

CONFIRMATION OF SELECT AMPHETAMINES BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

3.1 POLICY

This test method may be used to confirm the presence of amphetamine (AMP), methamphetamine (METH), ephedrine (EPD), 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.

3.2 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.

3.3 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 subjected to chemical derivatization with heptafluoro butyric acid anhydride (HFBA).

Measured volumes of the extracts are injected into a GC where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, selected-ion-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.

3.4 SPECIMENS

3.4.1 The specimen volume is 1mL.

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

3.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

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

3.5 REAGENTS, MATERIALS AND EQUIPMENT

3.5.1 REAGENTS

- 3.5.1.1 Acetonitrile
- 3.5.1.2 n-butyl chloride
- 3.5.1.3 Certified blank blood
- 3.5.1.4 Deionized water (DI H₂O)
- 3.5.1.5 Ethyl acetate
- 3.5.1.6 Heptafluoro butyric acid anhydride (HFBA)
- 3.5.1.7 Hydrochloric acid (HCl, concentrated)
- 3.5.1.8 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. Adjustments to final volume are permitted as long as the proportions are maintained.

- 3.5.1.9 Iso-octane
- 3.5.1.10 Methanol
- 3.5.1.11 Sodium borate decahydrate (Na₂B₄O₇ • 10H₂O)
- 3.5.1.12 0.13M Sodium borate solution (saturated)

In a 100 mL flask, dissolve 4.9 g Na₂B₄O₇ • 10H₂O in approximately 75 mL DI H₂O. Dilute to 100 mL with DI H₂O 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.

3.5.2 MATERIALS

- 3.5.2.1 Autosampler vials, inserts and caps
- 3.5.2.2 Disposable 16 x 100mm tubes
- 3.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures
- 3.5.2.4 Disposable pipette tips
- 3.5.2.5 Disposable safety closures for 16 x 100mm tubes
- 3.5.2.6 GC column (Phenomenex ZB-DRG-1; 10 m x 0.180 mm i.d. x 0.180 µm film thickness, or equivalent)
- 3.5.2.7 Laboratory glassware (graduated cylinders, flasks)
- 3.5.2.8 Volumetric glassware (flasks)

3.5.3 EQUIPMENT

- 3.5.3.1 Agilent GC (6890 or equivalent)
- 3.5.3.2 Agilent MS (5973 or equivalent)
- 3.5.3.3 Calibrated, adjustable piston pipettes
- 3.5.3.4 Centrifuge
- 3.5.3.5 Evaporator (Biotage, formerly Zymark, TurboVap)
- 3.5.3.6 Oven, dry-bath, or wet-bath
- 3.5.3.7 pH Meter and/or indicating pH paper
- 3.5.3.8 Rotary mixer
- 3.5.3.9 Vortex mixer

3.6 STANDARDS, CALIBRATORS AND CONTROLS

3.6.1 STANDARDS

- 3.6.1.1 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, positive controls and the working internal standard.
- 3.6.1.2 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-d₁₁ (AMP-d₁₁): 0.1 mg/mL
 - c. Methamphetamine: 1.0 mg/mL
 - d. Methamphetamine-d₁₄ (METH-d₁₄): 0.1 mg/mL
 - e. Ephedrine: 1.0 mg/mL
 - f. Ephedrine-d₃ (EPD-d₃): 0.1 mg/mL
 - g. Pseudoephedrine: 1.0 mg/mL
 - h. Pseudoephedrine-d₃ (PSED-d₃): 0.1 mg/mL
 - i. MDA: 1.0 mg/mL
 - j. MDA-d₅: 0.1 mg/mL
 - k. MDMA: 1.0 mg/mL
 - l. MDMA-d₅: 0.1 mg/mL
- 3.6.1.3 Working standard (10 ng/μL)
 - a. Using calibrated pipettes, measure 250 μL each of AMP, METH, EPD, 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.

3.6.1.4 Working internal standard (1 ng/μL, 3 ng/μL)

- a. Using calibrated pipettes, measure 250 μL each of AMP-d₁₁, METH-d₁₄, MDA-d₅ and MDMA-d₅ stock internal standards, and 750 μL each of EPD-d₃ and PSED-d₃ stock internal standard, 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 for all compounds except EPD-d₃ and PSED-d₃ which have concentrations of 3 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.

3.6.2 CALIBRATORS

- 3.6.2.1 Calibrators are prepared in certified blank whole blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 3.7 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.

3.6.3 CONTROLS

3.6.3.1 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.)

3.6.3.2 Positive Controls

- a. Two 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 3.6.1.3.
- e. The preparation of the positive whole blood controls is detailed in 3.7 SAMPLE PREPARATION. Alternatively, quality control 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.

3.7 SAMPLE PREPARATION

- 3.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e. Calibrator, control, case sample)
- 3.7.2 Place 2 mL of the sodium borate solution into each tube.
- 3.7.3 Add 1 mL of certified blank whole blood into each of the five calibrator tubes, the two positive control tubes and the negative control tube(s).
- 3.7.4 Prepare a 1:10 dilution of the working standard. (1 ng/μL)
 - a. Using calibrated pipettes, combine 0.1 mL of the working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.
- 3.7.5 Using the working standard and the prepared dilution, spike the calibrators according to the following table.

Calibrator Description	Volume (μL) Added	Working Standard
Calibrator 1 (50 ng/mL)	50	1 ng/μL
Calibrator 2 (100 ng/mL)	100	1 ng/μL
Calibrator 3 (500 ng/mL)	50	10 ng/μL
Calibrator 4 (1000 ng/mL)	100	10 ng/μL
Calibrator 5 (2000 ng/mL)	200	10 ng/μL

- 3.7.6 Prepare a 1:10 dilution of the control working standard. (1 ng/μL)
 - a. Using calibrated pipettes, combine 0.1 mL of the control working standard with 0.9 mL of acetonitrile or methanol in a labeled tube.
 - b. Cap and vortex mix. This dilution shall be disposed of after control preparation.
- 3.7.7 Using the control working standard and the prepared dilution, spike the positive controls according to the following table.

Control Description	Volume (μL) Added	Control Working Standard
Control 1 (150 ng/mL)	150	1 ng/μL
Control 2 (750 ng/mL)	75	10 ng/μL

- 3.7.8 If in-house positive controls are being used, transfer 1 mL of each into their labeled tubes.
- 3.7.9 Sample 1 mL of each case sample into its respective tube.

- 3.7.10 Add 100 μ L of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL for all internal standards except EPD-d₃ and PSED-d₃ which are 300 ng/mL.
- 3.7.11 Cap the tubes and briefly vortex mix. Let the tubes stand for 5 minutes.
- 3.7.12 Add 2 mL of n-butyl chloride to each tube.
- 3.7.13 Cap the tubes and place on a rotary mixer for 20 minutes.
- 3.7.14 Centrifuge the tubes for 10 minutes at 3500 rpm.
- 3.7.15 Transfer the n-butyl chloride layer to clean, labeled 10 mL centrifuge or screw cap tubes.
- 3.7.16 Add 100 μ L of 1% HCl in methanol to each tube. Do not omit this step as the recovery of the volatile amines will be reduced.
- 3.7.17 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C. Extracts must be completely dry for efficient chemical derivatization.
- 3.7.18 In a fume hood, add 50 μ L ethyl acetate and 50 μ L HFBA to each tube and immediately cap. Minimize the time that HFBA is exposed to the atmosphere.
- 3.7.19 Incubate the tubes for 10 minutes at 60-70°C.
- 3.7.20 Remove the tubes from heat and cool to room temperature. Alternatively, transfer the tubes to a centrifuge and spin for 2 minutes at 2000 rpm.
- 3.7.21 Transfer the tubes to the evaporator and evaporate to dryness at 40°C. Make sure the extracts are evaporated to dryness before reconstitution.
- 3.7.22 Reconstitute the extracts by the addition of 50 μ L ethyl acetate to each tube. Briefly vortex mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.
- 3.7.23 Transfer the extracts to labeled glass autosampler vials and cap.

3.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is AMINES.M for each line.

3.9 DATA ANALYSIS

- 3.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.
- 3.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.
- 3.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

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

3.10 CRITERIA FOR BATCH ACCEPTANCE

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

3.10.1 Calibrators and calibration curves

- 3.10.1.1 Chromatographic peaks for AMP, METH, EPD, PSED, MDA, MDMA and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).
- 3.10.1.2 Retention times shall be within $\pm 2\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 3. These are inclusive ranges.
- 3.10.1.3 Quantitative results for AMP, METH, EPD, PSED, MDA and MDMA in each calibrator shall be within $\pm 20\%$ of their target values with the exception of calibrator 1 which shall be within $\pm 25\%$ of its target. These are inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.
- 3.10.1.4 The calibration curves for AMP, METH, EPD, PSED, MDA and MDMA shall have correlation coefficients ≥ 0.99 .
- 3.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

3.10.2 Controls

- 3.10.2.1 The negative control(s) shall not identify AMP, METH, EPD, PSED, MDA or MDMA above their respective limits of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.
- 3.10.2.2 Positive controls
 - a. Chromatographic peaks for AMP, METH, EPD, PSED, MDA, MDMA and internal standards shall appear symmetrical.
 - b. Retention times shall be within $\pm 2\%$ and ion ratios shall be within $\pm 20\%$ of those in calibrator 3 for each compound in a positive control. These are inclusive ranges.
 - c. Quantitative results for AMP, METH, EPD, PSED, MDA and MDMA in each control shall be within $\pm 20\%$ of their target values. These are inclusive ranges. Result comparison will use whole integer, truncated results in units of ng/mL.
 - d. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
 - e. At least one positive control must meet these criteria for all compounds for the batch to be accepted.

3.11 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.

- 3.11.1 Any chromatographic peaks for AMP, METH, EPD, PSED, MDA or MDMA shall appear symmetrical.
 - 3.11.2 The retention times for any reportable compounds are $\pm 2\%$ and the ion ratios are within $\pm 20\%$ of those in calibrator 3. These are inclusive ranges.
 - 3.11.3 The quantitative result for each identified compound must be within the dynamic range of the test method.
 - 3.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.
 - 3.11.5 If any target compound in a given case sample is outside of the dynamic range it does not invalidate the result for other compounds.
- 3.12 REPORTING
- 3.12.1 Results are reported in units of milligrams per liter (mg/L).
 - 3.12.2 The whole integer, truncated results are converted from ng/mL to mg/L.
 - 3.12.3 Converted results are truncated to two significant figures for reporting.
 - a. For example: ephedrine 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.
 - 3.12.4 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.
- 3.13 METHOD PERFORMANCE
- 3.13.1 Limit of detection: 5 ng/mL (0.005 mg/L; METH, EPD, PSED), 10 ng/mL (0.010 mg/L; AMP, MDA, MDMA)
 - 3.13.2 Lower limit of quantification: 50 ng/mL (0.05 mg/L)
 - 3.13.3 Dynamic range: 50 ng/mL to 2000 ng/mL (0.05 - 2.0 mg/L)
 - 3.13.4 Upper limit of quantitation: 2000 ng/mL (2.0 mg/L)
- 3.14 TRACEABILITY
- 3.14.1 Traceability of the reference materials to SI units is provided through the certificates of analysis provided by the approved reference material supplier.

APPENDIX A
 INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet		Oven / Column	
Mode	Split	Carrier Gas Mode	Constant Flow
Inlet Liner	4mm splitless w/ glass wool plug	Carrier Gas Flow	1.0 mL/min
Temperature	180° C	Initial Temperature	70° C
Split Ratio	3:1	Initial Time	1.00 min
Gas Type	Helium	Ramp Rate 1	20° C/min
Gas Saver	On	Final Temp 1	130° C
Gas Saver Flow	15 mL/min	Ramp Rate 2	30° C/min
Gas Saver Time	2.00	Final Temp 2	270° C
Autosampler		Final Time	0.33 min
Injection Volume	2.0 µL	Transfer Line Temp	280 ° C
Solvent Wash A	3 (Isooctane)		
Solvent Wash B	3 (Ethyl acetate)		
Sample Pumps	2		

MASS SPECTROMETER

Solvent Delay	3.00 min	MS Quad Temperature	150° C
EM Offset	Set in tune	MS Source Temperature	230° C
Resolution	Low	Dwell Time	50 msec
Signals			
	Ions	Ion ratios	
Amphetamine	240, 192, 118	192/240, 118/240	
Amphetamine-d ₁₁	244, 128	128/244	
Methamphetamine	254, 210, 118	210/254, 118/254	
Methamphetamine-d ₁₄	261, 213	213/261	
Ephedrine	254, 210, 344	210/254, 344/254	
Ephedrine-d ₃	257, 213	213/257	
Pseudoephedrine	254, 210, 344	210/254, 344/254	
Pseudoephedrine-d ₃	257, 213	213/257	
MDA	240, 375, 162	375/240, 162/240	
MDA-d ₅	244, 380	380/244	
MDMA	254, 389, 210	389/254, 210/254	
MDMA-d ₅	258, 394	394/258	

