Title: (TX-39 SOP) Quantitative Analysis of Biological Materials for Cocaine and Its Metabolites using Liquid Chromatography/Mass Spectrometry (LC/MS)

1. Introduction

Cocaine is a naturally occurring stimulant that is found in the leaves of the Erythroxylon coca plant. The primary metabolites of cocaine in humans are benzoylecgonine and methylecgonine. Cocaethylene is another biotransformation product of cocaine that is produced when cocaine and ethanol are ingested within the same time period. Cocaine is rapidly metabolized within the body (half-life of ~90 minutes) by hydrolysis of its methyl ester group to form benzoylecgonine (BE). Because of the rapid breakdown, BE is usually the major detectable analyte in biological fluids as a result of cocaine ingestion. It may not be unusual that the parent compound, cocaine, is not detected at all within blood or urine specimens but metabolites are detected. Samples that require confirmation by liquid chromatography/mass spectrometry (LC/MS) are extracted from buffered, diluted sample aliquots by adsorption onto solid phase extraction (SPE) columns. Cocaine and/or metabolites that may be present are then eluted from the SPE columns, dried, and reconstituted before being analyzed using an LC/MS instrument. Quantitations are performed using a multipoint calibration graph with deuterated internal standards, if available. Matrix-specific (e.g., blood) positive and negative controls are extracted and analyzed in each analytical batch. The presence of cocaine and/or its metabolites may be confirmed in blood, or serum/plasma.

Based on the DSS laboratory's MOU with the state OCME, cocaethylene will be evaluated for postmortem samples and related antemortem samples where screening methods, if used, had indicated their presence.

2. Scope

This procedure can be used for the quantitative determination of cocaine and/or its metabolites (e.g., cocaine, benzoylecgonine, cocaethylene) within biological specimens (e.g., blood, serum).

3. Principle

Biological specimens are analyzed for the presence of drugs of abuse (DOA) and/or their metabolites by extraction using solid phase extraction (SPE) columns. Final extracts are analyzed by LC/MS using electrospray ionization (ESI) and can involve a combination of targeted ion monitoring and full scan analysis modes.

4. Specimens

This procedure uses biological fluid(s) such as blood, serum, and/or plasma. Quantitative analyses are usually applied to blood (or serum/plasma). Blood sample collection tubes containing proper anticoagulant and preservative (i.e., 'gray-tops' containing potassium oxalate and sodium fluoride) should be used. All samples should be received with proper documentation, have been submitted within properly sealed containers (i.e., prevent sample loss, contamination, or deleterious change), and have been stored appropriately. Once samples have been received by Toxicology Unit staff they will be properly stored within either a refrigerator or freezer. Typically 0.25 mL of sample is consumed during the analysis but varying volumes may be used, as necessary. Dilution of samples due to limited specimen or due to

suspicion of high drug or metabolite concentration is acceptable but documentation of such dilutions will be found within the appropriate casefiles.

5. Equipment/Materials/Reagents

5.1 General laboratory glassware (Calibrated glassware shall be used to prepare control and calibrator solutions)

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

5.3 Vortex mixer

5.4 Sonicator

5.5 Automatic pipettes (with disposable tips)

5.6 Positive pressure solid phase extraction device – SPEWare Cerex, 48 sample (or equivalent)

5.7 Sample concentrator with nitrogen – SPEWare Cerex 48 heated (or equivalent)

5.8 Centrifuge

5.9 pH paper (or equivalent)

5.10 Trace B Extraction Columns – SPEWare (or equivalent)

5.11 Liquid Chromatograph/Mass Spectrometer (Shimadzu LCMS-8030, ThermoElectron LTQ, or equivalent)

5.12 HPLC column – Kinetex, phenyl hexyl, 2.6 µm, 100Å, 50 mm x 4.6 mm (Phenomenex), or equivalent)

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

5.14 Autosampler vials (LC/MS grade 1.8mL or equivalent)

5.15 Acetic acid, glacial ($CH_3COOH_{(l)}$, Reagent grade or equivalent)

5.16 Acetonitrile (CH_3CN, Reagent grade, LC/MS Grade, or equivalent)

5.17 Ammonium formate (NH_4CHOO, Reagent grade or equivalent)

5.18 Formic acid (HCOOH, Reagent grade or equivalent)

5.19 Methanol (MeOH, Reagent grade, LC/MS Grade, or equivalent)

5.20 Sodium acetate trihydrate ($NaCH_3COO \cdot 3H_2O$, Reagent grade or equivalent)

5.21 Sodium bicarbonate ($NaHCO_3$; Reagent grade or equivalent)

5.22 Sodium carbonate (Na_2CO_3 ; Reagent grade or equivalent)

5.23 Water (Mobile Phase) (LC/MS Grade ; Optima, or equivalent)

5.24 Certified Reference Materials (Cocaine, Benzoylecgonine, Cocaethylene, Cocaine D_3 and Benzoylecgonine D_3 ; 1 mg/mL) (Cerilliant, Lipomed, or equivalent)

5.25 Ammonium Formate $_{(aq)}$: ($NH_4CHOO_{(aq)}$; 5M; 31.5% (w/v)): Can be prepared by dissolving 3.15 g of ammonium formate in 10 mL of water. Stable for one (1) year in glass container when refrigerated.

5.26 Sodium Acetate Buffer $_{(aq)}$ (0.07M; $NaCH_3COO$; pH~4.5): Can be prepared by combining 5.86 g of sodium acetate with 3.24 mL of glacial acetic acid in a 1 L volumetric cylinder and diluting to volume with water. Stable for one (1) year in glass container while at room temperature.

5.27 Sodium Bicarbonate $_{(aq)}$ (0.1M; $NaHCO_3$; 0.84% (w/v); pH~8): Can be prepared by dissolving 4.2 g of sodium bicarbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for one (1) year in glass container while at room temperature.

5.28 Sodium Carbonate $_{(aq)}$ (0.1M; Na_2CO_3; 1.1% (w/v); pH~11): Can be prepared by dissolving 5.3 g of sodium carbonate in water within a 500 mL volumetric flask and diluting to volume with water. Stable for one (1) year in glass container while at room temperature.

5.29 Bicarbonate/Carbonate Buffer $_{(aq)}$ (pH~9): Can be prepared by transferring 0.1M sodium bicarbonate $_{(aq)}$ solution into a beaker and checking pH (should be pH~8). Adjust the pH to ~9 using the 0.1M sodium carbonate $_{(aq)}$ solution and pH paper (or equivalent). Stable for one (1) year in glass container while at room temperature.

5.30 Solid Phase Extraction Elution Solution (e.g., 50 mL):
{Dichloromethane (CH_2Cl_2): IPA: NH_4OH (80: 18: 2)}.
Can be prepared by adding 9 mL of isopropanol to 1 mL of ammonium hydroxide within a 50 mL volumetric cylinder. To this mixture add 40 mL of methylene chloride and mix. This solution will be prepared when needed for use.

5.31 MeOH $_{(aq)}$ (20% (v/v)): Can be prepared by adding 80 mL of water and 20 mL of methanol to a 100 mL volumetric cylinder and mixing. Stable for one (1) month in glass container while at room temperature.

5.32 Mobile Phase A – 0.01 %(v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$:
Can be prepared by mixing 50 µL of formic acid with 0.5 mL of 5M ammonium formate in a 500 mL volumetric cylinder, diluting to volume with water, and mixing well. Store in glass at room temperature. Stable for one (1) week while in a closed state.
{Note: 5mM $NH_4CHOO_{(aq)}$ is equivalent to 0.0032 % (w/v) $NH_4CHOO_{(aq)}$.}

5.33 Mobile Phase B – (MeOH or CH_3OH). Store in glass at room temperature – Follow manufacturer specification for stability.

Note: Volumes for reagent preparations can be adjusted using appropriate ratios in order to account for the number of samples that are to be extracted within specific batches (~2 mL are needed for each extraction sample).

6. Standards and Controls

Positive Controls, Negative Control, and Calibrators:
Can be purchased (e.g., UTAK Labs) or generated in-house (e.g., spiked/un-spiked blank blood). Store frozen, refrigerated, or obtain fresh. If purchased, the stability should be determined by the manufacturer. Appropriate positive and negative controls will be extracted and analyzed with each assay or batch. When possible, control and calibrator solutions will be matrix-matched.

Internal Standard Analytes:
Purchased from suitable vendor(s) in solutions or neat solids (e.g., 0.1 mg/mL solution). Storage and stability is determined by manufacturer(s). Additional deuterated compounds may be used. If purchased solutions vary in concentration than what has typically been used, analysts may adjust the preparation volumes to account for differences. Any variations will be noted on solution preparation worksheets and, if such worksheets are not linked to case files, variations will be noted in case files (e.g., notes, summary sheets).

Preparation of Standards, Controls, and Calibrators:

Internal Standard(s) {Cocaine-D_3 and Benzoylecgonine-D_3}

6.1 Internal Standard (I.S.) Stock Solution (10 µg/mL Cocaine-D_3; 25 µg/mL Benzoylecgonine-D_3): Can be prepared by adding 100 µL of the 1 mg/mL Cocaine-D_3 and 250 µL of the 1 mg/mL Benzoylecgonine-D_3 into a 10 mL volumetric flask and diluting with MeOH and mix. Stable in freezer for 1 year.

6.2 Internal Standard (I.S.) Working Solution (1.0 µg/mL Cocaine-D_3; 2.5 µg/mL Benzoylecgonine-D_3): Can be prepared by adding 1000 µL of the Internal Standard Stock Solution into a 10 mL volumetric flask and diluting with MeOH and mix. Stable in freezer for 1 year.

Note: If other drugs/metabolites are to be validated then the same concentrations of drug and deuterated analog will be utilized, respectively).

Controls

6.3 Negative Control Blood:

Purchased (e.g., Diagnostics Products Corporation, UTAK Labs) or donated (e.g., American Red Cross, hospital blood bank). Store frozen or refrigerated. If purchased or donated then the stability should, when possible, be determined by manufacturer. A negative control blood should be extracted and analyzed with each appropriate batch.

6.4 Positive Control Solutions (In-House ; 1 mg/mL (prepared from reference standard (e.g., from Lipomed or equivalent)) {Cocaine, Cocaethylene, and Benzoylecgonine}

6.4.1 Positive Control Working Solution 1: (10 µg/mL of Cocaine/Cocaethylene/BE ; In-House (prepared from reference standard (e.g., from Lipomed or equivalent))

Combine 100 µL of each 1 mg/mL reference standards into a 10mL volumetric flask, bring to a final volume with methanol, and mix. Stable in freezer for 1 year.

6.4.2 Positive Control Working Solution 2: (1 µg/mL of Cocaine/Cocaethylene/BE ; In-House (prepared from reference standard (e.g., from Lipomed or equivalent))

Pipette 1 mL of the 10 µg/mL Positive Control Solution 1 into a 10 mL volumetric flask, bring to a final volume with methanol, and mix. Stable in freezer for 1 year.

Calibrators

Solutions used for calibrations should be prepared using as few dilutions as possible and will be from certified reference materials (CRM). While this procedure lists specific analytes, equivalent reference standards may be substituted, if needed. Calibrators should be from different suppliers than those used for control solutions, when possible. If the same supplier is used for both, then calibrator and control solutions should be prepared from solutions containing differing lot numbers or prepared by separate analysts. When purchased standards are not 1 mg/mL concentrations, then appropriate adjustments will be made and a second analyst should confirm calculations, dilutions, and other preparatory work so as to ensure quality. Such changes will be recorded within appropriate case notes, batch preparation documents, and/or reagent log books.

6.5 Calibrator Solutions (In-House ; 1 mg/mL (prepared from reference standard (e.g., from Cerilliant or equivalent)) {Cocaine, Cocaethylene, and Benzoylecgonine}

6.5.1 Calibrator Working Solution 1: (10 µg/mL of Cocaine/Cocaethylene/BE ; In-House (prepared from reference standard (e.g., from Cerilliant or equivalent))

Combine 100 µL of each 1 mg/mL reference standards into a 10mL volumetric flask, bring to a final volume with methanol, and mix. Stable in freezer for 1 year.

6.5.2 Calibrator Working Solution 2: (1.0 µg/mL of Cocaine/Cocaethylene/BE ; In-House (prepared from reference standard (e.g., from Cerilliant or equivalent))

Pipette 1 mL of the 10 µg/mL Calibrator Solution 1 into a 10 mL volumetric flask, bring to a final volume with methanol, and mix. Stable in freezer for 1 year.

7. Procedure

7.1 Add 0.25 mL of specimen, unless directed to analyze with dilutions, into properly labeled screw-capped test tubes. Within each batch of samples ensure that the correct samples transfer into the correct tubes. Cap after each sample transfer or otherwise reduce the risk of cross-contamination between samples (e.g., by moving tubes after each addition).

7.2 Prepare Calibrator and Controls

Calibrators and Controls are prepared in 0.25 mL of drug-free whole blood.

Calibrator (Concentration)	Volume of Matrix	Volume of Working Solutions
Cal 1 (20 ng/mL)	0.25 mL	5 µL of Calibrator Working Solution 2
Cal 2 (50 ng/mL)	0.25 mL	12.5 µL of Calibrator Working Solution 2
Cal 3 (100 ng/mL)	0.25 mL	25 µL of Calibrator Working Solution 2
Cal 4 (200 ng/mL)	0.25 mL	5 µL of Calibrator Working Solution 1
Cal 5 (500 ng/mL)	0.25 mL	12.5 µL of Calibrator Working Solution 1
Cal 6 (1000 ng/mL)	0.25 mL	25 µL of Calibrator Working Solution 1
Negative Control (0 ng/mL)	0.25 mL	0 µL
Low Control (40 ng/mL)	0.25 mL	10 µL of Positive Control Working Solution 2
Medium Control (150 ng/mL)	0.25 mL	37.5 µL of Positive Control Working Solution 2
High Control (400 ng/mL)	0.25 mL	10 µL of Positive Control Working Solution 1

7.3 Add 50 µL of Internal Standard (I.S.) Working Solution into each sample test tube, cap, and vortex-mix (e.g., ~10 seconds).

7.4 Add 1 mL of 0.10 M sodium acetate buffer (pH ~4.5) into each sample test tube and cap.

7.5 Add 500 µL of water to each sample test tube and cap.

7.6 Vortex-mix each sample test tube (e.g., ~10 seconds) and then sonicate for at least 15 minutes.

7.7 Centrifuge all tubes for ~10 min at ~5000 rpm.

7.8 Precondition the SPE columns (do not allow sorbent to dry):

7.8.1 Methanol (1 mL)

7.8.2 Water (1 mL)

7.9 Add the samples to properly labeled SPE columns:

7.9.1 Slowly decant the supernatant from the samples (use caution to avoid debris) to the column

7.9.2 Use pressure to push through the samples at 1-2 mL/minute.

7.10 Sequentially perform the following wash/rinse on each SPE column:

7.10.1 Bicarbonate/Carbonate $Buffer_{(aq)}$ (1 mL ; pH~9)

7.10.2 Water (1 mL)

7.11 Dry the columns for ~10 minutes using maximum pressure (e.g., between 60-80 psi).

7.12 During this 10 minute window (or earlier) actions such as the labeling of the collection tubes along with placing them into the appropriate order within the SPE collection rack for elution collection can be performed.

7.13 Once the SPE columns are dry replace the plastic waste tray with the SPE collection rack containing the collection tubes. Ensure that each tube is placed under their corresponding SPE column for elution.

7.14 Elute the SPE columns:

7.14.1 Add 2 mL of the Solid Phase Extraction Elution Solution (80:18:2 CH_2Cl_2: IPA: NH_4OH) to each SPE column.

7.14.2 Collect eluent using gravity or low pressure (<3 psi)

7.15 Remove the top SPE column rack and transfer the collection rack from the SPE manifold to the sample concentrator. Ensure concentrator parts are clean and free of contamination.

7.16 Evaporate all samples to dryness at ~<40º C.

7.17 Reconstitute each extract sample with 500 µL of $MeOH_{(aq)}$ (20% (v/v))

7.18 Vortex-mix and transfer elution solutions into properly labeled auto sampler vials containing sample inserts.

7.19 If samples are not to be immediately analyzed they should be tightly capped, protected from light, and placed into a refrigerated environment (i.e. autosampler rack on instrument or refrigerator).

Note: The word 'immediately' refers to instrumental sequences being started on the same day.

7.20 Inject on LC/MS and analyze appropriately.

Setting-up Instrument with Samples:

7.21 Ensure the appropriate instrumental quality assurance/quality control (QA/QC) procedures were performed. The instrument must have passing QA/QC results prior to preparing and loading of samples.

7.22 Prepare the sequence and enter the samples in the appropriate order. Negative controls will be analyzed prior to evidentiary samples. Blank samples (i.e., those containing just $MeOH_{(aq)}$ (20% (v/v))) may be analyzed in-between evidentiary samples to avoid carry-over and shall be analyzed after the highest calibrator is injected.

7.23 Place the labeled autosampler vials in the appropriate order within the instrument.

7.24 Save the sequence to the day's date. Ensure lab number, operator/analyst's name(s), and instrument name are recorded within each sample.

7.25 Sequence Verification:

7.25.1 Print the sequence list.

7.25.2 Check that the physical placement of the autosampler vials and the vial positions within the instrument's sequence list match.

7.25.3 Once the check has been completed place an indication of the sample check (e.g., 'sequence checked' or 'sequence verified') on the sequence page along with Analyst's initials and date.

7.26 Print the instrument method and include both the method and the sequence printouts with the batch documents.

7.27 Begin the sequence and analyze the samples.

8. Instrumental Parameters

The following are the typical operating parameters for the instrument used in this procedure. With documented approval from the Lead Examiner and Assistant Director (or higher), the instrument conditions may be modified to adjust or improve the procedure. Documentation of such changes must be included with batch data so that any instrumental parameter change can be associated with data and casework until this procedure has been updated. For more specific parameters, see method printout(s) attached to this procedure or contained within appropriate instrument binders.

HPLC Parameters:

Mobile Phase A: 0.01 % (v/v) $HCOOH_{(aq)}$ and 5mM $NH_4CHOO_{(aq)}$
Mobile Phase B: $MeOH_{(l)}$ (neat)
HPLC Column: Kinetex, 2.6 µm Phenyl-Hexyl, 100 Å, 50 mm x 4.6 mm (Phenomenex or equivalent)
Column Temperature: 40 °C
Constant Flow: 0.6 mL/min.
Autosampler Temperature: 15 °C
Injection Volume: 5 µL
Needle Wash: 500 µL before and after aspiration

Gradient Program: Initial 75% A and 25% B; Flow 0.6 mL/min

Time (min.)	Mobile Phase A [0.01 %(v/v) $HCOOH_{(aq)}$ + 5mM $NH_4CHOO_{(aq)}$] (%)	Mobile Phase B $MeOH_{(neat)}$ (%)
Initial	75	25
4.00	5	95
5.00	5	95
5.01	75	25
7.00	STOP	STOP

Mass Spectrometer Parameters:

Ionization Source	Heated Electrospray Ionization (HESI)*	**Polarity**	Positive Ion
HESI Gases	Nitrogen	**Scan Type**	MRM & Full Scan
Ion Spray Potential	+4.5 kV	**Resolution**	Unit

*Only on the LCMS-02 instrument – the LCMS-01 instrument only has ESI

9. Decision Criteria

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

9.1 If a solvent blank was injected, it must be reviewed for possible carryover.

9.1.1 The solvent blank shall not contain any analyte measured by this assay which meets reporting criteria (retention time and peak shape). If an analyte is present in the solvent blank and the following injection, this analyte shall not be reported. Upon re-injection of the solvent blank and the corresponding sample, if the solvent blank acceptance criteria are met, proceed with analysis.

9.1.2 If a case specimen exceeds the 2000 ng/mL and a solvent blank was not run immediately after it, repeat or reinject (with a solvent blank prior) the next case specimen if that specimen is positive for the analyte that exceeded 2000 ng/mL.

9.2 Chromatography

All chromatographic peaks for the analytes of interest should show good chromatographic characteristics, with reasonable peak shape, width, and resolution. For low concentrations of an analyte (e.g., ≤5 ng/mL), there may be transitions that are not optimal. In order to be determined as acceptable, a chromatographic peak in a sample should compare favorably to the same analytes chromatographic peak in a known sample which has been analyzed on the same system and in the same, or subsequent, analytical timeframe.

9.3 Retention Time (RT)

The retention time of a peak of interest should be within 0.1 minute of the retention time of a reference standard (i.e., calibrator or positive control).

9.4 Mass Spectrometry

Ion ratios should compare favorably to ion ratios of an extracted calibrator or positive control at a comparable concentration (e.g., positive control). Generally, ion ratios are within the limits as specified within the Section procedure related to mass spectral comparisons. NOTE: With the exceptin of the internal standard, it is recognized that some ion ratios are concentration dependent; thus, concentrations at the ends of the calibration curve may not be within the updated ratios and may be acceptable.

9.5 Batch Acceptance:

In order for a batch to be acceptable:

9.5.1 No analytes of interest will be detected in the Negative Control.

9.5.2 Significant carry-over will be brought to the attention of the lead Examiner to determine if evidentiary samples have been negatively impacted. If so, re-analyses will occur and sample re-extraction may be necessary. Appropriate case documentation will accompany these instances within affected case files to record events.

9.5.3 Quantitative values greater than the upper limit of quantitation can be reported as "greater than" and no uncertainty value will be associated with that result.

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Analytes of interest are considered those compounds that are being reported.

9.5.4 All applicable analytes of interest within Positive Controls, as well as internal standards, will be identified.

9.5.5 Quantitative results of positive controls must fall within ±20% of the analytes target concentration. If one drug does not demonstrate acceptable quantitative results, all other drugs that are acceptable may be reported.

9.5.6 Statistical data of positive controls should be recorded and evaluated within appropriate charts.

10. Calibration

Calculations are performed by the applicable instrument software.

A correlation coefficient should be ≥ 0.990 when using deuterated internal standards. If the correlation coefficient is lower than 0.990 then approval from the appropriate lead analyst or higher must occur and, if deemed acceptable, justification must accompany the applicable data. For Cocaethylene, a corresponding deuterated internal standard is not used, a correlation coefficient ≥ 0.98 is acceptable. Following a weighted linear regression, reprocessed calibrators shall be within 20% of their target value. Calibrators shall not be removed from calibration graphs without approval from the lead Examiner or higher. Such removal of calibration points will be appropriately documented within batches and within each case file (e.g., case notes, summary sheets). Calibration data are used for the analysis of each batch and are done independently.

Such instances may involve reporting results as 'greater than the highest calibrator' may be acceptable and would avoid dilution and re-analysis. The lower limit of quantitation (LLOQ) is the concentration of the lowest calibrator. The upper limit of quantitation (ULOQ) is the concentration of the highest calibrator.

11. Limitations

Limits of Detection (LOD), Lower Limits of Quantitation (LLOQ), Upper Limits of Quantitation (ULOQ):

Analyte	LOD – Blood (ng/mL)	LLOQ – Blood (ng/mL)	ULOQ – Blood (ng/mL)
Benzoylecgonine	20	20	1000
Cocaethylene	20	20	1000
Cocaine	20	20	1000

12. Stability Post-Extraction

Calibrators, controls or case specimens may be re-injected within 24 hours for LCMS-01 or 96 hours for LCMS-03 of being injected with a solvent blank prior. The chromatograms for both injections are saved with the data packet. A positive control must be re-injected with case specimens to verify that the curve is still acceptable if the sequence was completed.

If a case specimen(s) is re-injected after 24 hours for LCMS-01 or 96 hours for LCMS-03, the calibrators and controls shall be re-injected along with the case specimen(s). NOTE: This would be considered a new batch and prepared separately from the original runs.

If a case specimen(s) was inadvertently not injected and it is more than 48 hours since the first calibrator was injected, you shall re-inject the calibrators, controls and the reagent blank with the case specimen(s). NOTE: This would be considered a new batch analytical run and prepared separately from the original runs.

13. Safety

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

14. References

In-House validation documentation

Solid-Phase Extraction Method for Trace B Columns. SPEWARE: Baldwin Park, CA.

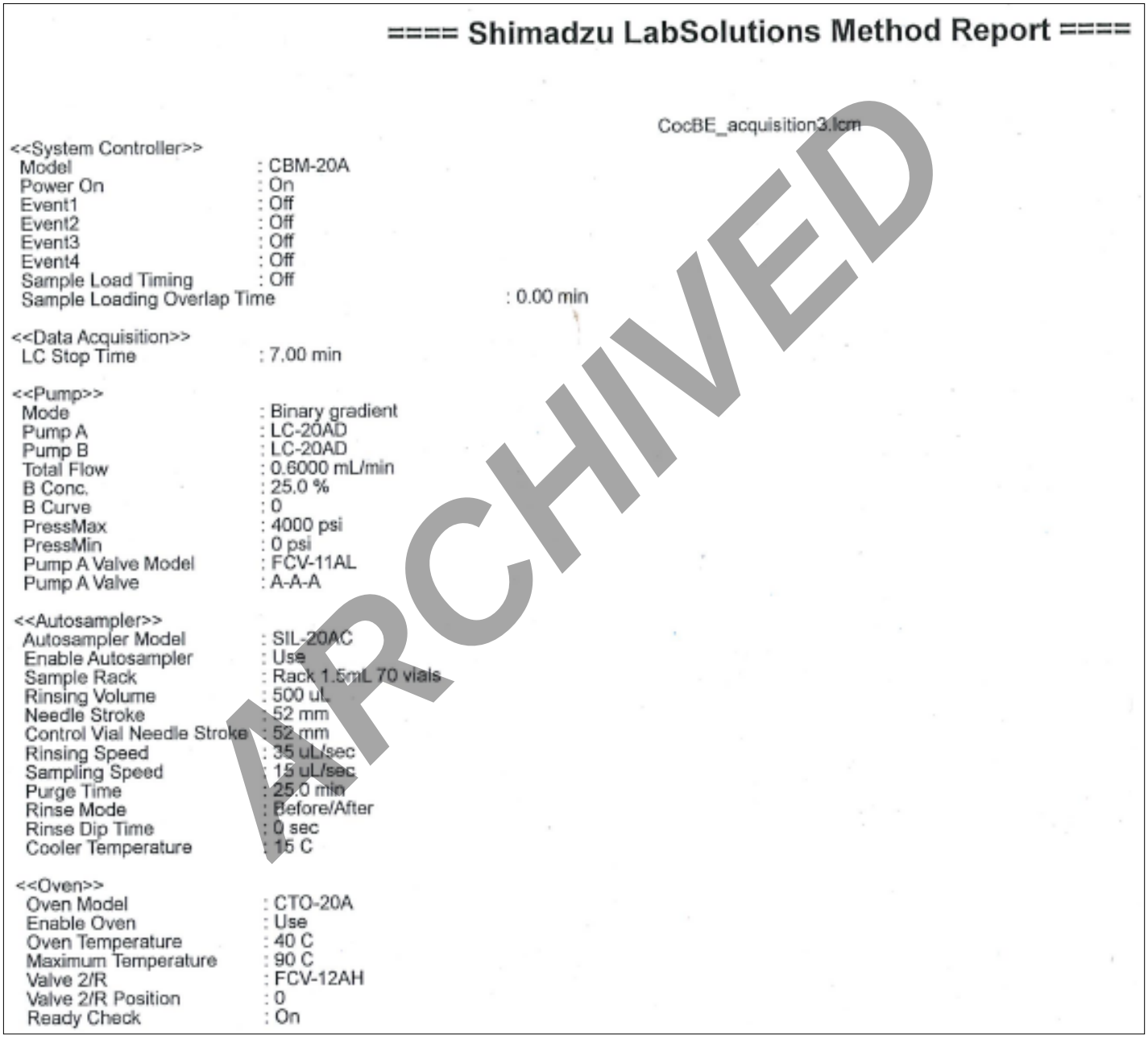
Mule, S. J.; Casella, G. A. J. Anal. Toxicol. 1988, 12, 153-155.

Jeanville, P.M.; Estape, E.S.; et al. J. Am. Soc. Mass Spectrom. 2000, 11, 257-263.

Jeanville, P.M.; Estape, E.S.; et al. J. Anal. Toxicol. 2001, 25, 69-75.

15. Appendix

LCMS-01 (Shimadzu):



==== Shimadzu LabSolutions Method Report ====

CocBE_acquisition3.lcm

<<System Controller>>
Model : CBM-20A
Power On : On
Event1 : Off
Event2 : Off
Event3 : Off
Event4 : Off
Sample Load Timing : Off
Sample Loading Overlap Time : 0.00 min

<<Data Acquisition>>
LC Stop Time : 7.00 min

<<Pump>>
Mode : Binary gradient
Pump A : LC-20AD
Pump B : LC-20AD
Total Flow : 0.6000 mL/min
B Conc. : 25.0 %
B Curve : 0
PressMax : 4000 psi
PressMin : 0 psi
Pump A Valve Model : FCV-11AL
Pump A Valve : A-A-A

<<Autosampler>>
Autosampler Model : SIL-20AC
Enable Autosampler : Use
Sample Rack : Rack 1.5mL 70 vials
Rinsing Volume : 500 uL
Needle Stroke : 52 mm
Control Vial Needle Stroke : 52 mm
Rinsing Speed : 35 uL/sec
Sampling Speed : 15 uL/sec
Purge Time : 25.0 min
Rinse Mode : Before/After
Rinse Dip Time : 0 sec
Cooler Temperature : 15 C

<<Oven>>
Oven Model : CTO-20A
Enable Oven : Use
Oven Temperature : 40 C
Maximum Temperature : 90 C
Valve 2/R : FCV-12AH
Valve 2/R Position : 0
Ready Check : On

LCMS-01 (Shimadzu) (Cont'd):

<<LC Time Program>>

Time	Module	Command	Value	Comment
0.50	Column Oven	CTO.RVR	1	
4.00	Pumps	B.Conc	95	
5.00	Pumps	B.Conc	95	
5.01	Pumps	B.Conc	25	
6.50	Column Oven	CTO.RVR	0	
7.00	Controller	Stop		

<<MS Parameter>>

CID Gas :Use the Data in the Tuning File
Conversion Dynode :Use the Data in the Tuning File
Initial Valve Position :-
--Segment 1 Event 1--
Acquisition Mode :MRM
Polarity :Positive
Start Time :0.000 min
End Time :6.000 min
Compound Name :BE-D3_postrl
Ch1 (Precursor m/z) 293.05 (Product m/z) 171.15 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -11.0 (CE) -22.0 (Q3 Pre Bias) -12.0
Ch2 (Precursor m/z) 293.05 (Product m/z) 105.10 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -11.0 (CE) -33.0 (Q3 Pre Bias) -21.0
Ch3 (Precursor m/z) 293.05 (Product m/z) 77.15 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -11.0 (CE) -53.0 (Q3 Pre Bias) -29.0
Event Time :0.069 sec
Q1 Resolution :Unit
Q3 Resolution :Unit
Micro Scan Width :0.00 u
Interface Volt. :Use the Data in the Tuning File
DUIS Corona Needle Volt. :Use the Data in the Tuning File
Pause Time / Dwell Time :Set Value of Each Channel (Pause Time & Dwell Time)
DL Bias :Use the Data in the Tuning File
Qarray Bias :Use the Data in the Tuning File
Qarray RF :Use the Data in the Tuning File
Q1 Prerod Bias :Set Data
Q3 Prerod Bias :Set Data
--Segment 1 Event 2--
Acquisition Mode :MRM
Polarity :Positive
Start Time :0.000 min
End Time :6.000 min
Compound Name :Cocaine-D3
Ch1 (Precursor m/z) 307.20 (Product m/z) 85.10 (Pause Time) 3.0 (Dwell Time) 50.0 (CE) -35.0
Ch2 (Precursor m/z) 307.20 (Product m/z) 105.00 (Pause Time) 3.0 (Dwell Time) 50.0 (CE) -35.0
Ch3 (Precursor m/z) 307.20 (Product m/z) 185.10 (Pause Time) 3.0 (Dwell Time) 50.0 (CE) -35.0
Event Time :0.159 sec
Q1 Resolution :Unit
Q3 Resolution :Unit
Micro Scan Width :0.00 u
Interface Volt. :Use the Data in the Tuning File
DUIS Corona Needle Volt. :Use the Data in the Tuning File
Pause Time / Dwell Time :Set Value of Each Channel (Pause Time & Dwell Time)
DL Bias :Use the Data in the Tuning File
Qarray Bias :Use the Data in the Tuning File
Qarray RF :Use the Data in the Tuning File
Q1 Prerod Bias :Use the Data in the Tuning File
Q3 Prerod Bias :Use the Data in the Tuning File
--Segment 2 Event 3--
Acquisition Mode :MRM
Polarity :Positive
Start Time :0.000 min

LCMS-01 (Shimadzu) (Cont'd):

End Time :6.100 min
Compound Name :Cocaethylene_postoarl
Ch1 (Precursor m/z) 318.05 (Product m/z) 196.10 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -12.0 (CE) -22.0
(Q3 Pre Bias) -14.0
Ch2 (Precursor m/z) 318.05 (Product m/z) 82.15 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -12.0 (CE) -34.0
(Q3 Pre Bias) -17.0
Ch3 (Precursor m/z) 318.05 (Product m/z) 105.10 (Pause Time) 3.0 (Dwell Time) 20.0 (Q1 Pre Bias) -12.0 (CE) -39.0
(Q3 Pre Bias) -11.0
Event Time :0.069 sec
Q1 Resolution :Unit
Q3 Resolution :Unit
Micro Scan Width :0.00 u
Interface Volt. :Use the Data in the Tuning File
DUIS Corona Needle Volt. :Use the Data in the Tuning File
Pause Time / Dwell Time :Set Value of Each Channel (Pause Time & Dwell Time)
DL Bias :Use the Data in the Tuning File
Qarray Bias :Use the Data in the Tuning File
Qarray RF :Use the Data in the Tuning File
Q1 Prerod Bias :Set Data
Q3 Prerod Bias :Set Data
--Segment 3 Event 4--
Acquisition Mode :MRM
Polarity :Positive
Start Time :0.000 min
End Time :6.200 min
Compound Name :BE
Ch1 (Precursor m/z) 290.00 (Product m/z) 168.10 (Pause Time) 1.0 (Dwell Time) 10.0 (Q1 Pre Bias) -14.0 (CE) -21.0
(Q3 Pre Bias) -18.0
Ch2 (Precursor m/z) 290.00 (Product m/z) 77.15 (Pause Time) 1.0 (Dwell Time) 10.0 (Q1 Pre Bias) -14.0 (CE) -51.0
(Q3 Pre Bias) -14.0
Ch3 (Precursor m/z) 290.00 (Product m/z) 105.10 (Pause Time) 1.0 (Dwell Time) 10.0 (Q1 Pre Bias) -14.0 (CE) -30.0
(Q3 Pre Bias) -21.0
Event Time :0.033 sec
Q1 Resolution :Unit
Q3 Resolution :Unit
Micro Scan Width :0.00 u
Interface Volt. :Use the Data in the Tuning File
DUIS Corona Needle Volt. :Use the Data in the Tuning File
Pause Time / Dwell Time :Set Value of Each Channel (Pause Time & Dwell Time)
DL Bias :Use the Data in the Tuning File
Qarray Bias :Use the Data in the Tuning File
Qarray RF :Use the Data in the Tuning File
Q1 Prerod Bias :Set Data
Q3 Prerod Bias :Set Data

LCMS-01 (Shimadzu) (Cont'd):

--Segment 4 Event 5--

Acquisition Mode	:MRM				
Polarity	:Positive				
Start Time	:0.000 min				
End Time	:6.300 min				
Compound Name	:Cocaine_postoarl				
Ch1 (Precursor m/z) 304.05 (Q3 Pre Bias) -13.0	(Product m/z) 182.15	(Pause Time) 1.0	(Dwell Time) 10.0	(Q1 Pre Bias) -12.0	(CE) -21.0
Ch2 (Precursor m/z) 304.05 (Q3 Pre Bias) -17.0	(Product m/z) 82.10	(Pause Time) 1.0	(Dwell Time) 10.0	(Q1 Pre Bias) -12.0	(CE) -32.0
Ch3 (Precursor m/z) 304.05 (Q3 Pre Bias) -15.0	(Product m/z) 77.15	(Pause Time) 1.0	(Dwell Time) 10.0	(Q1 Pre Bias) -12.0	(CE) -55.0
Event Time	:0.033 sec				
Q1 Resolution	:Unit				
Q3 Resolution	:Unit				
Micro Scan Width	:0.00 u				
Interface Volt.	:Use the Data in the Tuning File				
DUIS Corona Needle Volt.	:Use the Data in the Tuning File				
Pause Time / Dwell Time	:Set Value of Each Channel (Pause Time & Dwell Time)				
DL Bias	:Use the Data in the Tuning File				
Qarray Bias	:Use the Data in the Tuning File				
Qarray RF	:Use the Data in the Tuning File				
Q1 Prerod Bias	:Set Data				
Q3 Prerod Bias	:Set Data				
<<MS Program>>					
<<Analog Table>>					
<<Interface>>					
Interface	:DUIS - DUIS				
Interface Temperature	:350 C				
DL Temperature	:250 C				
Nebulizing Gas Flow	:2.00 L/min				
Heat Block	:400 C				
Drying Gas	:On				
Drying Gas Flow	:15.00 L/min				

LCMS-02 (LTQ):

```
Instrument Method:  COCBE_V2_031522_HESI.meth

<Title not set>
Program for Dionex Chromatography MS Link

; Please click "Wizard" to start the
; Chromeleon Program Wizard.
; It will guide you through program creation.
       %C.Equate =                    "H2O/FormicAcid/AmmoniumFormate"
       ColumnOven.TempCtrl =          On
       ColumnOven.Temperature.Nominal =  40.0 [°C]
       ColumnOven.Temperature.LowerLimit =  5.0 [°C]
       ColumnOven.Temperature.UpperLimit =  110.0 [°C]
       EquilibrationTime =            0.5 [min]
       ColumnOven.ReadyTempDelta =    5.0 [°C]
       Pressure.LowerLimit =          0 [psi]
       Pressure.UpperLimit =          10000 [psi]
       %D.Equate =                    "MeOH"
       DrawSpeed =                    3.333 [µl/s]
       DrawDelay =                    1 [ms]
       DispSpeed =                    20.000 [µl/s]
       DispenseDelay =                0 [ms]
       WasteSpeed =                   32.000 [µl/s]
       SampleHeight =                 2.000 [mm]
       InjectWash =                   Both
       WashVolume =                   100.000 [µl]
       WashSpeed =                    33.333 [µl/s]
       LoopWashFactor =               2.000
       PumpDevice =                   "Pump"
       InjectMode =                   Normal
       Sampler.TempCtrl =             On
       Sampler.Temperature.Nominal =  15.0 [°C]
       Sampler.Temperature.LowerLimit =  4.0 [°C]
       Sampler.Temperature.UpperLimit =  45.0 [°C]
       Sampler.ReadyTempDelta =       1.0 [°C]

 0.000 Wait                           ColumnOven.Ready and Pump.Ready and Sampler.Ready and
PumpModule.Ready
       ;Chromeleon sets this property to signal to Xcalibur that it is ready to start a
run.
       ReadyToRun =                   1
       ;Xcalibur sets this property to start the run or injection.
       Wait                           StartRun
       Flow =                         0.600 [ml/min]
       %B =                           0.0 [%]
       %C =                           75.0 [%]
       %D =                           25.0 [%]
       Wait                           ColumnOven.Ready and Pump.Ready and Sampler.Ready and
PumpModule.Ready
       Inject
       InjectResponse =               1
       ;Chromeleon sets this property to signal the injection to Xcalibur.
       ;Depending on your system configuration it might be necessary to manually insert
       ;a "Relay" command below in order to send the start signal to the MS.
       ;Typical syntaxes:
       ;Pump_Relay_1.Closed  Duration = 2.00
       ;UM3PUMP_Relay1.On    Duration = 2.00
```

LCMS-02 (LTQ) (Cont'd):

```
Instrument Method:  COCBE_V2_031522_HESI.meth

        Flow =                  0.600 [ml/min]
        %B =                    0.0 [%]
        %C =                    75.0 [%]
        %D =                    25.0 [%]

  4.000 Flow =                  0.600 [ml/min]
        %B =                    0.0 [%]
        %C =                    5.0 [%]
        %D =                    95.0 [%]

  5.000 Flow =                  0.600 [ml/min]
        %B =                    0.0 [%]
        %C =                    5.0 [%]
        %D =                    95.0 [%]

  5.010 Flow =                  0.600 [ml/min]
        %B =                    0.0 [%]
        %C =                    75.0 [%]
        %D =                    25.0 [%]

  7.000 Flow =                  0.600 [ml/min]
        %B =                    0.0 [%]
        %C =                    75.0 [%]
        %D =                    25.0 [%]

 7.500 InjectResponse =         0
        End
```

LCMS-02 (LTQ) (Cont'd):

```
Instrument Method:  COCBE_V2_031522_HESI.meth

                        LTQ XL Instrument Method

Creator: LTQ XL
Last modified: 4/22/2022 by LTQ XL

MS Run Time (min): 7.00

Sequence override of method parameters not enabled.

Divert Valve:  in use during run

                        Divert Time (min)   Valve State
                        =================   ===========
                                0.00          To Waste
                                3.00          To Source
                                5.00          To Waste

Contact Closure:  not used during run

Syringe Pump:  not used during run

MS Detector Settings:

Real-time modifications to method not enabled

Stepped collision energy not enabled

Additional Microscans:
        MS2     0       0
        MS3     0       0
        MS4     0       0
        MS5     0       0
        MS6     0       0
        MS7     0       0
        MS8     0       0
        MS9     0       0
        MS10    0       0

Segment 1 Information

Duration (min):         7.00
Number of Scan Events: 6
Tune Method:            COC_HESI_600uL_Pos_031422

Scan Event Details:
 1:  ITMS + c norm o(280.0-320.0)
       CV = 0.0V
 2:  ITMS + c norm !corona !pi ·( 290.10)->o(75.0-300.0)
       MS/MS:  AT   CID CE 35.0%   Q 0.250   Time 5.000   IsoW 1.0
       CV = 0.0V
 3:  ITMS + c norm !corona !pi ·( 304.20)->o(80.0-300.0)
       MS/MS:  AT   CID CE 35.0%   Q 0.250   Time 5.000   IsoW 1.0
F      CV = 0.0V2 8:39:21 AM                                Page 3 of 4
 4:  ITMS + c norm !corona !pi ·( 318.10)->o(85.0-300.0)
```

LCMS-02 (LTQ) (Cont'd):

Instrument Method: COCBE_V2_031522_HESI.meth

LTQ XL Instrument Method

```
      MS/MS:  AT   CID CE 35.0%   Q 0.250   Time 5.000   IsoW 1.0
      CV = 0.0V
 5:  ITMS + c norm ·( 307.20)->o(80.0-300.0)
      MS/MS:  AT   CID CE 35.0%   Q 0.250   Time 5.000   IsoW 1.0
      CV = 0.0V
 6:  ITMS + c norm ·( 293.20)->o(80.0-300.0)
      MS/MS:  AT   CID CE 35.0%   Q 0.250   Time 5.000   IsoW 1.0
      CV = 0.0V

Custom Data Dependent Settings:
      Not enabled
```

Rev. #	History
1	New document