Opiates by Supported Liquid Extraction (OPSLE) for Analysis by LC-MS/MS

1.0 Purpose - This procedure specifies the required elements for the extraction and quantitation of opiates and opioids using supported liquid extraction (SLE) for LC-MS/MS analysis.

2.0 Scope – This procedure applies to Toxicology in the Raleigh, Triad, and Western locations of the State Crime Laboratory.

3.0 Definitions – See Toxicology Definitions List

4.0 Equipment, Materials and Reagents

4.1 Equipment

- Centrifuge
- Mechanical Pipettes
- Class A Volumetric flasks
- TurboVap or equivalent evaporator

4.2 Materials

- Test tubes (16 x 125, 16 x 150 mm) with caps
- ISOLUTE SLE+ columns
- Glass Conical test tubes
- Vortexer
- Pipette tips
- LC vials with pre-slit septa caps

4.3 Reagents

- Ammonium Hydroxide
- Dichloromethane
- Isopropanol
- Methyl tert-butyl ether (MTBE)
- Methanol, HPLC grade or higher
- Water, HPLC grade or higher
- Negative Blood

4.4 Primary Reference Standards

• 6-Acetylmorphine (6-AM)	• 6-Acetylmorphine-D6 (6-AM-D6)
• Codeine	• Codeine-D6
• Fentanyl	• Fentanyl-D5
• Hydrocodone	• Hydrocodone-D3
• Hydromorphone	• Hydromorphone-D6
• Meperidine	• Meperidine-D4
• Methadone	• Methadone-D9
• Morphine	• Morphine-D6
• Oxycodone	• Oxycodone-D6

• Oxymorphone	• Oxymorphone-D3
	• Tramadol

4.5 Prepared Reagents – Refer to Toxicology Solution Prep Guidelines for instructions on how to prepare the reagents required by this procedure.

4.6 Prepared Standards –Standards may be prepared by the Forensic Scientist in any amount provided that the component ratios are kept constant. If using a 100 µg/mL reference standard solutions in place of a 1.0 mg/mL solution, multiply the volume pipetted by 10.

4.6.1 Opioid Internal Standard Solution (OIS)

4.6.1.1 Prepare the stock solution in methanol in a 100 ml volumetric flask. The final concentration of the opioids will be 0.10/0.50/1.0 µg/mL.

4.6.1.1.1 Pipette 100 µL of the 100 µg/mL Fentanyl-D5 reference standard solution into the volumetric flask.

4.6.1.1.2 Pipette 50 µL of the 1.0 mg/ml 6-Acetylmorphine-D6, Hydromorphone-D6, Oxycodone-D6, and Oxymorphone-D3 reference standard solutions into the volumetric flask.

4.6.1.1.3 Pipette 100 µL of the 1.0 mg/ml Codeine-D6, Hydrocodone-D3, Meperidine-D4, Methadone-D9, and Morphine-D6 reference standard solutions into the volumetric flask.

4.6.1.1.4 QS to volume with methanol.

4.6.1.2 Lot number: Eight digit format year/month/day + OIS

4.6.1.2.1 Example: 20241201OIS

4.6.1.3 Expiration: One year.

4.6.1.4 Storage: Freezer.

4.6.1.5 QC check: Successful control check.

4.6.2 Opioid Stock Calibration Solution (OSC)

4.6.2.1 Prepare the stock solution in methanol in a 10 ml volumetric flask. The final concentration of the opioids will be 1.0/5.0/10/15/20/25 µg/mL.

4.6.2.1.1 Pipette 10 µL of the 1.0 mg/mL Fentanyl reference standard solution into the volumetric flask.

4.6.2.1.2 Pipette 50 µL of the 1.0 mg/ml 6-Acetylmorphine, Hydromorphone, and Oxymorphone reference standard solutions into the volumetric flask.

4.6.2.1.3 Pipette 100 µL of the 1.0 mg/ml Codeine, Hydrocodone, Morphine, and Oxycodone reference standard solutions into the volumetric flask.

4.6.2.1.4 Pipette 150 µL of the 1.0 mg/mL Meperidine reference standard solution into the volumetric flask.

4.6.2.1.5 Pipette 200 µL of the 1.0 mg/mL Methadone reference standard solution into the volumetric flask.

4.6.2.1.6 Pipette 250 µL of the 1.0 mg/mL Tramadol reference standard solution into the volumetric flask.

4.6.2.1.7 QS to volume with methanol.

4.6.2.2 Lot number: Eight digit format year/month/day + OSC

4.6.2.2.1 Example: 20241201OSC

4.6.2.3 Expiration: One year.

4.6.2.4 Storage: Freezer.

4.6.2.5 QC check: Successful calibration (see **5.2**).

4.6.3 Opioid Calibration Working Solution (OCW)

4.6.3.1 Dilute 50 µL of the **OSC** solution with 950 µL of Methanol

4.6.3.2 Expiration: Prepare Daily.

4.6.3.3 QC check: N/A

4.6.4 Opioid Stock Verification Solution (OV)

4.6.4.1 Prepare the stock solution in methanol in a 100 ml volumetric flask. The final concentration of the opioids will be 0.25/1.25/2.5/5.0 µg/mL.

4.6.4.2 The Verification Stock solution shall be prepared using standards from different manufacturers or different lot numbers from the ones used to prepare the calibration solution.

4.6.4.2.1 Pipette 25 µL of the 1.0 mg/mL Fentanyl reference standard solution into the volumetric flask.

4.6.4.2.2 Pipette 125 µL of the 1.0 mg/ml 6-Acetylmorphine, Hydromorphone, and Oxymorphone reference standard solutions into the volumetric flask.

4.6.4.2.3 Pipette 250 µL of the 1.0 mg/ml Codeine, Hydrocodone, Morphine, and Oxycodone reference standard solutions into the volumetric flask.

4.6.4.2.4 Pipette 500 µL of the 1.0 mg/mL Meperidine, Methadone, and Tramadol reference standard solution into the volumetric flask.

4.6.4.2.5 QS to volume with methanol.

4.6.4.3 Lot number: Eight digit format year/month/day + OV

4.6.4.3.1 Example: 20241201OV

4.6.4.4 Expiration: One year.

4.6.4.5 Storage: Freezer.

4.6.4.6 QC check: Successful control check.

5.0 Procedure

5.1 Allow all solutions and samples to be analyzed to equilibrate to room temperature.

5.2 Calibration and Control Sample Preparation

5.2.1 Calibrator Preparation

5.2.1.1 Pipette the following volumes of negative blood, the **OSC,** and the **OCW** into the appropriately labeled test tubes.

Amount of Neg. Blood (µL)	Amount of OSC (µL)	Amount of OCW (µL)	Final Concentration of Opioids (ng/mL)
490		10	1.0/5.0/10/15/20/25
475		25	2.5/12.5/25/37.5/50/62.5
450		50	5/25/50/75/100/125
430		70	7/35/70/105/140/175
400		100	10/50/100/150/200/250
490	10		20/100/200/300/400/500
480	20		40/200/400/600/800/1000

5.2.2 Positive Control Preparation

5.2.2.1 Pipette the following volumes of the **OV** and negative blood into an appropriately labeled test tube.

Amount of Neg. Blood (µL)	Amount of OV (µL)	Final Concentration of Opioids (ng/mL)
490	10	5/25/50/100
460	40	20/100/200/400

5.2.3 Negative Control Preparation

5.2.3.1 Add 0.5 mL of negative blood to the labeled screw cap tubes.

5.2.4 An extraction batch will include at least two negative and two positive controls. Case specimens shall be bracketed by one of each.

5.2.5 Control samples shall make up at least 10 % of an extraction batch.

5.3 Maintenance

5.3.1 Add water to the TurboVap if needed.

5.4 Sampling

5.4.1 Allow all solutions and samples to be analyzed to equilibrate to room temperature.

5.4.2 Pipette 0.5 mL of case specimens to be analyzed into a labeled screw cap tube.

5.4.2.1 Ensure that all body fluids are homogenous.

5.4.2.2 If a homogenous sample cannot be obtained, a notation shall be made in the worksheet detailing the condition of the sample and its handling.

5.5 Extraction Procedure

5.5.1 Add 50 µL of the internal standard solution (OIS) to each calibrator, control, and case specimen to be analyzed.

5.5.2 Add 500 µL of 0.1 % ammonium hydroxide (aq) to each sample and vortex.

5.5.3 Centrifuge samples for five minutes.

5.5.4 Pipette 750 µL of sample onto labeled ISOLUTE SLE+ column and allow to absorb onto the column.

5.5.5 Add 2.5 mL 95:5 Dichloromethane/Isopropanol to each column and allow to elute into a clean labeled conical tube.

5.5.6 Add 2.5 mL MTBE to each column and allow to elute into the same tube as **5.5.5**.

5.5.7 Repeat **5.5.6**.

5.5.8 Evaporate to dryness using a TurboVap at 40 °C.

5.5.9 Add 100 µL of methanol and vortex.

5.5.10 Add 400 µL of Mobile Phase A and vortex.

5.5.11 Centrifuge for 5 minutes.

5.5.12 Transfer reconstituted specimens to labeled LC vials and cap.

5.5.13 Analyze samples on a LC-MS/MS as specified in the Toxicology Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) procedure.

5.6 Data Processing and Calibration/Control Acceptance Criteria

5.6.1 Process the run using the OPSLE method.

5.6.1.1 Ensure that the boxes are checked to update the retention times and ion ratios of the analytes and their internal standards.

5.6.2 Examine the calibration samples for outliers. See 5.6.5.1 and 5.6.5.2.

5.6.3 If manual integration of a compound is needed, the chromatogram showing the integration prior to manual integration shall be printed and included with the data as well. The reason for the manual integration shall be documented on the chromatogram.

5.6.4 Save the data with the name of the procedure and the extraction date added to the end.

5.6.4.1 Example: OPSLE20241201

5.6.5 Calibration Curve Acceptance Criteria

5.6.5.1 Evaluate the curve by back-calculating the calibrator concentrations against the curve. Values of +/- 25 % from the target concentration are acceptable for the lowest calibrator. All other calibrators shall be within 20 % of the target concentration.

5.6.5.2 The qualifier ion ratios for an analyte/internal standard in the calibrators shall be within +/- 20 % of the analyte ion ratios determined by the average of the calibration sample ion ratios.

5.6.5.3 A maximum of two calibration points may be dropped from the curve with cause (e.g., statistical outlier, laboratory accident, fails **5.6.5.1**, **5.6.5.2**).

5.6.5.3.1 If the low calibration point is dropped, this will change the LOQ and may require a repeat analysis for case specimens whose quantitation is between the lowest extracted calibrator and the new LOQ.

5.6.5.3.2 If the high calibration point is dropped, this will change the ULOQ and may require a repeat analysis for case specimens whose quantitation is between the highest extracted calibrator and the new ULOQ.

5.6.5.3.3 If the two lowest or two highest calibration points for an analyte are dropped, the run fails for that analyte and the extraction must be repeated.

5.6.5.4 The calibration curves for each analyte shall show a coefficient of determination (r^2) of 0.985 or greater.

5.6.5.5 The absolute value of the x-intercept shall be less than the lowest extracted calibrator.

5.6.5.6 If a calibration curve for an analyte fails to meet the criteria in **5.6.5.4** or **5.6.5.5**, the extraction shall be repeated.

5.6.6 Quality Control Acceptance Criteria

5.6.6.1 Each analyte in a positive control shall give a quantitation within +/- 20 % of the expected concentration.

5.6.6.2 The qualifier ion ratios for an analyte/internal standard in the controls shall be within +/- 20 % of the analyte ion ratios determined by the average of the calibration sample ion ratios.

5.6.6.3 The negative control fails for an analyte if there is an integrated peak for both transitions at the expected retention time that is greater than 20 % of the lowest calibrator area of that analyte and meets the requirement in **5.6.6.2.**

5.6.7 The failure of an analyte to meet the criteria in **5.6.5** and **5.6.6** does not invalidate the acceptability of another analyte.

5.6.8 Create a data packet for the run, including the following quality control data:

- Summary page with FA workstation reference
- Completed extraction worksheet
- LC-MS/MS sequence list
- Approved LC-MS/MS system check
- Experiment, Method and Calibration Report
- Quantitation reports of all calibrators and controls

5.6.9 The Quality Control data packet will be named beginning with “OPSLE” (capitalization optional), followed by eight digit format year/month/day ending with the instrument name. A suffix may be added to differentiate multiple runs.

5.6.9.1 Example: OPSLE20241201-QQQ-R-X

5.6.10 All quality control data packets shall be administratively and technically reviewed prior to use of the associated case data for reporting.

5.6.11 The reviewed data packet shall be uploaded into the workstation managed files. The review and approval will be indicated by signing the summary page prior to uploading to FA.

5.6.12 Control Charting

5.6.12.1 Complete the Toxicology Control Chart Form.

5.7 Sample Acceptance Criteria

5.7.1 Analyte and Internal Standard Identification Criteria

5.7.1.1 The qualifier ion ratios shall be within +/- 20 % of the target value.

5.7.1.2 The retention time shall not differ from the target value by more than 2.0 %.

5.7.2 Quantitative Acceptance Criteria

5.7.2.1 The internal standard area must be within 50 % - 200 % of the average internal standard area of the controls.

5.7.2.1.1 If the internal standard area is less than 50 %, the quantitative results may be used if the signal to noise (S/N) calculated by the instrument software is greater than 10:1. If not, the case shall be re-analyzed to confirm the quantitation, sample volume permitting.

5.7.2.1.2 If the internal standard area is greater than 200 %, the case shall be re-analyzed to confirm the quantitation, sample volume permitting.

5.7.2.1.3 If the internal standard area fails to meet acceptance criteria and there is insufficient volume remaining, the data may be evaluated qualitatively with documented approval from the Toxicology Technical Leader.

5.7.2.2 The signal to noise (S/N) of each analyte to be quantitated shall be greater than 10:1.

5.7.2.3 The quantitation result shall be equal to or greater than the LOQ of each analyte to be reported.

5.7.3 Qualitative Acceptance Criteria

5.7.3.1 If there is insufficient sample volume remaining, the data may be reported qualitatively only if the following acceptance criteria are met:

5.7.3.1.1 Calibration curve is acceptable.

5.7.3.1.2 Quality control acceptance criteria in **5.6.6.2** and **5.6.6.3**.

5.7.3.1.3 Case sample acceptance criteria in **5.7.1** and **5.7.2**.

5.7.4 If an analyte meets the criteria in **5.7.1** and the corresponding internal standard fails to meet the same criteria; the case sample will be re-analyzed, sample volume permitting. If there is insufficient volume remaining, the data may be evaluated qualitatively with documented approval from the Toxicology Technical Leader.

5.7.5 If an analyte meets the criteria listed in **5.7.2,** but fails to meet the criteria in **5.7.1**, the case sample will be re-analyzed. The data may not be used qualitatively for that analyte.

5.7.6 If an analyte meets the criteria in **5.7.1** and **5.7.2** but failed to meet the acceptance criteria listed in **5.6.6.1**, the case shall be re-analyzed, sample volume permitting.

5.7.6.1 If there is insufficient volume remaining the data may be reported qualitatively, provided all acceptance criteria in **5.7.3** are met.

5.8 Calculations

5.8.1 Reported Measurement uncertainty calculation: see **5.10.6.**

5.8.2 Percent Difference Calculation:
|(standard retention time – analyte retention time)| / (standard retention time) * 100

5.9 Uncertainty of Measurement

5.9.1 The current process uncertainty for each analyte quantitated by this procedure is located in the Toxicology Reporting Index.

5.9.2 The uncertainty of measurement shall be reported in the same units as the compound quantitation.

5.10 Reporting

5.10.1 Opioids identified by LC-MS/MS analysis shall have a positive indication from an initial analysis of another aliquot. Refer to the Drug Toxicology Reporting procedure for reporting of Opioids.

5.10.2 All quantitative results will be truncated and reported to two significant figures.

5.10.3 All calculated measurement uncertainties will be rounded and reported to the same level of significance as the quantitative results.

5.10.4 If a case sample is re-extracted, the average of all acceptable quantitations will be reported.

5.10.5 The full analyte result is used to calculate the associated measurement uncertainty (with coverage factor k=2, 95.45% coverage probability).

5.10.6 Example Reporting Statement and Uncertainty determination.

5.10.6.1 Fentanyl full quantitation = 5.45 ng/mL

5.10.6.2 Multiply the full quantitation by the current process uncertainty (see 5.9.1).

5.10.6.2.1 5.45 ng/mL * 0.36 (36%) = 1.962 ng/mL.

5.10.6.3 The Fentanyl result is truncated to 5.4 ng/mL, and the associated uncertainty is rounded to 2.0~~1.1~~ ng/mL for reporting.

5.10.6.4 Report: Fentanyl – 5.4 +/- 2.0 ng/mL at a coverage probability of 95.45%

5.10.7 Case Samples with opioid concentrations that exceed the upper level of the calibration curve will be reanalyzed with a lower sample volume or after dilution with the proper matrix to bring it within the calibration range.

5.11 Record the following in the case record:

- Quantitation report of the sample

6.0 Limitations- n/a

7.0 Safety

7.1 Refer to the Laboratory Safety Manual.

8.0 References

- Biotage Application Note AN875 – Extraction of a Drugs of Abuse Pane from Whole Blood Using Isolute SLE+ Prior to UPLC-MS/MS Analysis
- OPSLE Method Validation

9.0 Records

- Quality Control data packet
- Case record
- Toxicology Control Chart Form

10.0 Attachments

- Attachment 1 - Safety Precautions for Particularly Hazardous and Extremely Hazardous Substances

Revision History		
Effective Date	Version Number	Reason
12/02/2024	1	Original Document

Appendix 1: Safety Precautions for Particularly Hazardous and Extremely Hazardous Substances

Methylene Chloride/Dichloromethane **DANGER: PARTICULARLY HAZARDOUS SUBSTANCE**	
	**HEALTH 2** **FLAMMABILITY 1** **REACTIVITY 1**
Detection of Release	Clear colorless liquid. Ether like odor
Signs/Symptoms of Exposure	Serious eye irritation; skin irritation; may cause drowsiness or dizziness.
PEL	ACGIH (TLV) – 50 ppm; OSHA Specifically Regulated Chemicals/Carcinogens – (PEL) 25 ppm
Associated Hazards	Serious eye and skin irritation; suspected of causing cancer
Controls	Use under fume hood. Avoid contact with skin, eyes and clothing. Wash hands before breaks and immediately after handling the product. Use eye protection. Handle with gloves. Wear lab coat. Gloves: Fluorinated rubber (break through time = 148 minutes)
Safe handling, storage, disposal	Avoid contact with skin and eyes. Avoid inhalation of vapor or mist. Keep in a tightly closed container. Containers which are opened must be carefully resealed and kept upright to prevent leakage. Dispose of in Hazardous Chemical Waste.
Emergency Procedures (2.2)(4.1)(6)	**Eye Contact:** Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician. **Inhalation Exposure:** If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician. **Ingestion:** Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician. **Skin Contact:** Wash off with soap and plenty of water. Consult a physician. **Spills:** Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Small contained spill: wearing appropriate PPE, collect with absorbent material, and place in container. Dispose in Hazardous Chemical Waste. Large spills: Evacuate area and call 911 (Haz Mat).