CONFIRMATION OF SELECT AMPHETAMINES BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

39.1 **METHOD**

This test method may be used to confirm the presence of amphetamine (AMP), methamphetamine (METH), pseudoephedrine (PSED), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in biological specimens.

The targeted compounds and internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological specimens by liquid-liquid extraction (LLE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

39.2 **SPECIMENS**

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Matrix-matching of the full calibration curve and all positive control levels is not required for quantitation of target compounds in serum or liver (tissue) homogenate samples, as determined through evaluation of alternative matrix (serum, liver homogenate) during method validation (see 39.4.3.2).

39.3 **REAGENTS, MATERIALS AND EQUIPMENT**

39.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used, unless otherwise specified.

- Acetonitrile (ACN), reagent grade and LC-MS grade
- n-butyl chloride
- Certified blank blood and/or other biological matrices
- DI H_2O (laboratory general-use) and LC-MS grade H_2O (or equivalent from a high-purity filtration system)
- Formic acid (concentrated)
- 0.1% Formic acid in LC-MS grade H_2O

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade H_2O and mix. Store the solution in an amber glass bottle at room temperature for up to one year.

  NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.
- Hydrochloric acid (HCl, concentrated)

- 1% Hydrochloric acid in methanol

  Add 45 mL methanol to a graduated cylinder. Carefully add 0.5 mL concentrated HCl and bring total volume to 50 mL with methanol and mix. Store the solution in a glass bottle at room temperature for up to 1 month.

- Methanol (MeOH), reagent grade and HPLC grade
- Sodium borate decahydrate ($Na_2B_4O_7 \bullet 10H_2O$)
- 0.13M Sodium borate solution (saturated)

  In a 100 mL flask, dissolve 4.9 g $Na_2B_4O_7 \bullet 10H_2O$ in approximately 75 mL DI H_2O. Dilute to 100 mL with DI H_2O and mix thoroughly (may require low heating). The weighed contents may not go completely into solution. This is normal. Store the solution in a glass bottle at room temperature for up to 6 months.

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

39.3.2 MATERIALS

- Polypropylene autosampler vials with integrated inserts and caps
- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- HPLC Column, Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7µM particle size, or equivalent
- Laboratory glassware (graduated cylinders, flasks)

39.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series or equivalent)
- Agilent MS-MS with API-ES source (6420 or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heating plate, rotary mixer, vortex mixer)

39.4 **STANDARDS, CALIBRATORS AND CONTROLS**

39.4.1 STANDARDS

- Working standard (WS): 10 ng/µL
- Working control standard (QC): 10 ng/µL
- Working internal standard (IS): 1 ng/µL

39.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 39.5 SAMPLE PREPARATION.

39.4.3 CONTROLS

39.4.3.1 At least one negative whole blood control and three positive whole blood controls are tested with every batch, prepared as described in 39.5.

39.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

NOTE: Serum is an appropriate matrix match for vitreous specimens.

39.4.3.3 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls. When the batch contains more than 20 specimens, a positive control must be analyzed mid-run.

39.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

39.5 **SAMPLE PREPARATION**

39.5.1 Label a clean extraction tube (16 x 100 mm recommended) for each member of the test batch. (i.e., calibrator, control, case sample).

39.5.2 Add 2 mL sodium borate solution into each tube.

39.5.3 Using a calibrated pipette, add 0.5 mL of certified blank whole blood into each of the calibrator tubes, the positive control tubes and the negative control tube(s).

39.5.4 Prepare a 1:10 dilution of the working standard. (1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

39.5.5 Prepare a 1:100 dilution of the working standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

39.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 (10 ng/mL)	50	0.1 ng/µL	1:100
Calibrator 2 (25 ng/mL)	125	0.1 ng/µL	1:100
Calibrator 3 (50 ng/mL)	25	1 ng/µL	1:10
Calibrator 4 (100 ng/mL)	50	1 ng/µL	1:10
Calibrator 5 (500 ng/mL)	25	10 ng/µL	WS
Calibrator 6 (1000 ng/mL)	50	10 ng/µL	WS

39.5.7 Prepare a 1:10 dilution of the working control standard. (1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

39.5.8 Prepare a 1:100 dilution of the working control standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation

39.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the working control standard and prepared dilution.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 (30 ng/mL)	150	0.1 ng/µL	1:100
Control 2 (400 ng/mL)	20	10 ng/µL	QC
Control 3 (800 ng/mL)	40	10 ng/µL	QC

39.5.10 Using a calibrated pipette, sample 0.5 mL of each case sample into its respective tube.

39.5.11 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL.

39.5.12 Briefly vortex mix. Let the tubes stand for 5 minutes.

39.5.13 Add 4 mL of n-butyl chloride to each tube.

39.5.14 Cap the tubes and place on a rotary mixer for 20 minutes.

39.5.15 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

39.5.16 Transfer the n-butyl chloride layer to clean, labeled 10 mL centrifuge or screw cap tubes.

39.5.17 Add 100 µL of 1% HCl in methanol to each tube and briefly vortex-mix. Do not omit this step as the recovery of the volatile amines will be reduced.

39.5.18 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

39.5.19 Reconstitute the extracts by the addition of 100 µL 0.1% formic acid in LC-MS grade H_2O to each tube and briefly vortex-mix. If necessary, cap the tubes and centrifuge for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

39.5.20 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

39.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – AMINES (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor for PSED

  quadratic, 1/a weighting factor for AMP, MDA, MDMA, MET
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4
- Result comparisons – all units in ng/mL

  Cal 1: truncated to one decimal place (acceptable range ±25%; 7.5 – 12.5 ng/mL)

  Cal 2-6, Ctl 1-3: truncated, whole integer values (acceptable range ±20%)

  NOTE: Quadratic calibration curves must include 6 calibration points; removal of one or more calibration points will prohibit quantitative reporting. Removal of more than one calibration point from the linear PSED curve will prohibit quantitative reporting.

39.7 REPORTING

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), truncated to two significant figures.

39.8 METHOD PERFORMANCE

- Limit of detection: 1 ng/mL (0.001 mg/L; AMP, METH, MDA, MDMA)

  5 ng/mL (0.005 mg/L; PSED)
- Lower limit of quantification: 10 ng/mL (0.01 mg/L)
- Dynamic range: 10 – 1000 ng/mL (0.01 – 1.0 mg/L)
- Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)
- Upper limit of linearity: 1500 ng/mL (1.5 mg/L; PSED, MDA, MDMA)

  1000 ng/mL (1.0 mg/L; AMP, METH)

39.9 REFERENCES

- A. Black, in-house method development.
- J. Hudson, J. Hutchings, R. Wagner, Amphetamines, Phentermine, and Designer Stimulant Quantitation Using an Agilent 6430 LC/MS/MS, *Agilent Application Note 5991-5059EN*, June 2015.
- J. Hudson, J. Hutchings, R. Wagner, Validation of a Quantitative Method for Amphetamines, Phentermine, and Designer Stimulants Using an Agilent 6430 LC/MS/MS, *Agilent Application Note 5991-5129EN*, June 2015.
- Pat Friel, Agilent Technologies, Inc.
- Virginia Department of Forensic Sciences, Amphetamines Quantitation/Confirmation method.

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Amphetamine
Amphetamine-d_{11} (AMP-d_{11})
MDA
MDA-d_5 (MDA-d_5)
MDMA
MDMA-d_5 (MDMA-d_5)
Methamphetamine
Methamphetamine-d_{14} (MET-d_{14})
Pseudoephedrine
Pseudoephedrine-d_3 (PSED-d_3)

APPENDIX B
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.500 mL/min
Solvent A	0.1% Formic Acid (in LC-MS grade H_2O)
Solvent B	ACN (LC-MS grade)
Initial composition	97% (A), 3% (B)
0 – 6.0 min	%B increased to 90%
Hold time	1.0 min (90%B)
7.0 – 8.0 min	%B decreased to 3%
Re-equilibration	4.0 min
Autosampler	
Column temp	50° C
Injection volume	5.0 µL
Injection flush-port	Active
Flush-port time	15 sec
Flush-port solvent	75:25/ HPLC grade MeOH:LC-MS grade H_2O

MASS SPECTROMETER

Ion mode	(+) MRM	Nebulizer gas	Nitrogen
Time filter width	0.05 min	Nebulizer pressure	50 psi
Dynamic MRM	2.0 min	Drying gas	Nitrogen
Cycle time	500 ms	Drying gas flow	12 L/min
Ion source	ESI	Drying gas temp	350° C
		Capillary voltage	4kV

Compounds	MRM Transitions
Pseudoephedrine	166.1→148.1/91.1
Pseudoephedrine-d_3	169.1→151.1/115
Amphetamine	136.1→91.1/119.1
Amphetamine-d_{11}	147.2→98.1/130.1
MDA	180.1→163.1/105.1
MDA-d_5	185.1→168.1/110.1
Methamphetamine	150.1→91.1/119.1
Methamphetamine-d_{14}	164.2→98.1/130.1
MDMA	194.1→163.1/105.1
MDMA-d_5	199.1→165.1/107.1

LIST OF CHANGES

Revision Date	Description	Page Number
2/16/17	Method approved by Washington State Toxicologist. See DRA dated 2/8/17. Method released for use in evidentiary testing as of 2/16/17.	All
6/12/17	Specified use of calibrated pipettes for measurement of blank blood, specimens, and standards throughout SAMPLE PREPARATION in 39.7. Other minor edits throughout.	3-9
11/12/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 39.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 39.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Target compound/internal standard list added in APPENDIX A, with test method parameters moved to APPENDIX B. Formatting and minor edits throughout.	All
4/1/20	Edited NOTE in section 39.3.1; moved filtration information to NOTE in prep of 0.1% formic acid (no filtration required for prep with LC-MS grade H_2O). Changed references for "LC-MS grade DI H_2O" to "LC-MS grade H_2O." NOTE regarding specific grade of H_2O and solvents used was removed from 39.5 (covered in 39.3.1). Use of mid-run control added in 39.4.3.3. Specified use of HPLC grade MeOH and LC-MS grade H_2O for flush port solvent in instrument parameters. Other minor edits throughout.	All