HEALTH & ENVIRONMENTAL TESTING LABORATORY
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LC-MS/MS ANALYSIS, ACCEPTANCE, & REPORTING CRITERIA STANDARD OPERATING PROCEDURE

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Maine HETL- Forensic Toxicology

1. Summary:

This document describes data acquisition protocols as well as the toxicology quality guidelines for the analysis, evaluation, acceptance procedures, and reporting of toxicology results for Shimadzu LC-MS/MS methodology, unless otherwise specified in a specific test methods SOP.

Case notes and comments shall be documented in the case file by the analyst. Minor and major deviations or exceptions 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.

This procedure is to be used in conjunction with the Forensic Chemistry Toxicology Section Individual methods SOPs.

2. Definitions

Calibrators: Laboratory fortified samples 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 consisting from 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.

Internal Standard(s): Compound(s), most commonly compound of interest matched deuterated equivalents. 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 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 or a deuterated compound of interest. 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, 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, 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.

3. Evidence Handling and Preservation

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
- All glassware and tools to be used will be clean
- Test tubes, capillary pipettes, Pasteur pipettes, etc are used only once, then discarded
- To prevent cross contamination of samples, only one case will be opened by the analyst at a time
- Reagents and solvents will be kept in closed containers when not being used in the analysis
- The evidence will be under constant control by the analyst.

3.1 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 Form will be filled out (i.e., internal chain of custody).
- The analyst will initial stickers bearing the Lab 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 label at the time of kit opening (if known).
- The analyst will verify all identification numbers and names agree with the Chain of Custody Receipt.
- The collection kit and all specimens will be labeled with the lab identification number, name of the subject (if known) and the analyst's initials.
- All paperwork contained in the kit will be labeled with the laboratory identification number and initialed by the analyst.
- The analyst will verify the case information provided with the kit matches the HETL folder, sample information from the Laboratory Blood Analysis Request Form submitted with the sample and all StarLIMS labels. Any discrepancy shall be noted by the analyst within the case folder.
- 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.
- The sample kit (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 and will be retained until filled in the laboratory. All filled storage boxes will be placed in long term storage, for a period of at least six months, until being returned to the submitter or destroyed.
- 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 a plastic tube container, the seal 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. Laboratory Consumables

For all Blood Drug Testing Program 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 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

4.1 Whole Blood Matrix Quality Assurance

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

Upon receiving a new lot number of vendors certified negative sample matrix an aliquot of the lot specific matrix shall be taken and run per each relevant tests standard operating procedure as a blank sample. A blank sample is a compound of interest free 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 with acceptable results exhibiting no method interferences caused by manufacturing contaminants or interfering compounds. Records containing the results, pass/fail status, and lot number of the certified negative sample matrix shall be maintained.

4.2 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 2 mL of DI water and the liquid contents of the PI pad to one tube and vortex.
3. Transfer the resulting mixture to a second tube and vortex.
4. Label an autosampler vial with identification number.
5. Transfer a portion of the mixture to the labeled autosampler vial with insert.
8. 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 on of the screening methods the mixture shall be injected and analyzed with the respective confirmation method.
9. 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 with acceptable results exhibiting no method interferences caused by manufacturing contaminants or interfering compounds. Records containing the results, pass/fail status, and lot number of the blood collection kits shall be maintained.

5. Guidelines for Confirming Positive Results

Prior to identification of an unknown in a subject sample, review and acceptance of the applicable calibration curve, quality controls, and internal standards/recovery compounds shall be performed to ensure suitability of an unknown in a subject sample

The detection of drugs and drug metabolites should be confirmed by a second technique. 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

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.

6. 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	
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	Primary use is to evaluate bias. The evaluation of bias will be done after the calculation of combined standard uncertainty.
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 LCMS 8030: MS 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.

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 error rate 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 and duplicates are tracked in either a Microsoft Excel Spreadsheet or StarLIMS. 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.

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 significant digits 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 significance.

7. Batch Analysis

It is common practice to group samples into batches; the following section illustrates step by step suggested instructions on how this is set up on the LC-MS/MS as well as the protocol for data analysis, unless otherwise specified in a specific test methods SOP.

Prior to running a batch, a mobile phase blank shall be run to flush the system, this is performed for maintenance purposes and 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.

If mixes and quality control or calibrators and quality controls analysis fail to meet the acceptance criteria a copy of the bench sheet and sequence table documenting the reason for batch failure shall be included in each affected case sample file.

Each auto sampler vial shall be checked by the analyst in comparison to the instrument sequence upon placing into and removing from auto sampler rack; this shall be documented on the instrument sequence table with the date and analyst's initials.

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).

8. Data Acquisition

8.1 Shimadzu LC-MS/MS 8030– *Suggested procedures*

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 your user name and password
- If you want to check your mixes, calibration or QC **before the run is finished** it will not allow opening an incomplete batch file. In this case File/open and change the file type from batch (*.lcb) to data file (*.lcd). Then select one data file in your batch- Insight will automatically pull in the associated calibration data files. You can look at the mixes, calibration, and QC (or sample) but do not save until you are able to pull the entire batch file over.
- If the batch has finished acquisition- 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.
- View/compound- will show the sample batch on the left and the highlighted sample on the right.
- View/cal curve- will display the calibration curve for the highlighted compound
- View/ survey- toggles between compound details and survey view. Survey shows all compounds for the highlighted sample for quick survey and compound details shows a single compound with its associated Internal standard.
- **Edit** a peak- right click and
    - 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.
    - Update retention time and ion ratios- on a middle calibration data point, select each compound and update each RT & ion ratio then click on the apex of the peak.
- Start with the calibration or mixes analysis. Review each peak and update retention times and ion ratios using a mid-level calibrator.
- 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. This way the batch, analysis method and project are saved together in a file with the date name.
- Edit/integrate batch- This will reintegrate all data files using the modified calibration curve. Check save manual integrations if needed.
- Analyze each data file and each compound.
- Edit/Table -If you need to change a dilution factor, sample name or comment. Make changes and select edit table again. The file that was changed will need to be reprocessed (Edit/ integrate sample) if any changes would alter the results.

- 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.
- If in the future, you want the raw data then File/open and select the batch file. If you want to open the reviewed batch then File/open and select DAML project.

Exporting Data to STARLIMs

- Once the Insight project batch has been fully analyzed and all data approved by the chemist, select view and change from Compound view to Summary view.
- Right click on the summary results and select properties. The orientation should be samples vs compounds. The fixed columns should be data file name, Flags, Sample ID, Sample Name (in that order). The comparison columns should be concentration.
- Select File then Export. A window will appear confirm that Output to File is selected and Format Options consist of Delimiter: Comma and Columns: By View.
- Click the browse button, select data transfer flash drive location and name file the same as the project batch file (Example: THC091819EAF).
- Export
- Properly eject the data transfer flash drive.
- In STARLIMs select Results Entry: By Run.
- Select relevant batch and Results Tab.
- Click Capture Data and browse to select your data summary file from the flash drive.
- Double check the data is correct and that all results are filled in
- Under test workflow steps select “finish results”

Exporting Data to Excel LCMSMS Area Calculator

- Once the Insight project batch has been fully analyzed and all data approved by the chemist, select view and change from Compound view to Summary view.
- Right click on the summary results and select properties. The orientation should be samples vs compounds. The fixed columns should be data file name, Flags, Sample ID, Sample Name (in that order). The comparison columns should be area.
- Select File then Export. A window will appear confirm that Output to File is selected and Format Options consist of Delimiter: Comma and Columns: By View.
- Click the browse button, select data transfer flash drive location and name file the same as the project batch file with an Area designation (Example: THC091819EAFArea).
- Export
- Properly eject the data transfer flash drive.
- Open Excel File: Shimadzu LCMSMS Area Calculator (Stored as a read only document on the K drive).

- Select Data Tab and import data From Text/CSV
- Import and Load, this shall create a new Excel sheet.
- Highlight necessary rows, copy, and paste values into Area Sheet
- Select respective Test Code Sheet, evaluate data and print.
- Close Excel file, do not save. (This is a read only document meant to be used as a calculation tool, if necessary the Insight project file may be re-opened, the data exported, and re-evaluated again using this tool)

8.2 Aglient LC-MS/MS 6470A– *Suggested procedures*

Starting a sequence from a template:

- Open Masshunter Data Acquisition go to Worklist tab
- New batch, select your template
- Change: Sample names, Sample IDs, Sample type (standard, control, unknown), Level #s, Dilution factor (if any), comments, 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 your 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

Exporting Data to Excel LCMSMS Area Calculator

- Once the batch has been fully analyzed and all data approved by the chemist, select Display multiple compounds/samples in batch table with compounds going left to right.
- Right click on batch table and select Add/Remove Columns-remove all until you are only left with ISTD Compound Results areas.
- Select File-Export-Export Table and select Excel file (.xlsx)
- Select data transfer flash drive location and name file the same as the project batch file with an Area designation (Example: THC091819EAF Area).
- Export
- Properly eject the data transfer flash drive.
- Open Excel File: Agilent LCMSMS Area Calculator (Stored as a read only document on the K drive).
- Select Data Tab and import data From Text/CSV
- Import and Load, this shall create a new Excel sheet.
- Highlight necessary rows, copy, and paste values into Area Sheet
- Select respective Test Code Sheet, evaluate data and print.
- Close Excel file do not save. (This is a read only document meant to be used as a calculation tool if necessary, the Insight project file may be re-opened, the data exported, and re-evaluated again using this tool)

9. Data Analysis Acceptance Criteria

9.1 Calibration Curve

A calibration curve shall be run for each new sequence. Routine quantitative curves shall consist of a non-forced linear weighted 1/A calibration curve with a coefficient of determination (r2) of ≥.99 and all positive quality controls being within the LOQ. An acceptable calibration curve shall consist of a minimum of 5 calibration points unless otherwise stated in a specific method SOP.

The calibration curve shall be evaluated by back-calculating calibrator concentrations against the curve. Values of ±30% from the expected concentration are acceptable for calibration points below the lower limit of quantitation, whereas values of ±20% from the expected concentration are acceptable for the range of quantitation unless otherwise indicated in a specific method SOP. In addition, the compound of interest peak signal to noise ratio must be ≥3 to be deemed a quantifiable peak signal and not baseline noise. These calculations to determine quantitative values and signal to noise ratios shall be performed by the data analysis computer software.

Qualifier ion ratios shall be updated for each batch using a mid-point calibrator. Expected ion ratios for all the quantitative blood drug methods are <±30% ion ratio difference to the set ratio within the quantitative range. 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.

If LOQ calibrators 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. If a sample volume is quantity not sufficient to permit a re-extraction and analysis then the change to LOQ shall reported to the customer on the Certificate of Analysis as either “Not detected at (LOQ)” or “Detected, < or >LOQ”.

In the event that more than three calibrators need to be excluded from the calibration curve and a sample has been consumed results may be reported out on a case by case basis for that sample only. Supervisor approval of the calibration curve and case sample results is required and will be documented using the “Deviation Request Form”.

9.2 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 StarLIMS and evaluated to detect trends.

Quantitative analysis: 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.

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. Internal standard areas should be consistent with the response found in the associated calibrators and positive controls. No integrated peaks should be present but may be deemed acceptable the peak in question: does not correspond with any of the retention times for the compounds of interest, calculated concentration is less than the lower limit of detection/quantification, or there is no qualitative ion present at the expected retention time.

Positive Quality Controls shall consist of blank matrix samples fortified respectfully at low, medium, and high levels of each test methods calibration range. These fortified blank matrix samples are extracted and analyzed with each batch at a frequency of one set of positive quality controls per a maximum batch size of twenty samples. If a batch of greater than twenty samples is extracted and analyzed, then additional set(s) of positive quality controls shall be required. Internal standard areas should be consistent with the response found in the batch’s calibrators and the negative control. Calculated concentrations must be within ±20% of the expected value. In addition, the compound of interest peak signal to noise ratio must be ≥3 to be deemed a quantifiable peak signal and not baseline noise.

If any of the positive control(s) exceed the range of acceptability for a compound(s) of interest exhibiting a concentration of < ±30% of the expected value, any subject sample(s) not exhibiting the aforementioned compound(s) of interest can be reported without remedial action.

If any of the positive control(s) exceed the range of acceptability for a compound(s) of interest and a subject sample(s) exhibit the aforementioned compound(s) of interest, remedial action is required which may include reinjection or re-extraction.

If any of the positive control(s) exceed the range of acceptability for a compound(s) of interest exhibiting a concentration of >±30% of the expected value, remedial action is required which may include reinjection or re-extraction.

9.3 Qualitative Quality Controls

A corresponding Mix shall be run with each new sequence, 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 frame of reference for case samples. Mixes shall consist of blank matrix samples fortified at respective positive concentrations. These fortified blank matrix samples are extracted and analyzed with each batch and shall be deemed acceptable for a compound of interest when the compound of interest consists of a peak present at the expected retention time and the signal to noise ratio is >3. A qualitative batch shall consist of respective mixes and a negative quality control for every twenty samples.

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 mixes in every batch to monitor for carryover. Recovery compound areas should be at least >50% the response found in the associated mixes. No integrated peaks should be present but may be deemed acceptable if the peak in question does not correspond with any of the retention times for the compounds of interest or the calculated concentration is less than the lower limit of detection.

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.

9.4 Internal Standards & Recovery Compounds

Internal standards are required for all quantitative chromatographic assays and deuterated internal standards for the compounds of interests are preferred. In the event that a matching deuterated internal standard is not available for a particular compound of interest then a differing deuterated internal standard shall be used with similar extraction and chromatographic properties to the compound of interest.

Internal standard recovery as measured by peak area shall be monitored for calibrators, quality controls, and case specimens within a batch on the Calibration Batch (calibrators and quality controls) and Data Summary (case samples) respectfully. It is noted that the internal standard recovery of the case samples should be >-50% as compared to the most recent calibrators and quality controls prepared with certified reference material.

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

2. Re-extraction and reanalysis of the sample

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 with authorization from the Forensic Laboratory Director as “unsuitable for _____ analysis.”

A recovery compound is required for all qualitative chromatographic assays, this consists of a compound of interest that would be highly unlikely to be found in case samples (for example: Reserpine). The recovery compound as measured by peak area shall be monitored for mixes, negative quality controls and case samples within a batch. It is noted that the

recovery compound of the negative quality controls and case samples should be >50% as compared to the average most recent mixes prepared with certified reference material.

9.5 Chromatogram Identification & Quality Control

The detections of drugs should be confirmed (when possible) by a second technique based on a different instrument methodology, extraction method, and/or chemical principle. If a second technique is not possible then the confirmation must be performed on a different aliquot of the same specimen or from a second specimen.

Select Ion Monitoring (SIM) Identification: As part of the qualitative procedure when SIM is used for identification of a compound of interest the retention time match and compound ion match is required. Please note SIM may be backed up with an MRM for select difficult to detect compounds. For compounds of interest with a SIM and MRM the presence in the respective Mix 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.

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 and one internal standard transition for the compound of interest is required.

In the qualitative method A, B, or C analysis, a compound of interest shall be identified by comparison of ion peak retention time to that of the compound of interest expected retention time. The retention time of the compound of interest will be within ±0.2 minutes of the expected time as compared to the most recent respective mix prepared with certified reference material to be deemed acceptable. A presumptive positive value must also have a signal to noise value of ≥3:1 and a calculated concentration of the respective compound of interest's lower limit of detection. 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. For all other compounds of interest within the qualitative method, the lower limit of detection shall be considered approximate and that, if there is a peak seen at the expected retention time, with a signal to noise ration of >3, and a calculated approximated concentration close to the respective lower limit of detection the compound of interest may be deemed positive based on analyst discretion. In addition, expected ion ratios for all the confirmatory blood drug methods are <±30% ion ratio difference to the set ratio. 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, 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.

In quantitative analysis a compound of interest shall be identified by comparison of its quantifying ion peak retention time to that of the compound of interest expected retention time. The retention time of the compound of interest will be within ±0.2 minutes of the expected time as compared to the most recent calibration prepared with certified reference material to be deemed acceptable. A verification of the target compound shall be established by the presence of the target compound's qualifier ion peak within ±0.2 minutes of the expected time as compared to the most recent calibration prepared with certified reference material to be deemed acceptable. In addition, expected ion ratios for all the quantitative blood drug methods are <±30% ion ratio difference to the set ratio within the quantitative range. 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.

A positive value must also have a signal to noise value of ≥3:1. If not, the case sample may be re-analyzed, volume permitting. If volume not permitting (QNS) re-extraction and analysis compounds not meeting confirmation criteria will not be reported.

Quantitative values are calculated by measuring the area of characteristic ion transitions 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.

Auto Integration is set up in the instrument data analysis method to have the software correctly integrate most of the peaks. There are conditions in which the analyst experience will require the use of manual integration. This shall be used sparingly, and sound scientific principals shall be followed for correct peak integration to insure 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 and if improper auto-integration was performed by the computer software as deemed by analyst experience then manual integration shall be utilized.

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 as well as notated in the Mode column of the data summary sheet.

A peak shall never be integrated unreasonably below or above the baseline.

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

All un-integrated batches shall be available for review in the Insight software program as the .lcm batch file.

10. Reinjection, Dilution, & Re-extraction Documentation

In the event that a case sample is reinjected, or a dilution re-extraction and analysis is performed the unused original data shall be documented as data not used and provide information as to why it was unacceptable. The reinjected data results shall be labeled as "reinjection". 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.

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 within the quantitative range must be within ±20% and the lowest concentration detected shall be reported.

11. 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. While 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 it must be

evaluated and confirmed on all samples that exhibit results greater than the specific methods ULOQ. Appropriate actions are as follows:

1. If the case sample following the sample exhibiting the >ULOQ concentration does not exhibit the compound of interest no further action is required.
2. If the case sample following the sample exhibiting the >ULOQ concentration does exhibit the compound of interest at >LOD reinject the Negative Quality Control, one Positive Quality Control, and the sample exhibiting the >ULOQ concentration followed by a mobile phase blank. 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 lowest calibrator 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 lowest calibrator then the case samples following the >ULOQ concentration effected sample(s) require re-extraction and analysis.

12. References

Taylor, B.N. and Kuyatt, C.E. National Institue of Standards and Technology (NIST) Technical Note 1297: Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results. 1994 Edition.

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.

Report from the Scientific Working Group for Forensic Toxicology (SWGTOX). Journal of Analytical Toxicology 2013; 37:452-474. Standard Practices for Method Validation in Forensic Toxicology.

Raposo, F. Trends in Analytical Chemistry 77 (2016) 167-185. Evaluation of analytical calibration bases on least squares linear regression of instrumental techniques: A tutorial review.

SOFT/AAFS 2006 version Forensic Toxicology Laboratory Guidelines.

13. Integration Appendix

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.

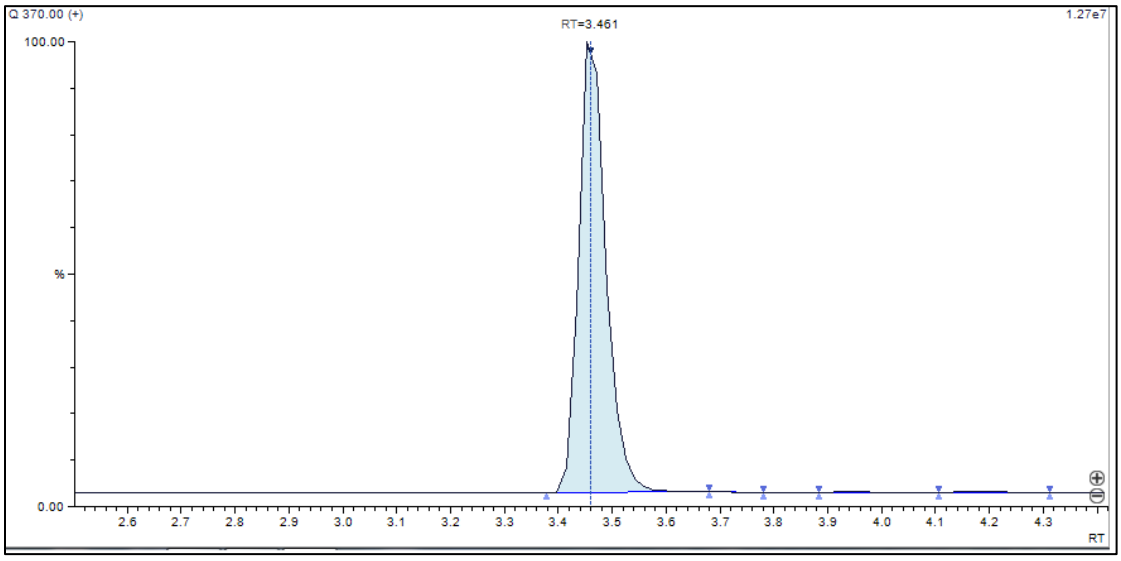
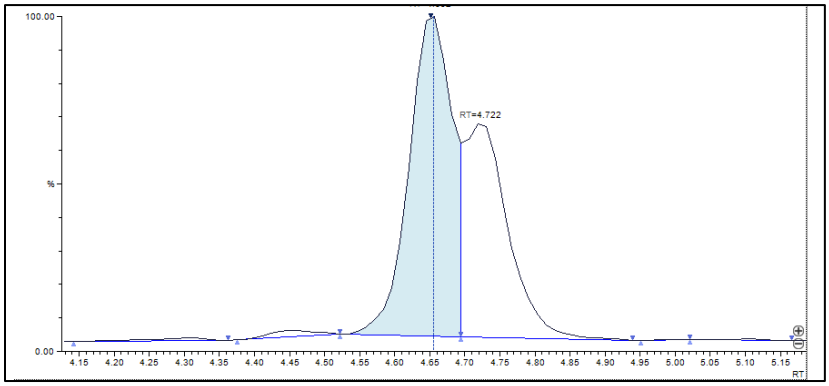


Figure 2: Properly integrated coeluting peak.

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.



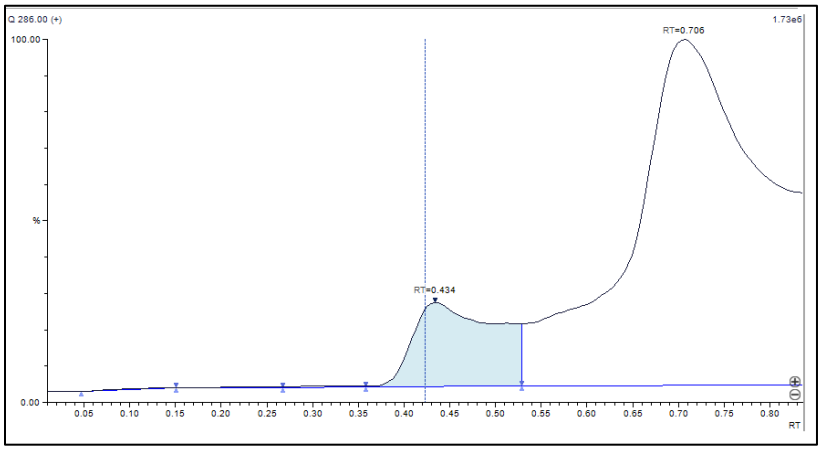


Figure 2A: Properly integrated co-eluting peak with a rising baseline.

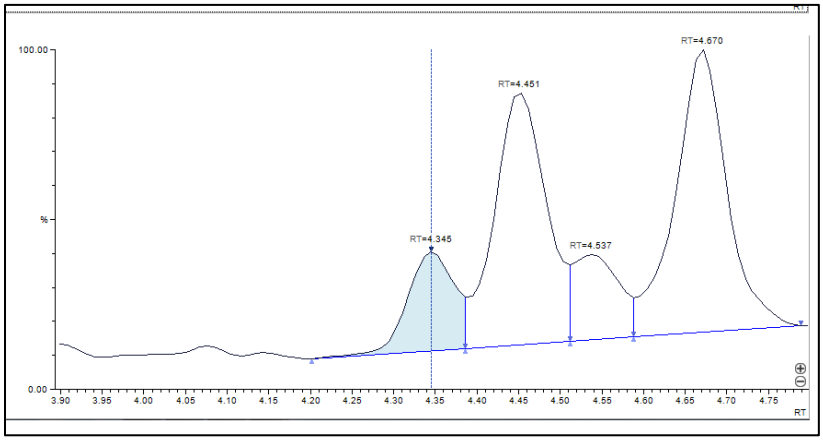


Figure 3: Uniform integrations.

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.

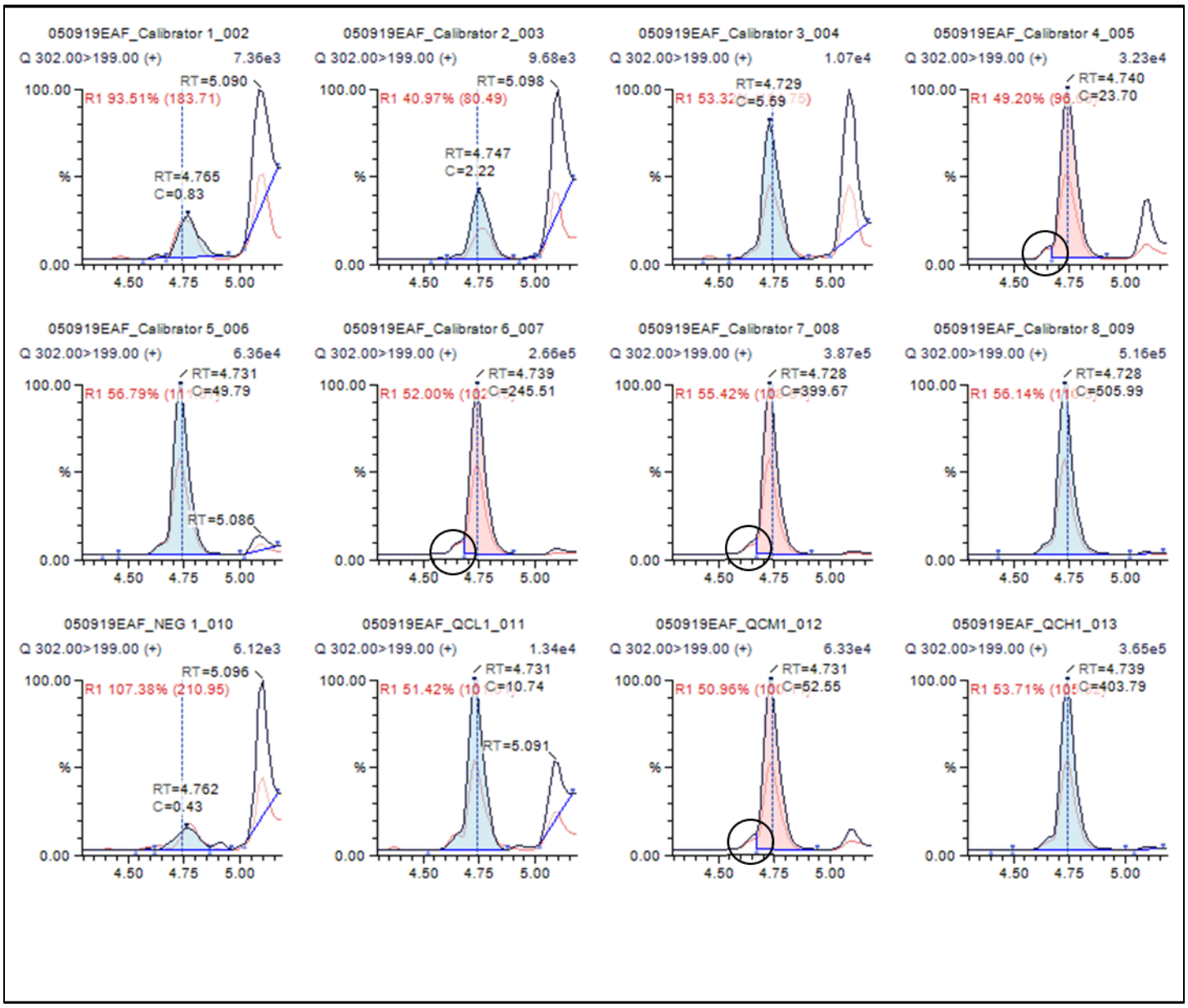


Figure 4: Baseline Noise Example.

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.

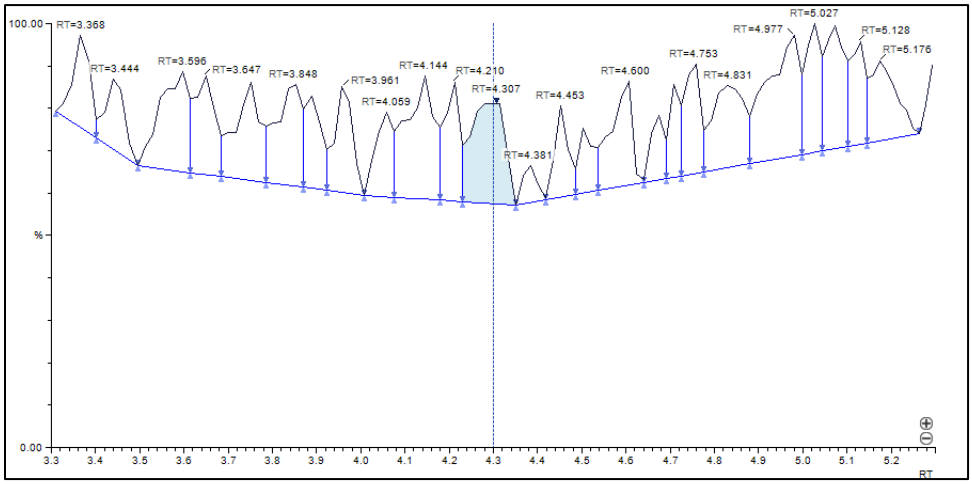


Figure 5: Peak Fronting

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)

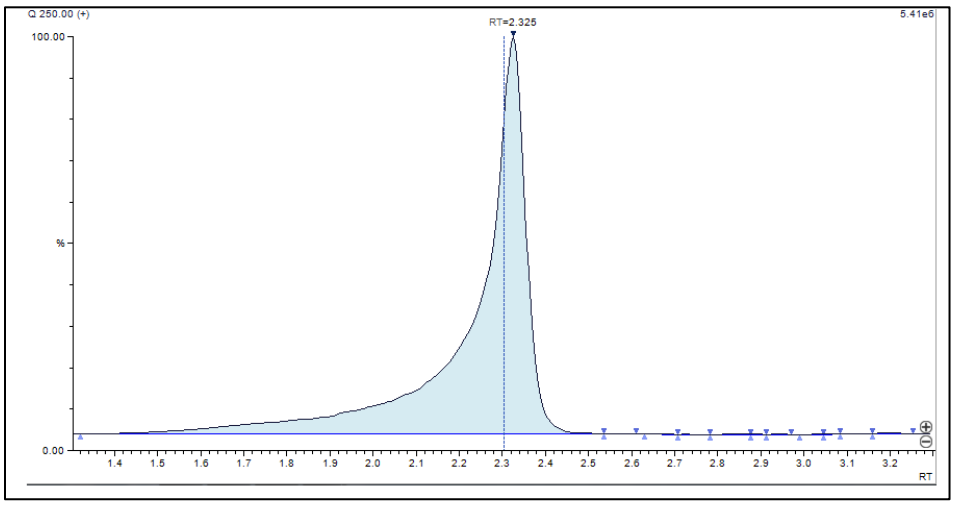


Figure 6: Peak Tailing

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.

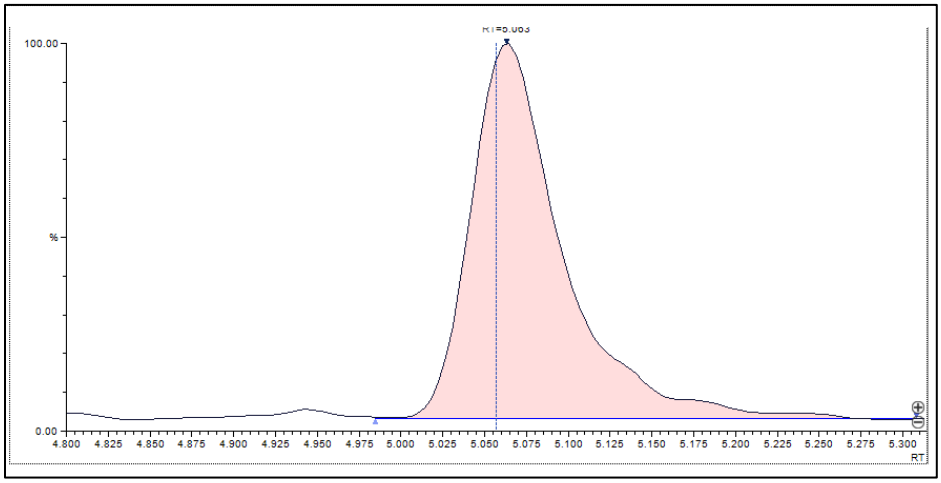


Figure 7: Improper Peak Shaving

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.

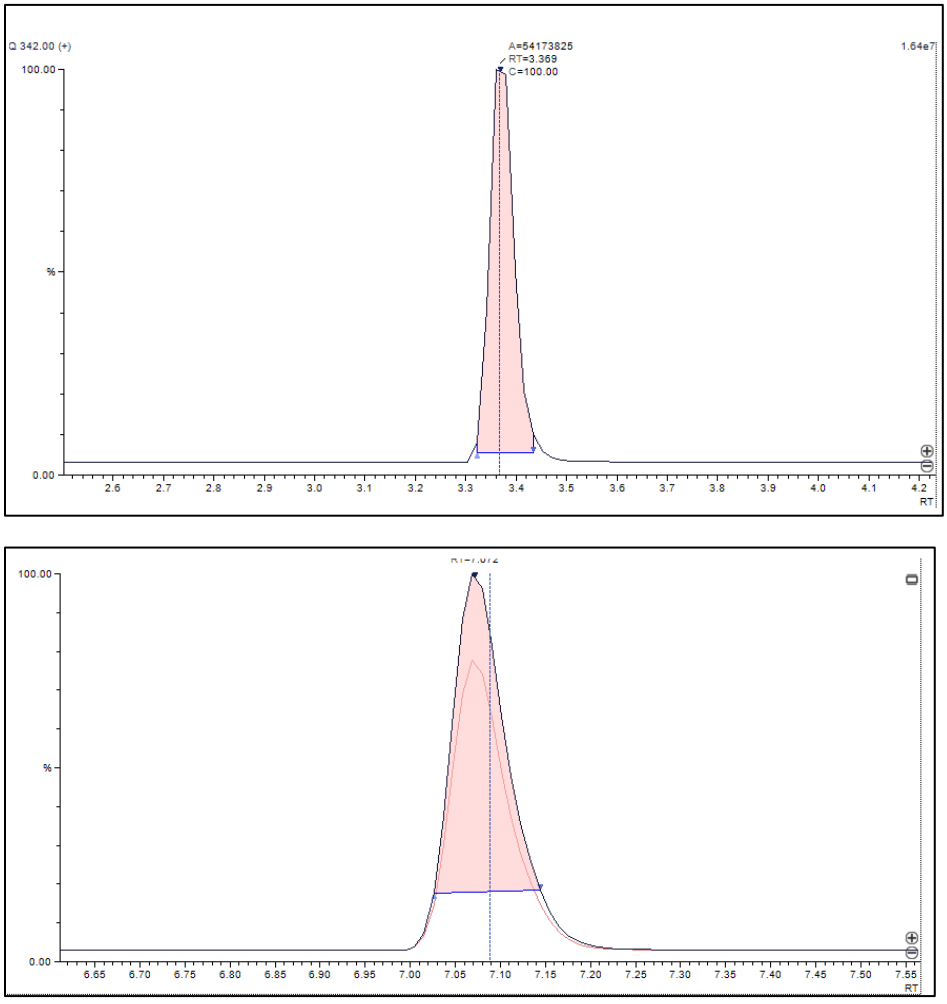
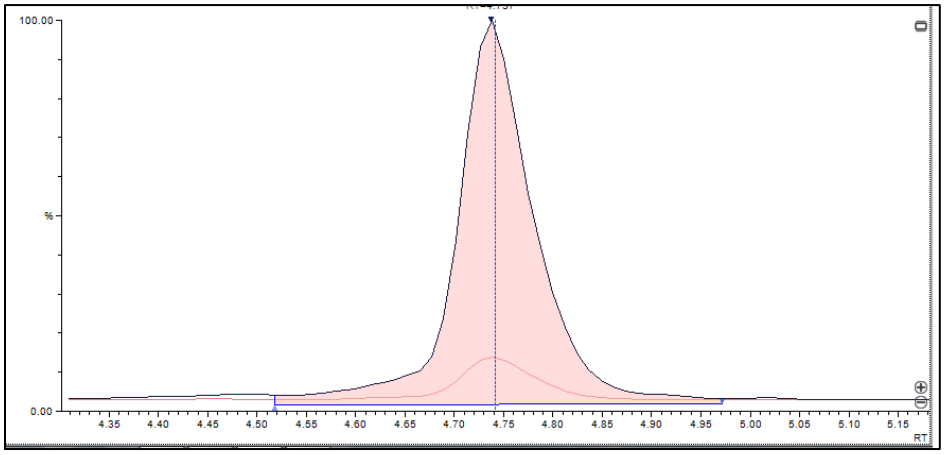


Figure 8: Improper Peak Enhancing

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.



14. Revision:

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.