
    - Select Manual Tune and then the Standard Sample button this will start the flow of Tuning Solution and peaks should become visible, once peaks become visible and show acceptable chromatography select Tuning Results and select “No” to saving the tune file at this time.
    - In Labsolutions Data Acquisition File drop down tab select New Tune File and then save as “AutotuneDate” Example: Autotune061720
    - In LabSolutions Data Acquisition Main Tab select Autotuning Conditions to confirm that “positive tune only” is selected and all remaining parameters are selected.
    - Once you are back in the Autotune window select Autotuning Start (and confirm positive mode (+) is selected) this will start the Autotune, this runs for ~20-30 minutes. Upon completion save and print the tune file.

- Remove Tune Solution bottle and replace with bottle of methanol.
- Select Manual Tune and then the Standard Sample button this will start the flow of the methanol, once peaks disappear the tuning capillary has been flushed and is ready for storage. Select Tuning Results and select "No" to saving this file this will bring you back to the Autotune menu.
- Disconnect the tuning capillary and place into storage in the Tune Solution bottle chamber.
- Reconnect the LC flow capillary to the MS.

- **Autotune Agilent:**
  - Activate MS.
  - In MassHunter Data Acquisition Context select Tune. This shall change the window display to tuning.
  - Select Autotune tab and then select Autotune, this will start the Autotune and automatic Checktune. Upon completion print the Autotune and Checktune file.
  - Once a successful Autotune has been performed in MassHunter Data Acquisition Context select Acquisition. This shall change the window display to Acquisition.

Generator Test: As part of the monthly generator test all instruments and equipment in the laboratory are shut down and unplugged. Prior to the generator test the LC-MS/MS must be vented, shut down, and unplugged from all outlets the following steps are the manufacturers recommended procedure for this process.

- **Venting & Shutting Down: Shimadzu 8030 LCMSMS**
  - Confirm that the LC stack and MS are shut down.
  - In LabSolutions 'Main' left tab select 'System Control' then 'Advanced' options.
  - Close CID valve.
  - Place DL plug into DL hole in electrospray chamber (DL plug is stored in tune bottle chamber & has the appearance of an Allen wrench).
  - Stop Turbo pump, allow instrument to sit for one hour.
  - Stop Rotary pump
  - Open Vent valve.
  - In LabSolutions 'File' tab select 'Exit'
  - Once all computer windows are closed shut down computer switch LC stack and MS off (MS switch located on back of instrument) and unplug everything.
- **Venting & Shutting Down: Agilent 6470A LCMSMS**
  - Confirm that the LC stack and MS are shut down.
  - In Masshunter Data Acquisition right click on the MS and select Vent instrument. A message window will open once the instrument is ready to be shut off.
  - Once the message window appears exit Mass Hunter Data Acquisition and any other open software.
  - Once all computer windows are closed shut down computer switch LC stack and MS off and unplug everything.
- **Starting the MS from "cold": Shimadzu 8030 LCMSMS**
  - Plug all equipment in, turn on computer and switch LC stack, Nitrogen generator, and MS on.
  - Confirm DL plug is in DL hole in electrospray chamber.
  - In LabSolutions 'Main' left tab select 'System Control' then 'Advanced' options.
  - Close Vent valve.
  - Start Rotary pump
  - Once the PG vacuum has reached x10^1 start the Turbo pump, the light next to the start button will stop flashing when the Turbo pump is up to the correct speed.
  - Once the Turbo pump light has stopped flashing and is a solid green open the CID valve and remove the DL plug.

- **Starting the MS from "cold": Agilent 6470A LCMSMS**
    - Plug all equipment in, turn on computer and switch LC stack, Nitrogen generator, and MS on. Note: Nitrogen generator needs to be on and running prior to turning MS on.

Shimadzu LC-MS/MS 8030– Suggested guidance in the following tasks.

- **Starting a sequence from a template:**
    - Open Labsolutions go to Main tab / Real time Batch
    - File, new batch, select your template
    - Change: Sample names, Sample IDs, Sample type (standard, control, unknown), Level #s, Dilution factor (if any), comments
    - Check: method file, vial #s (you can right click fill down or fill in series)
    - Add lines if needed (right-click, add line- it will prompt for how many)
    - Delete any extra lines (highlight, right-click delete)
    - Batch settings (found on top drop down or from right clicking)- change folder to today's date with the appropriate test panel. If you do not want to shut down after this batch then go to shutdown tab and deselect the "shutdown" button. When you leave settings, the software will prompt you to create the new folder for the new folder for this date.
    - File- save batch as todays date under the new folder for the date using the format: TestDateInitials
    - Start Real time Batch
    - Edit batch while running- under real time batch hit "edit batch/restart" button, do editing than make sure to hit button again to restart.
    - You can watch the MS data file in Realtime by clicking on Acquisition/Instrument Parameters and the MS tab. Click on instrument parameters again to return.
- **Data Analysis**
    - Open Insight software and enter username and password
    - File/open and select your batch files. All files associated with the batch will be opened. You can sort by clicking the top column heading.
    - Then File/Save as and name the LCMS method file (*.lcm) and DAML project file (*.damlp) with the analysis panel code + the analysis date + analyst initials. For example: NAR050119NMI.
    - Start with the calibration or mix sample. Review each peak and update retention times and ion ratios using a mid-level calibrator for each compound (right click each compound and select: Update- RT & ion ratio then click on the apex of the peak).
    - Analyze each data file and each compound.
    - Edit a peak- right click on the chromatogram
        - Manual identification and click on the correct integrated peak if the peak was misidentified.
        - Manual identification-horizontal to draw from baseline to baseline.
        - Manual integration-new baseline to draw a new baseline or horizontal to use existing baseline.
    - Review/Accept- This will accept each compound as complete
    - File/ Save again
    - Report/ HETL Batch report for all checked files and HETL sample report for a single file. The software will pull up the report for review before printing.

Agilent LC-MS/MS 6470A– Suggested guidance in the following tasks.

- **Starting a sequence from a template:**
    - Open Masshunter Data Acquisition go to Worklist tab

- New batch, select your template
- Add sampl: Sample names, Dilution factor (if any), and data acquisition file names (you can right click fill in series)
- Check: method file, vial #s
- Add lines if needed (right-click, add/insert sample)
- Delete any extra lines (highlight, right-click delete)
- Save As: batch as todays date under the new folder for the date using the format: TestDateInitials
- Print sequence
- Start run

- **Data Analysis**
  - Open MassHunter QQQ Quantitative Analysis software.
  - File/new batch and select your run folder and name batch file using the format: TestDateInitials. Select create and a Add Samples window will appear-select and add samples (note: samples that are in the process of acquisition cannot be added, the acquisition process must be completed for a sample to be added to a batch)
  - Edit a peak- left click on the integrated peak and drag.
  - Update retention times- on a middle calibration data point, Update/Update Retention times and select all/desired compounds to update
  - Analyze batch.
  - Update ion ratios- on a middle calibration data point, Update/Update Qualifier Ratios and select all/desired compounds to update
  - Start with the calibration or mixes analysis. Review each peak and update retention times and ion ratios using a mid-level calibrator.
  - Analyze each data file and each compound.
  - File/ Save.
  - Generate Report- Report/Generate-use Cal and Batch Reporting Template for quantitative methods and Batch Reporting Template for qualitative methods

Blood Drug Testing Menu & Reporting Limits

Blood Drug Testing Menu & Lower Reporting Limits	**Qualitative Tests**	**Quantitative Tests**
Analyte	**Lower Reporting Limit (ng/mL)**	**Lower Reporting Limit (ng/mL)**
Benzodiazepine Panel		
7-amino flunitrazepam	4*	4
7-aminoclonazepam	4*	4
a-Hydroxyalprazolam	4*	4
Alprazolam	4*	4
Clonazepam	4*	4
Diazepam	4*	4
Etizolam	4*	4
Flunitrazepam	4*	4
Lorazepam	4*	4
Nordiazepam	4*	4
Oxazepam	4*	4

Temazepam	4*	4
Zolpidem	4*	4
Cannabinoids Panel		
Δ9-THC	1*	1
OH-THC	1*	1
THC-COOH	5*	5
Narcotics Panel		
Fentanyl	0.5*	0.5
Norfentanyl	0.5*	0.5
Sufentanil	0.5*	0.5
6-Acetylcodeine	5*	5
6-acetylmorphine (6-MAM)	5*	5
Codeine	5*	5
Heroin	5*	5
Hydrocodone	5*	5
Hydromorphone	5*	5
Methadone	5*	5
Morphine	5*	5
Norhydrocodone	5*	5
Noroxycodone	5*	5
Oxycodone	5*	5
Oxymorphone	5*	5
Tramadol	5*	5
EDDP	5*	5
Meprobamate	5*	5
O-Desmethyltramadol	5*	5
Trazodone	5*	5
Buprenorphine Panel		
Buprenorphine	1*	0.5
Norbuprenorphine	1*	0.5
Stimulants Panel		
Amphetamine	10*	10
Benzoylecgonine	10*	10
Cocaethylene	10*	10
Cocaine	10*	10
Ketamine	10*	10
MDMA	10*	10
Methamphetamine	10*	10
Methylphenidate	10*	10
Norketamine	10*	10
PCP	10*	10
Qualitative B Panel		
Olanzapine	10	NA
Lamotrigine	10	NA
Dextromethorphan	10	NA

Carisoprodol	10	NA
Propoxyphene	10	NA
Meperidine	10	NA
Clonidine	10	NA
Risperidone	10	NA
Triazolam	10	NA
Citalopram	10	NA
Chlordiazeoxide	10	NA
Sertraline	10	NA
Venlafaxine	10	NA
Fluoxetine	10	NA
Nortriptyline	10	NA
Mirtazapine	10	NA
Amitriptyline	10	NA
LSD	10	NA
Norcodeine	10	NA
Zopiclone	10	NA
Diphenhydramine	10	NA
Doxepin	10	NA
Carbamazepine	10	NA
Imipramine	10	NA
Cyclobenzaprine	10	NA
Desipramine	10	NA
Cyclobenzaprine	10	NA
Qualitative A Panel (only)		
3-methylfentanyl	0.5	NA
Butyrylfentanyl HCL	0.5	NA

* Qualitative Screen Reporting Limit. (Qualitative methods A, B, & C produce semi-quantitative results that are derived from a one-point calibration forced through zero curve, to achieve approximate quantitative results that are only to be used by the analyst as a guide for approximating values. The approximate quantitative values shall never be reported out on any certificates of analysis.)

8. References

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- *Determination of Δ9-THC in Whole Blood using Gas Chromatography-Mass Spectrometry. Chu and Drummer. JAT 26 November/December 2002.*
- *Development and validation of an automated liquid-liquid extraction GC/MS method for the determination of THC, 11-OH-THC, and free THC-carboxylic acid (THC-COOH) from blood serum. Purschke, Heinl, Lerch, Erdmann, and Veit. Analytical and Bioanalytical Chemistry 408: 4379-4388 April 2016.*
- *An Efficient, Robust Method for the Determination of Cannabinoids in Whole Blood by LC-MS-MS. Tiscione, Miller, Shan, Sprague, and Yeatman. JAT 40:639-648 July 2016.*
- *Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC-MS-MS. Coles, Kushnir, Nelson, McMillin, and Urry. JAT 31 January/February 2007.*
- *Accurate Pain Management Analysis in Under 5 Min on Raptor Biphenyl Superficially Porous Particle LC Columns. Lupo, Kahler, and Connolly. The Application Notebook: Pharma/Drug Discovery 20 September 2015*
- *ASB Standard 120, First Edition. 2019. Standard for the Analytical Scope and Sensitivity of Forensic Toxicology Testing in Impaired Driving Investigations*
- *ASB Standard 098, First Edition. 2020. Standard for Mass Spectral Data Acceptance in Forensic Toxicology*
- *ASB Standard 054, First Edition. 2020. Standard for a Quality Control Program in Forensic Toxicology Laboratories*
- *ASB Standard 113, First Edition. 2020. Standard for Identification Criteria in Forensic Toxicology*
- *Journal of Analytical Toxicology, 2021; 45:529-536. Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities-2021 Update*
- *ASB Standard 152, First Edition. 2021. Standard for the Minimum Content Requirements of Forensic Toxicology Procedures*
- *ASB Standard 053, First Edition. 2020. Standard for Report Content in Forensic Toxicology*

9. Revision Table:

REVISED BY	REV#	DATE	Revisions

LN	1	11/18/19	Section 8.4 – To reflect changes made following an addendum study the internal standard acceptance criteria for samples was changed from being +/- 50% of the calibrators to >-50% of the calibrators and quality controls. The paragraph stating the steps to take if the sample falls outside of this criteria was updated to include a list of options.
LN	2	11/20/19	Section 9 – Criteria for reporting compounds when sample has been diluted was added.
EAF	3	12/10/19	Section 8.2-To reflect changes made following Positive Control Acceptance evaluation dated December 2019.
EAF	4	12/18/19	Added to Section 9: "In the event that a compound of interest is screened and confirmed by extracting and analyzing two separate aliquots in two separate extraction batches using a quantitative method, acceptable results must be within ±20% and the lowest concentration detected shall be reported. "
EAF	5	2/13/20	Added to 8.5 qualitative methods acceptance criteria qualifier ion ratio information for confirmation methods.
EAF	6	10/9/2020	Annual Review: Definitions: Positive QC removed "second sourced", combined LLOQ and LLOD definitions, added RL definition. Laboratory consumables- reorganized wording. Guidelines for confirming positive results: added language/definitions for "same samples", added language regarding suitability of an unknown in a subject sample. Batch analysis: added notation regarding mobile phase blanks, added date to vial check requirements, removed "copy of calibration reports and sequence tables shall be included in each case file" as they are included in the associated batch files. Chromatogram identification and quality control: added Mix compounds with MRM the MRM must have a S/N >3, added wording to chromatogram examples "include but are not limited to". Peak tailing included working "limited example" as the example shown is not considered gross.

EAF	7	3/15/2021	Reworded Laboratory Consumables section specifically Whole Blood Matrix Quality Assurance and Blood Collection Kit Quality Assurance. Added Evidence Handling and Preservation Section. Added statement to Uncertainty of Measurement section regarding “if no K=2 is provided..” Added Agilent LC-MS/MS (suggested procedures) to Data Acquisition section. Updated Data Analysis Acceptance Criteria-Calibration Curve R2 to ≥0.99. Fixed spacing in section 13: Integration Appendix.
EAF	8	3/26/2021	9.2 changed >+/-30% to “exceeding the range of acceptability”
EAF	9	5/17/2021	Updated batch analysis: Pre-run sequence check to be performed by secondary individual.
EAF	10	7/30/21	Updated Blood Collection Kits Quality Assurance section to remove adding PI prep pad to liquid injected on instrument following Quality Issue Reporting form 7/29/21 and Preventative Action form 7/29/21.
EAF	11	10/27/21	Annual Review: Updated all S/N ratios to 3.3, added statement regarding recovery compound “not in blood drug testing menu”. Section 6 UoM added Agilent to instruments used for analysis. Section 7 reworded maintenance blanks "shall be run to prime and flush the system, this is performed for maintenance purposes and the data will not be analyzed", reworded documentation of failed batches to match current clerical practices. Section 8 updated Agilent exporting data to excel LCMSMS area calculator from csv file to excel file. Section 9 updated QC wording to "recorded in an excel QC tracking document or StarLIMS and evaluated to detect trends", added S/N requirements for negative controls, 9.4 added wording to reinjections "after visual examination of the vial insert for breakage or air bubbles.", 9.5 removed reference to Shimadzu Insight and changed to data analysis software. Section 10 changed so that acceptable results must be within the method’s uncertainty of measurement. Section 11 updated to include current practice of using screen to predetermine need for dilutions and running mobile phase blanks after high concentration undiluted samples.

EAF/LN	12	04/22/2022	Section 3 – Clarified evidence handling requirements during testing. Section 3.1 – guidance for ensuring proper seal upon opening kit, including initials added. Clarified that analyst opening kit is responsible for initialing stickers. Included guidance for minor vs major discrepancies. Added requirement for analyst opening kit to add box location to excel sheet and test codes in LIMS. Section 4 – added guidance for assigning expiration dates for consumables that do not come with a date. Section 7 – added batch sequence check sheet to requirement. 9.1 – Added requirement for deviation comment to be included on report. Grammar and spelling throughout.
EAF	13	06/29/2022	Created Reporting Criteria section and added postmortem testing, removed reference to Shimadzu in section 1 summary.
EAF	14	10/05/2022	Updated batch sequence check to include the individual data acquisition method for each sample.
EAF	15	11Jan23	Major revision: dihydrocodeine cut from Qual A and Narcotics panels, added direction for photographing evidence, additional internal standard acceptance criteria added for all panels, document renamed to Blood Drug Procedures and merged all blood drug SOPs into this SOP.

EAF/LN	16	30Jun23	In all extraction procedures changed "Pulse vortex 1 minute" to "Pulse vortex ~1 minute" Corrected small typo/missing information in extract procedures/bench sheets and made it so they match each other. Updated in Qual C limitations to include delta-9-THC in following statement: "Carboxy-delta-9-THC and delta-9-THC have a partially resolve isomer that has a retention time after the compound of interest, this results in two chromatograms that are partially merged. Carboxy-delta-9-THC and delta-9-THC retention times are earlier than the partially resolved isomer and is the first chromatograph peak. " Low volume samples updated comment to: “Unable to perform blood drug testing due to low sample volume.” (Cannot use the words "quantity insufficient" in STARLIMS) If a No Exam report is being utilized the result field on the report must say “No Exam” and the comment field on the report must say “Unable to perform blood drug testing due to low sample volume.” uncertainty of measurement reworded to "The expanded uncertainty of measurement shall always be rounded up to two decimal places and be reported as such in the test report with the coverage probability. In addition, the analytical test result and the rounded expanded uncertainty shall be reported to the same level of decimal places. " Reformatted spacing in document. Added: Homogenize the sample and break up any clots, where possible. If homogenized, sample must be placed on a rocker a second time for a minimum of ten minutes.
EAF	17	28Jul23	Added to Narcotics limitation: Norfentanyl-d5 qualifier ion ratios fluctuate outside of acceptance parameters on the Agilent 6470A. Any samples needing confirmation for Norfentanyl shall be run on the Shimdazu 8030.

EAF/LN	18	26Oct23	Evidence Handling: removed reference to blood kit box excel sheet To waste management section updated to “The liquid generated from the Hexane: Ethyl Acetate liquid-liquid extraction (Qualitative C, THC, and Buprenorphine methods) and the chloroform extraction (Qualitative A and B supernatant of water, methanol, and blood) is classified as dual waste (chemically and biologically hazardous) and is to be disposed of in the liquid waste hazardous waste stream.” And “Organic based solvent, stocks, and standards are to be disposed of in the liquid waste hazardous waste stream.” To Reporting Criteria added "An inventory table will be included on the report to indicate the number and types of tubes received, the collection information, the approximate volume of each tube, and an indication as to which tubes were tested. " Added screening limits to the table: Blood Drug Testing Menu & Reporting Limits and added the following associated *statement: * Qualitative Screen Reporting Limit. (Qualitative methods A, B, & C produce semi-quantitative results that are derived from a one-point calibration forced through zero curve, to achieve approximate quantitative results that are only to be used by the analyst as a guide for approximating values. The approximate quantitative values shall never be reported out on any certificates of analysis.) Changed any reference to "concurrently run positive control" to "the concurrently run certified reference materials" Added to dilution section: If a diluted and undiluted sample are analyzed any diluted results must be within ±20% of the undilute sample result for any concentrations that are within the quantitative range/adjusted quantitative range to be deemed acceptable. Diluted samples containing a concentration within the adjusted quantitative range that are not within ±20% of the undilute sample result shall be rejected for all compounds. If two diluted sample are analyzed the results within the respective adjusted quantitative ranges must be within 20% of each other for these two diluted samples to be deemed acceptable. Qualitative AB method limitation: added “Qualitative A & B can only be analyzed on the Agilent 6470A at this time. The Shimadzu 8030+ has decreased in sensitivity and a method optimization and validation is required for this instrument. “ Buprenorphine method limitations: added “The Agilent 6470A sheath gas flow is set too fast and the instrument is no longer able to maintain the flow rate to run the buprenorphine method. The buprenorphine testing method shall only be run on the Shimadzu 8030+ until a method optimization and validation can be performed on the Agilent 6470A.” Added requirements for expert letter.

EAF	19	31Oct2023	Clarified qualitative and quantitative negative control requirements, and added statement: “If a negative control does not meet the above parameters with regards to compounds of interest, all samples in the batch shall be evaluated and re-extracted only if a sample meets positive acceptance criteria for the compound(s) seen in the negative control.”
EAF	20	06Dec2023	Added to method limitations: Qualitative A: The following compounds were found to exhibit statistically significant ionization suppression which could produce a false negative result for a sample. 7-Aminoclonazepam, 7-Aminoclonazepam, Clonazepam, Morphine, 6-Acetylmorphine. And Qualitative A: The following compounds were found to exhibit statistically significant ionization enhancement which could produce a false positive preliminary screen result for a sample. These compounds are confirmed using a different method and would never be reported out as positive from only a preliminary screen result. Oxazepam, Alpha-Hydroxyalprazolam Replaced reserpine with zolpidem-d6 in the data acquisition table for Qualitative A Added the confirm batch names to each group in the reporting limits table. Removed Barbiturates from reporting limit table.
EAF	21	19Jan2024	Stimulants and Qualitative A data acquisition method, removed MDA and MDA-D5 All Quantitative Data Acquisition Methods: Combined compound of interest table with internal standards table and grouped compound of interest with associated internal standard. Removed Norfentanyl-d5 from Narcotics method limitations.
EAF/LN	22	19Jan2024	Added to blood kit opening section “For non-HETL collection materials, refer to the manufacture’s information or the below reference table: (Table containing guidance on different types of blood collection tubes” and “For continuity the volumes entered into the blood inventory worksheet and subsequent StarLIMS items received table shall adhere to the following format: (table containing guidance with different volumes)” Uncertainty of measurement: Added to uncertainty budget table: Bias: Taken from Type A Evaluation of Process reproductivity data-blood matrix QC sample. updated Quality control Samples: CRM-second source; uncertainty in the state reference value to be Type B evaluation. Sequence check: added "This shall be performed by the secondary individual physically checking each autosampler vial number to the instrument sequence and shall be documented by the following:" Added to evidence handling: If a non-HETL approved collection kit or materials were submitted, the WO# field in STARLIMS folder metadata shall be filled in with N/A. If the kit, upon opening, is found to contain non HETL approved collection materials this shall be noted on the inventory form and STARLIMS metadata shall be updated appropriately.