CONFIRMATION OF SELECT AMPHETAMINES BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

39.1 **POLICY**

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 samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

39.2 **PURPOSE**

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of select amphetamines present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compounds.

39.3 **PRINCIPLE**

The targeted compounds and internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, the specimens, now termed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion and multiple-reaction monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any target compound identified in a sample is determined from its calibration curve.

39.4 **SPECIMENS**

39.4.1 The specimen volume is 0.5 mL.

39.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

NOTE: Matrix-matching of calibrators and controls is not required for quantitation of target compounds in serum or tissue homogenate samples, as determined through evaluation of alternative matrix (serum, liver homogenate) during method validation.

39.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; in addition, the specimen may be analyzed at standard volume, as dictated by screening results, to ensure that concentrations of all target compounds present are within the dynamic range of the test method.

39.4.4 Analysis of larger specimen volumes must be approved and documented.

39.5 **REAGENTS, MATERIALS AND EQUIPMENT**

39.5.1 REAGENTS

39.5.1.1 Acetonitrile (ACN)

39.5.1.2 n-butyl chloride

39.5.1.3 Certified blank blood

39.5.1.4 Deionized water (DI H_2O)

39.5.1.5 Formic acid (concentrated)

39.5.1.6 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

39.5.1.7 Hydrochloric acid (HCl, concentrated)

39.5.1.8 1% Hydrochloric acid in methanol

Add 49 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. Adjustments to final volume are permitted as long as the proportions are maintained.

39.5.1.9 Methanol (MeOH)

39.5.1.10 Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)

39.5.1.11 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. Adjustments to final volume are permitted as long as the proportions are maintained.

39.5.2 MATERIALS

39.5.2.1 Autosampler vials (polypropylene with integrated inserts) and caps

39.5.2.2 Disposable 16 x 100mm tubes with closures

39.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

39.5.2.4 Disposable pipette tips

39.5.2.5 HPLC Column, Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7µM particle size, or equivalent

39.5.2.6 Laboratory glassware (graduated cylinders, flasks)

39.5.2.7 Solvent filters (0.45 µm pore size; reduced cellulose, other)

39.5.2.8 Volumetric glassware (flasks)

39.5.3 EQUIPMENT

39.5.3.1 Agilent HPLC (1100/1200 series or equivalent)

39.5.3.2 Agilent MS-MS with APCI/ESI source (6420 or equivalent)

39.5.3.3 Calibrated, adjustable piston pipettes

39.5.3.4 Centrifuge

39.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

39.5.3.6 pH Meter and/or indicating pH paper

39.5.3.7 Solvent filtration apparatus

39.5.3.8 Verified, adjustable repeater-pipette

39.5.3.9 Vortex mixer

39.6 **STANDARDS, CALIBRATORS AND CONTROLS**

39.6.1 STANDARDS

39.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards (which in turn are used to produce calibrators and positive controls) and the working internal standard.

39.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a.	Amphetamine:	1.0 mg/mL
b.	Amphetamine-d_{11} (AMP-d_{11}):	0.1 mg/mL
c.	Methamphetamine:	1.0 mg/mL
d.	Methamphetamine-d_{14} (METH-d_{14}):	0.1 mg/mL
e.	Pseudoephedrine:	1.0 mg/mL
f.	Pseudoephedrine-d_3 (PSED-d_3):	0.1 mg/mL
g.	MDA:	1.0 mg/mL
h.	MDA-d_5:	0.1 mg/mL
i.	MDMA:	1.0 mg/mL
j.	MDMA-d_5:	0.1 mg/mL

39.6.1.3 Working standard (10 ng/µL)

a. Using calibrated pipettes, measure 250 µL each of AMP, METH, PSED, MDA and MDMA stock standards into a 25 mL class-A volumetric flask.
b. Add acetonitrile or methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

39.6.1.4 Working internal standard (1 ng/µL)

a. Using calibrated pipettes, measure 250 µL each of AMP-d11, METH-d14, MDA-d5, MDMA-d5 and PSED-d3 stock internal standards and add to a 25 mL class-A volumetric flask.
b. Add acetonitrile or methanol to the flask to designated volume.
c. The final concentration of the working internal standard is 1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

39.6.2 CALIBRATORS

39.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 39.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure. If the matrix has not been verified as negative, a matrix blank must be included in the batch.

39.6.3 CONTROLS

39.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

39.6.3.2 Positive Controls

a. At least three positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
d. The control working standard (10 ng/µL) is prepared as described in 39.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 39.7 SAMPLE PREPARATION. Alternatively, quality assurance personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

39.7 **SAMPLE PREPARATION**

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

39.7.2 Add 2 mL sodium borate solution into each tube.

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

39.7.4 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.

39.7.5 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.

39.7.6 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	Working Standard
Calibrator 1 (10 ng/mL)	50	0.1 ng/µL
Calibrator 2 (25 ng/mL)	125	0.1 ng/µL
Calibrator 3 (50 ng/mL)	25	1 ng/µL
Calibrator 4 (100 ng/mL)	50	1 ng/µL
Calibrator 5 (500 ng/mL)	25	10 ng/µL
Calibrator 6 (1000 ng/mL)	50	10 ng/µL

39.7.7 Prepare a 1:10 dilution of the control working 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.

39.7.8 Prepare a 1:100 dilution of the control 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 control preparation

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

Control Description	Volume (µL) Added	Control Working Standard
Control 1 (30 ng/mL)	150	0.1 ng/µL
Control 2 (400 ng/mL)	20	10 ng/µL
Control 3 (800 ng/mL)	40	10 ng/µL

39.7.10 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes using a calibrated pipette.

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

39.7.12 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.

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

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

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

39.7.16 Centrifuge the tubes for 10 minutes at 3500 rpm.

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

39.7.18 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.

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

39.7.20 Reconstitute the extracts by the addition of 100 µL 0.1% formic acid 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.

39.7.21 Transfer the extracts to labeled polypropylene autosampler vials and cap.

39.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence or batch table by first setting the data path in MassHunter to the date of the test. After entering all vial locations and sample descriptions in the worklist, ensure that the method listing in the table is AMINES.M for each line. As needed, the sequence may conclude with an injection that rinses the column and puts the instrument in standby (e.g., using method RINSE.M or COLUMN1RINSE.M), or this may be done manually.

39.9 DATA ANALYSIS

39.9.1 Analysis of the batch data is conducted using MassHunter quantitative instrumental data analysis software.

39.9.2 Quantitative calculations are generated by internal standard, multi-point regression with a 1/a (inverse of concentration) weighting factor. Calibration curves for AMP, MDA, METH and MDMA are quadratic. The calibration curve for PSED is linear. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

39.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves (reports do not need to be generated for batch entries added for column rinse at the conclusion of acquisition).

39.9.4 Technical review of the batch is conducted according to the criteria listed below.

39.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

39.10.1 CALIBRATORS AND CALIBRATION CURVES

39.10.1.1 Chromatographic peaks for target compounds and internal standards shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

39.10.1.2 Retention times for target compounds and internal standards shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

39.10.1.3 Quantitative results for target compounds in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of their targets. These are inclusive ranges.

For calibrator 1 (target concentration 10 ng/mL), result comparisons will use values truncated after the first decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL). For target concentrations ≥10 ng/mL, result comparisons will use whole integer values in units of ng/mL.

39.10.1.4 The calibration curves for target compounds shall have a correlation coefficient ≥0.99.

39.10.1.5 Quadratic calibration curves must include 6 calibration points; removal of one or more calibration points will prohibit quantitative reporting. No more than one calibration point may be removed from the linear calibration curve for PSED; removal of more than one PSED calibration point will prohibit quantitative reporting.

39.10.1.6 The failure to meet any of these criteria for one compound does not invalidate the acceptability of the other compound.

39.10.2 CONTROLS

39.10.2.1 The negative control(s) shall not identify target compounds above their limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

39.10.2.2 Positive controls

a. Chromatographic peaks for target compounds and internal standards shall appear symmetrical.
b. Retention times for target compounds and internal standards shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for target compounds in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
d. All positive controls for a target compound must meet these criteria in order to report quantitative results for that compound.

39.10.2.3 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

39.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

39.11.1 Any chromatographic peak for target compounds, and peaks for internal standards, shall appear symmetrical.

39.11.2 The retention times for target compounds and internal standards are ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

NOTE: Ephedrine (not included in this test method) elutes approximately 0.1 minutes (outside ±2% criteria) prior to, and shares MRM transitions with, pseudoephedrine. A significant peak integrated at this earlier retention time may indicate the presence of ephedrine in a specimen.

39.11.3 The quantitative results for target compounds must be within the dynamic range of the test method. Results greater than the upper limit of quantitation may be reported qualitatively, provided that all other criteria for acceptance are met.

39.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

39.12 **REPORTING**

39.12.1 Results for target compounds are reported in units of milligrams per liter (mg/L).

39.12.1.1 The whole integer, truncated results are converted from ng/mL to mg/L.

39.12.1.2 Converted results are truncated to two significant figures for reporting.

a. For example: pseudoephedrine is measured as 516.73 ng/mL.

b. The unit conversion step truncates the result to 516 ng/mL and then represents the result as 0.516 mg/L.

c. The result is truncated to 0.51 mg/L (two significant figures) and reported.

39.12.2 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

39.13 **METHOD PERFORMANCE**

39.13.1 Limit of detection: 1 ng/mL (0.001 mg/L; AMP, METH, MDA, MDMA), 5 ng/mL (0.005 mg/L; PSED)

39.13.2 Lower limit of quantification: 10 ng/mL (0.01 mg/L)

39.13.3 Dynamic range: 10 – 1000 ng/mL (0.01 – 1.0 mg/L)

39.13.4 Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

39.13.5 Upper limit of linearity: 1500 ng/mL (1.5 mg/L; PSED, MDA, MDMA), 1000 ng/mL (1.0 mg/L; AMP, METH)

39.14 **TRACEABILITY**

Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

39.15 **REFERENCES**

39.15.1 A. Black, in-house method development.

39.15.2 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.

39.15.3 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.

39.15.4 Pat Friel, Agilent Technologies, Inc.

39.15.5 Virginia Department of Forensic Sciences, Amphetamines Quantitation/Confirmation method.

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow Rate	0.500 mL/min
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile
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
Column Temp	50° C
Autosampler	
Injection Volume	5.0 µL
Injection flush-port	Active
Flush-port time	15 sec
Flush-port solvent	75:25/ Methanol:DI 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	300 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