CONFIRMATION OF COCAINE, BENZOYLECGONINE AND COCAETHYLENE BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

1.1 METHOD

This test method may be used to confirm the presence of cocaine (COC), benzoylecgonine (BZE) and cocaethylene (CE) in biological samples. The target compounds and deuterated analog internal standards are isolated from biological matrices by solid phase extraction (SPE). Chemical derivatization is performed, converting BZE to the pentafluoropropyl ester, which is more suitable for gas chromatography (GC). The extracts are injected into a GC coupled to a mass spectrometer (MS) detector equipped with an electron ionization source.

1.2 SPECIMENS

The specimen volume is 1 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: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in serum/plasma specimens (see 1.4.3.4). Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in liver (tissue) homogenate specimens (see 1.4.2 and 1.4.3).

1.3 REAGENTS, MATERIALS AND EQUIPMENT

1.3.1 REAGENTS

NOTE: Organic solvents used are reagent grade.

- Acetonitrile (ACN)
- Ammonium hydroxide (NH_4OH, concentrated)
- Certified blank blood and/or other biological matrices
- Deionized water (DI H_2O), laboratory general-use
- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store the solvent in a glass flask/bottle at room temperature and use on date of preparation only.
- Ethyl acetate (EtAC)
- Hydrochloric acid (HCl, concentrated)
- 0.1M HCl

  To 400 mL DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store the acid in a glass bottle at room temperature for up to 6 months.
- Iso-octane
- Isopropanol (IPA)

- Methanol (MeOH)
- Methylene chloride (CH_2Cl_2, dichloromethane)
- 0.1M Phosphate buffer (pH6):

  Dissolve 1.7 g Na_2HPO_4 and 12.14 g $NaH_2PO_4 \bullet H_2O$ in 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to pH6 ± 0.5 with concentrated NaOH. Store the buffer in a glass bottle at room temperature for up to one year.
- Pentafluoro propionic anhydride (PFPA)
- 2,2,3,3,3-Pentafluoro-1-propanol (PFPOH)
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4)
- Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

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

1.3.2 MATERIALS

- Disposable extraction tubes (16 x 100mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206, 200mg/6mL), or equivalent
- GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 µm film thickness, or equivalent)
- Glass autosampler vials with inserts and caps
- Laboratory glassware (graduated cylinders, flasks)

1.3.3 EQUIPMENT

- Agilent GC (6890 or equivalent)
- Agilent MS (5973 or equivalent) with electron ionization source
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips
- General-use equipment (centrifuge, evaporator, heating block/oven, pH meter or paper, vacuum manifold, vortex mixer)

1.4 STANDARDS, CALIBRATORS AND CONTROLS

1.4.1 STANDARDS

- Working standard: 10 ng/µL
- Working control standard: 10 ng/µL
- Working internal standard: 1 ng/µL

1.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 1.5 SAMPLE PREPARATION. Quantitation in liver (tissue) homogenate specimens requires that a calibration curve be prepared in blank matrix. If testing only tissue homogenate specimens, a whole blood calibration curve is not required.

1.4.3 CONTROLS

1.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 1.5. For quantitative analysis of liver (tissue) homogenate specimens only, whole blood controls are not required.

1.4.3.2 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.

1.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch.

1.4.3.4 For quantitative analysis of serum/plasma specimens, matrix-matching of the full calibration curve and positive controls is not required. One negative control and one positive control must be included in the batch. Positive controls in both whole blood and/or serum serve to bracket serum/plasma case specimens and apply towards 10% of the batch.

1.4.3.5 For quantitative analysis of liver (tissue) homogenate specimens, matrix-matching of the full calibration curve and positive controls (to meet 10% and bracket specimens in that matrix) is required.

1.5 SAMPLE PREPARATION

1.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

1.5.2 Add 2 mL of 0.1M phosphate buffer (pH6) into each tube.

1.5.3 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

1.5.4 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.

1.5.5 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.

1.5.6 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	100	0.1 ng/µL	1:100
Calibrator 2 – 25 ng/mL	25	1 ng/µL	1:10
Calibrator 3 - 50 ng/mL	50	1 ng/µL	1:10
Calibrator 4 - 100 ng/mL	100	1 ng/µL	1:10
Calibrator 5 - 500 ng/mL	50	10 ng/µL	WS
Calibrator 6 - 1000 ng/mL	100	10 ng/µL	WS

1.5.7 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.

1.5.8 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions of the control working standard.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Control 1 - 30 ng/mL	30	1 ng/µL	1:10
Control 2 - 750 ng/mL	75	10 ng/µL	WS

1.5.9 Using a calibrated pipette, sample 1 mL of each case sample into its respective tube.

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

1.5.11 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

1.5.12 Place new SPE columns in the vacuum manifold.

1.5.13 Condition the SPE columns by passing each of the following solvents completely through under force of gravity.

a. 3 mL methanol
b. 3 mL DI H_2O
c. 2 mL 0.1M phosphate buffer (pH6)

1.5.14 Do not let columns dry out between each conditioning step.

1.5.15 Transfer the contents of each tube to its respective SPE column and allow them to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

1.5.16 Wash the SPE columns by passing each of the following solvents completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

a. 3 mL DI H_2O
b. 2 mL 0.1M acetate buffer (pH4.5)
c. 3 mL methanol

1.5.17 Dry the columns for 10 minutes under vacuum.

1.5.18 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

1.5.19 Pass 3 mL of elution solvent through each SPE column and collect the extracts.

1.5.20 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Extracts must be completely dry for efficient chemical derivatization.

1.5.21 In a fume hood, add 50 µL PFPOH and 50 µL PFPA to each tube and immediately cap. Minimize the time that PFPA is exposed to the atmosphere.

1.5.22 Incubate the tubes for 20 minutes at 55-60°C.

1.5.23 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.

1.5.24 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Make sure the extracts are evaporated to dryness before reconstitution.

1.5.25 Reconstitute the extracts by the addition of 50 µL 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.

1.5.26 Transfer the extracts to labeled glass autosampler vials with inserts and cap.

1.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – COCAINE (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cal 1: truncated to one decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL)

  Cals 2-6, Ctls 1-2: truncated, whole integer values in units of ng/mL

1.7 REPORTING

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

1.8 METHOD PERFORMANCE

- Limit of detection: 5 ng/mL (0.005 mg/L)
- Lower limit of quantification: 10 ng/mL (0.01 mg/L)
- Dynamic range: 10 – 1000 ng/mL (0.010 – 1.0 mg/L)
- Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Benzoylecgonine
Benzoylecgonine-d_3
Cocaethylene
Cocaethylene-d_3
Cocaine
Cocaine-d_3

APPENDIX B
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/ glass wool plug
Temperature	250° C
Split Ratio	2:1
Gas Type	Helium
Gas Saver	Off
Gas Saver Flow	N/A
Gas Saver Time	N/A
Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	3 (Isooctane)
Solvent Wash B	3 (Ethyl acetate)
Sample Pumps	2

Oven / Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.2 mL/min
Initial Temperature	150° C
Initial Time	2.00 min
Ramp Rate	15° C/min
Final Temperature	290° C
Final Time	0.67 min
Transfer Line Temp	280° C

MASS SPECTROMETER

Solvent Delay	6.00 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
Benzoylecgonine	300, 421, 316	421/300, 316/300
Benzoylecgonine-d_3	303, 424	424/303
Cocaine	182, 303, 198	303/182, 198/182
Cocaine-d_3	185, 306	306/185
Cocaethylene	196, 317, 272	317/196, 272/196
Cocaethylene-d_3	199, 320	320/199

LIST OF CHANGES

Revision Date	Description	Page Number
09/01/11	Method approved by Washington State Toxicologist. See DRA dated 8/17/11. Method released for use in evidentiary testing on 09/01/11.	All
2/04/16	Added wording for adjustment of prepared volumes in 1.5.1.8, 1.5.1.13, 1.6.1.3 and 1.6.1.4 and clarification to 1.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates in 1.6.1.3 and 1.6.1.4. Edited 1.12.3 to reflect that only two significant figures are used for reporting and added "Printed Copies are Uncontrolled" to footer. Other minor edits throughout.	All
7/10/17	Wording added to 1.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens, and standards throughout SAMPLE PREPARATION in section 1.7. Specified calibrator concentration/ranges in section 1.10.1.3. Edited section 1.10.2.2.e to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-9
7/9/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 1.2 SPECIMENS, 1.4.2 CALIBRATORS and 1.4.3 CONTROLS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. 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