FINAL REPORT
2019-DU-BX-0002
February 27, 2023

AWARD NUMBER	2019-DU-BX-0002
PROJECT TITLE	Combining LC/MS/MS Product-ion Scan Technology with GC/MS Analysis to Identify Drugs and Poisons in Postmortem Fluids and Tissues
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PROJECT PERIOD	01/01/2020 – 12/31/2022
AWARD AMOUNT	$372,438

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SUMMARY OF THE PROJECT

Major goals and objectives

The principal goal of this project was to develop an LC/MS/MS screening method for drugs and poisons in postmortem fluids and tissues from Medical Examiner cases to augment the current screening procedures using GC/MS. Ultimately this new procedure would replace the ELISA screening procedure that was unable to provide the sensitivity and specificity required. The secondary goal was to develop a method that generates more spectral detail to provide a higher confidence in the identification of a substance and eliminate misidentifications.

Preliminary research proved that this could be achieved by performing product-ion scanning at three different collision energies and combining these product scans into a library-searchable spectrum. The process would be triggered using known MRM transitions for each compound. If the MRM was present the product scan would be triggered, and the product-ion scans would be collected.

To accomplish these goals, funding was used to purchase a Shimadzu Model 8060 LC/MS/MS instrument with the required scanning speed and data collection rate to collect and collate the spectral data. In addition, the grant funding was used to purchase a centrifuge used in the sample preparation process and certified reference materials to be used in the preparation of the reference drug library.

Research questions

The essential goals were to:

1. Establish Instrument conditions to scan for 400 drugs in a single analysis, while retaining low detection limits and detailed spectra

2. Create a searchable spectrum library that includes relevant drugs found in a traditional toxicology screen that contained a searchable composite product-ion spectra.
3. Development of a rapid broad-scope sample preparation technique (extraction) of postmortem fluids and tissues suitable for a comprehensive screening method
4. Replace ELISA drug screening with the new method.
5. Correlate of test results with GC/MS testing method currently used.
6. Improve testing process and turnaround time using the new LC/MS/MS screening method.

Research design, methods, analytical and data analysis techniques

Project Design

1. Utilize several common substances routinely detected to develop the scanning conditions, suitable to assemble a reliable and repeatable product scanning spectrum.
2. Create a searchable library of 400 compounds combined product-ion spectra.
3. Develop a sample preparation technique that would yield the highest recovery across the scope of compounds while minimizing the chemical contamination and/or interferences.
4. Evaluate the method on spiked whole blood samples to determine effectiveness.
5. Validate the method using standard and acceptable procedures in forensic toxicology.
6. Evaluate the method by applying it to at minimum 100 cases previously screened with the older method and compare results.
7. Apply the method to the routine screening work and evaluate changes in case turnaround time, elimination of additional testing, and quality of reportable results.

Methods

Instrument optimization

Creating repeatable spectral data required optimized conditions for the instrument method. Shimadzu software allows for a large amount of control for each specific event. The goal was to create conditions that were stable and reproducible and would occur at specific retention time windows for each of the targeted compounds. Once this was achieved, a library based on standards could be developed.

The following optimizations were performed for every compound:

- collision energy (-V), pause time (msec), dwell time (msec) – common MRM parameters that can be manipulated to optimize signal intensity from a molecular product.
- Q1 pre bias (-V), Q3 pre bias (-V) – pre-quadrupole conditions that manipulate the oscillating path of ions, enhancing their signal under optimized conditions.
- Focus voltage (-V) – an applied voltage inside the source that directs ions from the corona needle into the de-solvation line.
- Event time (msec) – total amount of time it takes to scan for the ions instructed in a specific event. The instrument has a theoretical scan speed of 30,000 amu/second which was maximized by forcing lower event times (faster scanning) throughout all events.
    - MRM example: scan for precursor 286.14 and product 165.1 m/z with a 1 msec pause time and 7 msec dwell time = 8 msec event
    - Product ion scan example: scan from 50 to 296 m/z at a scan speed of 30,000 u/sec = 15 msec event
    - Maximizing scan speed across the hundreds of spectral events was crucial to create data with more than 10 data points across the peak.

- Dependent event conditions (product ion scans only) – the minimum intensity needed from a specified MRM to trigger product ion scans related to the same precursor ion. Product ion scans are a dependent event which means that they are not always collected. Product ion scans are only collected when the dependent event conditions are met.
- Auto exclude (product ion scans only) – an optional setting used only for product ion scans with an over-abundant (saturating) response. An automatic exclude period of X seconds instructs the instrument to only collect data on the product scan every X seconds during the run, even if event and loop time would normally cause it to happen sooner.
- Loop time (seconds) – sum of all event times over a specific period. Shortening event windows around their specified retention time lowers the amount of simultaneous mass spectral events, lowering the overall loop time. Theoretical loop time assumes that all events are always happening; however, creating trigger thresholds for product ion scans will lower loop time during a real run, as not all events will be triggered. Loop time and data points gathered are inversely related.
- Mass spectral events were created for each compound, consisting of two MRM transitions. Additionally, "Survey Event Mode" was utilized for each compound, which collected three product ion scans at different collision energies from the precursor ion once a signal threshold from the MRM was reached. The three product scans were merged into a single spectrum and compared to an in-house library made from certified reference materials.

Sample preparation

The goal of sample preparation was to develop an extraction procedure that was quick, required small sample volumes to reduce chemical contamination, had high recovery across the scope of compounds, and was repeatable. The following method met those criteria.

1. 200 µL of Post-mortem blood was fortified with pH 7 phosphate buffer and 10 µL of internal standard in a 5mL screw top glass tube.
2. Samples were vortexed, centrifuged, and loaded on to Biotage ISOLUTE SLE+ cartridges.
3. 4mL of 70:25:5 hexane:ethyl acetate:IPA, 2mL Hexane, and 2mL MTBE were used as elution solvents.
4. 100 µL of 1% hydrochloric acid in methanol was added to the elution tubes prior to evaporation to dryness in a TurboVap evaporator.
5. Residues were reconstituted in 50 µL 50:50 mobile phase A (5 mM ammonium formate + 0.1% formic acid in water) and mobile phase B (methanol) and gently vortexed.
6. 3 µL of sample was injected for instrument analysis with a Shimadzu LCMS-8060NX, triple quadrupole mass spectrometer.

Controls and Internal Standard

1. Internal standard solutions used to spike all samples included the following compounds.

Morphine–D3	Methamphetamine-D8	Oxycodone-D3
Cocaine-D3	7-Aminoclonazepam-D4	Fentanyl-D5
Midazolam-D4	Clonazepam-D4	THC-COOH-D9

2. Controls were prepared from certified reference standards and contained the 59 drugs of most common interest. The concentration of each was twice the established limit of detection. An aliquot of the control working solution was spiked into blood samples which are analyzed after every tenth sample in a batch.

Instrument conditions

- Chromatographic separation was achieved with a Restek Raptor Biphenyl 2.7µm 50x2.1mm
- LC mobile phase A: 5 mM ammonium formate + 0.1% formic acid in water mobile phase B: methanol
- Runtime 7.5 min

LC conditions: Shimadzu Model 8060NX LC/MS/MS

Mobile phase start	95:5 A:B
Pump pressure	~2800 psi
Flow rate	0.5 mL/min
Autosampler temp	10 °C
Oven temp	40 °C
Interface voltage	4 kV
Interface temp	400 °C
De-solvation temp	650 °C
DL temp	250 °C
Heat block temp	400 °C
Nebulizing gas flow	3 mL/min (Nitrogen)
Heating gas flow	10 mL/min (nitrogen)
Focus voltage	4 kV
CID gas	270 kPA (Argon)

Mobile phase gradient

Time (min)	Flow (mL/min)	A Conc (%)	B Conc (%)
0	0.5	95	5
0.5	0.5	95	5
6	0.5	5	95
6.5	0.5	5	95
6.51	0.5	95	5

Analytical and data analysis

After the completion of an analysis the Shimadzu .LCD (LC data files) files are imported into Shimadzu Insight Explore processing software. All mass spectral events related to a compound are displayed. The MRM ion signals are always collected and displayed. Product ion scans, when collected, are merged by the software, and searched against the library, displaying the highest similarity scores first.

The software flags during the process which are used to identify possible hits to help the analyst sort through the data file quickly. Flagging categorizes those compounds which had a product scan versus those that didn't. The analyst is generally interested in those which have library matches. Nine internal standards are checked to ensure the run was analyzed properly. Top library hits and peak integration can be changed as necessary by the analyst. The analyst checks peak shape, peak intensity, retention time, and match quality before making an identification. The files to be reported are printed and uploaded to the laboratory's LIMS.

This method is very sensitive and even though the laboratory has up-to-date instrumentation and analytical methods, generally not all identifications can be confirmed through another method. The analyst must be checking all components of the data (i.e., not just match quality) before making an identification to reduce false positives. Ionization suppression and enhancement are common issues in LC-MS analysis, especially when working with complex sample matrices like post-mortem fluids. Late eluting compounds such as THC and THC-COOH are more susceptible to these phenomena.

Expected applicability of results

The project presented an alternate approach for general screening and/or confirmation of targeted compounds in forensic toxicology. The results demonstrate its applicability to routine testing. The

applicability of the results will provide an effective method to use in conjunction with an additional screening method such as GC/MS. In addition, it will replace immunoassay screening normally used in the initial screening procedure. Sensitivity and specificity are the two significant factors that make this method significantly better than the current procedure. Traditional LC/MS/MS screening procedures rely on 2-3 MRM transitions to identify a compound. This was found to be insufficient as a qualitative tool because of the flood of new synthetic compounds with their multiple analogues that have very similar mass spectra and MRM transition. A more detailed spectra is necessary to distinguish these compounds. This detail is demonstrated with composite product ion spectra.

PARTICIPANTS AND OTHER COLLABORATING AGENCIES

Collaboration was obtained from scientists and engineers at Shimadzu Scientific Instruments

CHANGES IN APPROACH FROM ORIGINAL DESIGN AND REASON FOR CHANGE

There was only one significant change in the original project plan. Originally the goal was to include 400 compounds in the scope of the screen. However, this was adjusted at the beginning of the project to first include the compounds that could be detectable by the current ELISA screen. It was decided to narrow the scope to include the most common substances and over time add new compounds. This scope will expand over time to include more compounds to achieve the goal of 400. Increasing the scope of sample types was initially a goal to allow the application of this method to tissues as well as fluids. This part of the project is under way and once validated for tissue sample will expand the scope to include liver and brain tissue.

OUTCOMES

Activities/accomplishments

- Optimized the instrumental conditions of the Shimadzu Model 8060 LC/MS/MS to enable the collection and combining of three product ion scans resulting from three different collision energies. Detection was based on the presence of two MRM transitions for each compound.
- Created a searchable combined product ion spectral library of 60 frequently detected analytes.
- Created a SLE extraction procedure robust enough to handle post-mortem blood and fluids while also broad enough to handle a wide range of drug classes and pKa values. These drug classes included benzodiazepines, sympathomimetic amines, cocaine/cocaine metabolites, fentanyl/fentanyl analogues, opiates/opioids, and cannabinoids. The resulting limits of detection for these compounds exceeded other screening methods used in the laboratory.
- Validated the method according to ANAB/ANSI standards for postmortem fluids.
- Applied the method to routine work, eliminating the use of ELISA as an initial screening method, with a correlation to GCMS screening exceeding expectations.
- Implementation of the new method has resulted in a reduction in turnaround time, number of tests performed per case, and reduction in costs related to immunoassay testing.

Results and findings

LCMSMS testing has transformed the way initial screening is handled in the laboratory. The combination of LCMSMS and GCMS testing has improved the quality and processing time of forensic toxicology at the Miami-Dade County Medical Examiner Toxicology laboratory.

The original intent of this method was to replace ELISA as an initial routine screening tool and to supplement GC/MS in the normal screening of all cases. During the validation of the new method and its application to real cases to evaluate its efficacy, variations were found between the LC/MS/MS and GC/MS test results. The table below summarizes these variations.

	Screening outcome	Examples of drugs detected	Reasoning	Result
1	LCMSMS and GCMS results agree	Fentanyl, cocaine, benzoylecgonine, alprazolam	Drugs are in high enough concentration to be found on both methods	Screening is completed and case is sent for quantitation as necessary
2	LCMSMS finds additional drugs not found by GCMS	Methamphetamine, amphetamine, acetyl fentanyl, xylazine, Delta-9-THC and 11-nor-carboxy-THC	• Limit of detection on LCMSMS is much lower than GCMS. • Sample preparation for LCMSMS has better extraction recovery than the procedure for GCMS. • Compound analysis better on LC vs GC	Case is sent for additional confirmatory screening by LC/Ion Trap-MS or quantitation as necessary
3	GCMS finds additional drugs not found by LCMSMS	Diphenhydramine, ibuprofen, citalopram, trazadone	Library limitation on new LCMSMS method; currently does not have these drugs – see limitations section	Case is sent for additional confirmation screening by LC/Ion Trap-MS

See Attachment 1 for completed data sets representing three case sample reports using the LC/MS/MS method.

Improvements in testing quality

The utilization of LC/MS/MS in conjunction with GC/MS as an initial screening procedure for all cases has improved the quality of drug identification and specificity in routine testing. The composite spectra created by combining product ion spectrum has provided more detail and reduced the potential for false positives and negatives. Sensitivity and detection levels for these substances in postmortem samples using the LC/MS/MS method also exceeded expectations in most cases over the GC/MS method. The result is a greater confidence in reported results.

Example cases:

Case #	LCMSMS Results	GCMS Results	Case History
1	OXYMORPHONE OXYCODONE COCAINE **FENTANYL** ALPHA-HYDROXY ALPRAZOLAM **ALPRAZOLAM**	DESPROPIONYLFENTANYL **FENTANYL** **ALPRAZOLAM**	Decedent was found in a motel room unresponsive. No sign of foul play or trauma.
2	**ECGONINE METHYL ESTER** AMPHETAMINE METHAMPHETAMINE **BENZOYLECGONINE** **COCAINE** **COCAETHYLENE**	ECGONINE ETHYL ESTER **ECGONINE METHYL ESTER** **COCAINE** **COCAETHYLENE** **BENZOYLECGONINE** TAMOXIFEN	Decedent was the passenger in a fatal car accident off the highway.
3	PENTYLONE N,N-DIMETHYLPENTYLONE	IBUPROFEN METHYLPHENIDATE CHLORPHENIRAMINE	Decedent jumped from the rooftop of their apartment building. According to family, they were currently suffering from depression.
4	**FENTANYL** ALPRAZOLAM 11-NOR-9-CARBOXY-TETRAHYDROCANNABINOL DELTA-9-TETRAHYDROCANNABINOL XYLAZINE	**FENTANYL** DESPROPIONYLFENTANYL GABAPENTIN CETIRIZINE	Decedent found unresponsive in their car at a gas station. In the vehicle was a rolled-up dollar bill and foil paper but no narcotics were found.
5	**ECGONINE METHYL ESTER** **BENZOYLECGONINE** **COCAINE** **FENTANYL** MITRAGYNINE ALPRAZOLAM 11-NOR-9-CARBOXY-TETRAHYDROCANNABINOL PENTYLONE **N,N-DIMETHYLPENTYLONE**	**ECGONINE METHYL ESTER** **N,N-DIMETHYLPENTYLONE** NORFENTANYL **COCAINE** **BENZOYLECGONINE** **FENTANYL** SERTRALINE DESPROPIONYLFENTANYL	Decedent was found unresponsive on his couch after a potential cardiac event. Pending toxicology.
6	**ECGONINE METHYL ESTER** **ANHYDROECGONINE METHYL ESTER** **BENZOYLECGONINE** **COCAINE** FLUORO FENTANYL **FENTANYL** **PENTYLONE** **N,N-DIMETHYLPENTYLONE**	**ECGONINE METHYL ESTER** DESETHYLLIDOCAINE **PENTYLONE** DIPHENHYDRAMINE **N,N-DIMETHYLPENTYLONE** NORCOCAINE **COCAINE** CITALOPRAM NORCITALOPRAM **BENZOYLECGONINE**	Decedent found unresponsive in their room. History of depression and alcohol abuse. Prescribed Citalopram hydrobromide and risedronate sodium.

		FENTANYL **ANHYDROECGONINE METHYL ESTER** LIDOCAINE	
7	NALOXONE OXYCODONE BENZOYLECGONINE 7-AMINOCLONAZEPAM **FENTANYL** PENTYLONE **N,N-DIMETHYLPENTYLONE** XYLAZINE	CHLOROPHENYLPIPERAZINE LEVAMISOLE MIRTAZAPINE **FENTANYL** **N,N-DIMETHYLPENTYLONE**	Decedent found unresponsive on a bus bench. Fire rescue transported him where he was admitted for treatment; Hospital toxicology positive for cocaine.

Improvements in case processing time

Expediting case processing and reducing turnaround time was another goal of the project. In the third month of utilizing this new method the full impact has yet to be evaluated, however it is clear there has been a reduction in the number of follow up tests that were necessary in the past to properly identify individual or groups of drugs. Additional testing is necessary in some cases because of sensitivity differences or because there are compounds that are yet to be added to the new method, a problem that with time will be eliminated. As more compounds, both prescription drugs and NPS, get added to the library, the quality of our screening process will only continue to strengthen.

Elimination of immunoassay screening in the routine process was another goal of the project. Immunoassay screening such as ELISA (blood) and EMIT (urine) lacked specificity, sensitivity, and the necessary scope of compounds to be effective in the routine work. Post-mortem specimens often triggered false positives for assays like amphetamine and benzodiazepines which were pursued by additional testing with negative results. To date reductions in the use of these testing techniques have been demonstrated.

Pre-application of new LC/MS/MS method (Dec 12, 2022 – March 7, 2022)

ELISA tests	**EMIT tests**	**LCMSMS tests**
260	**408**	**0**

Post-application of new LC/MS/MS method (Dec 12, 2022 – March 7, 2023)

ELISA	EMIT	LCMSMS
20	**0**	**512**

Limitations

Scope of testing

Initial size of the library was limited to compounds that would cross-react with the ELISA immunoassay kits currently in use in the lab. Focusing on these 59 compounds was sufficient as a starting point to eliminate immunoassay screening, gradually expanding in scope as the method remains in use. As an accredited lab, methods must be validated within the ABFT/ANAB guidelines for use in genuine casework. Validation requirements are extensive, requiring hundreds of injections to establish limit of detection, carryover, ion suppression, endogenous/exogenous interferences, and other parameters for every single compound. Due to the time constraints and the laboratories desire to eliminate immunoassay testing as soon as possible, a smaller library than the initially proposed amount was created and validated.

Accreditation also recommends that for a targeted screen, every drug should be tested as part of the batch in a control mixture. This presented a difficult task to the lab to not only create and maintain a mixture of every drug in the screen as part of a control mixture, but also making sure that they are all simultaneously identifiable to reduce batch failures. At the current number of targeted compounds, the control is already beginning to hit the limits of feasibility in terms of maintenance and analysis. The laboratory will be exploring the options available to meet this accreditation requirement and continue to expand the scope of the screen.

Sample type

Currently validation has only been completed for the post-mortem blood matrix. This alone has nearly eliminated all ELISA and EMIT testing, with only a few other sample types requiring ELISA testing.

Additional studies are currently being conducted for liver homogenates and serums. Previous tests were done on liver homogenates during the method development stage, but it was deemed unsuitable for the SLE extraction procedure. The current GCMS extraction procedure, mixed-mode SPE, is being used for these specimens with the understanding that certain compounds like THC have exceptionally poor recovery.

Over the next twelve months the impact on case turnaround time and reductions in costs will be better understood and measured. Improvements in the scope of the method to include many more analytes will also be demonstrated in this time, reducing the reliance on additional confirmation testing which impacts both turnaround and cost. Based on the success of this new method to date, there is high confidence these two goals will be achieved.

ARTIFACTS

List of products

The following presentations were prepared from the project research data. Copy of the presentations can be obtained from the authors.

Giachetti, Alex "**Postmortem Screening by LC-MS/MS using Merged Product Ion Scanning**", presented at a Shimadzu sponsored workshop. 2022

Giachetti, A., Zaney, M., Kahl, J., Hime, G., Boland, D., **Raising the Quality of Initial Postmortem Screening to the Next Level by LC-MS/MS Merged Product Ion Scanning,** poster presentation at the 2022 Society of Forensic Toxicologists meeting.

Data sets generated

The data sets generated in this project are primarily instrument raw data of individual analyses generated during the development and validation of the method and data generated during the

use of method on real medical examiner case analyses. The data can be observed with the right Shimadzu data processing software.

Dissemination activities

Method development results have been shared with Shimadzu to assist in further software development of future versions to improve processing and reporting. Except for the two presentations cited above the results of the project have not been shared with any outside agencies to date.

In-house the method will be used in the routine processing of medical examiner cases to improve the quality of the initial screening process, reduce turnaround time, and reduce overall costs associated with discontinuing immunoassay testing and the reduced need for additional testing protocols.

Targeted Drug Screen by Supported Liquid Extraction and LC-MS/MS Analysis

1. Purpose

The purpose of this document is to describe the procedure for screening whole blood specimens for a select group of targeted analytes that are common in postmortem casework.

2. Scope

This document describes in detail the procedures for analyzing, processing, and reporting the presence of commonly identified analytes in whole blood specimens. This procedure is intended for all laboratory personnel performing this screen on case samples. A screen is performed on all cases in which whole blood specimens were submitted to the laboratory for routine testing.

3. Principle

Whole blood specimens are fortified with isotopically-labeled internal standards (ISTD) and then buffered to pH 7. All target analytes and ISTDs are isolated from biological matrices using supported liquid extraction (SLE) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

4. Clinical Significance

Not applicable

5. Specimen Requirements and Preparation

Whole blood specimens that are collected in tubes containing a preservative (e.g., 100 mg of sodium fluoride and 20 of mg potassium oxalate per 10 mL of blood) and stored between 2-8°C are preferred for analysis. Other specimens that can be tested include serum and urine; however, this is not recommended unless absolutely necessary when whole blood is not available for testing. A minimum of 200 µL are required for analysis.

6. Safety

The analysis of human biological specimens exposes the analyst to potentially biohazardous material, so the required safety practices referenced in the Toxicology Laboratory's Policy and Procedure Manual must be followed. Personal protective equipment such as safety glasses, gloves, and lab coats must be used when working with potentially hazardous materials. Disposal of all chemicals and biohazardous waste must be performed in accordance with the procedures referenced in the Laboratory's Policy and Procedure Manual.

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7. Laboratory Supplies

A. Instrumentation/Equipment

1. Shimadzu Nexera 40 Series LC Pumps, Autosampler, Column Oven, Degassing Units, and System Controller
2. Shimadzu LCMS-8060NX Triple Quadrupole Mass Spectrometer
3. UCT Positive Pressure Manifold with appropriate racks, supplied with nitrogen or air
4. SLE columns
   a. ISOLUTE SLE+, 400 µL sample volume, 820-0055-B-500, Biotage
5. Calibrated 0.5-10 µL, 20-200 µL, and 100-1000 µL adjustable volume pipettes with pipette tips
6. Calibrated repeater pipette with pipette tips
7. Laboratory glassware
   a. Gastight syringes
   b. Volumetric flasks with stoppers
   c. Volumetric pipettes with pipette bulb
8. Calibrated analytical balance
9. Vortex-mixer
10. Microcentrifuge
11. Biotage TurboVap sample evaporator, supplied with nitrogen
12. 2 mL snap-cap microcentrifuge tubes
13. 16x100 mm culture tubes with appropriate polyethylene or polypropylene caps
14. 15 mL, 20 mL, and 120 mL amber glass vials with PTFE-lined caps
15. 50 mL polypropylene tubes and caps
16. 2 mL amber and clear glass autosampler vials with blue and red colored caps containing PTFE septa
17. 350 µL clear glass pulled-point conical autosampler vial inserts
18. Other routine laboratory supplies including disposable plastic and glass transfer pipettes, test tube racks, lint-free wipes, absorbent wipes, etc.

B. Reagents/Chemicals/Solvents

1. Whole blood or defibrinated sheep blood (interchangeable) negative for target analytes preserved with potassium oxalate and sodium fluoride at pH 6-7, UTAK or Hemostat
2. Liquinox liquid detergent, Alconox
3. De-ionized (DI) water
4. Sodium hydroxide (NaOH), certified ACS
5. Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7\ H_2O$), certified ACS
6. Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$), certified ACS
7. Methanol (MeOH), HPLC grade
8. Acetonitrile (ACN), HPLC grade
9. Hexane (HX), HPLC grade

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10. Ethyl acetate (EtOAc), HPLC grade
11. Isopropanol (IPA), HPLC grade
12. Methyl *tert*-butyl ether (MTBE), HPLC grade
13. Hydrochloric acid (HCl), certified ACS Plus
14. Methanol (MeOH), LC/MS grade
15. Ammonium formate (AmF), LC/MS grade
16. Water, LC/MS grade
17. Formic acid (FA), LC/MS grade

C. Positive Control Reference Materials

Group 1			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
Fentanyl	F-013	Cerilliant	1 mg/mL

Group 2			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
7-Aminoclonazepam	A-916	Cerilliant	1 mg/mL
Alprazolam	A-903	Cerilliant	1 mg/mL
Cocaine	C-008	Cerilliant	1 mg/mL
Ketamine	K-002	Cerilliant	1 mg/mL
Methamphetamine	M-009	Cerilliant	1 mg/mL
MDMA	M-013	Cerilliant	1 mg/mL
Morphine	M-005	Cerilliant	1 mg/mL
Naloxone	N-004	Cerilliant	1 mg/mL
Oxycodone	O-002	Cerilliant	1 mg/mL

Group 3			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
(-)-Δ^9-THC	T-005	Cerilliant	1 mg/mL

Group 4			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
Diazepam	D-907	Cerilliant	1 mg/mL
Pseudoephedrine	P-035	Cerilliant	1 mg/mL
(-)-11-nor-9-Carboxy-Δ^9-THC	T-019	Cerilliant	1 mg/mL

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Group 5			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
Benzoylecgonine	B-004	Cerilliant	1 mg/mL

D. Internal Standard Reference Materials

Group 1			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
Fentanyl-D_5	F-001	Cerilliant	100 µg/mL

Group 2			
Analyte	**Part No.**	**Manufacturer**	**Concentration**
7-Aminoclonazepam-D_4	A-917	Cerilliant	100 µg/mL
Clonazepam-D_4	C-905	Cerilliant	100 µg/mL
Cocaine-D_3	C-004	Cerilliant	100 µg/mL
Methamphetamine-D_8	M-016	Cerilliant	100 µg/mL
Midazolam-D_4	M-918	Cerilliant	100 µg/mL
Morphine-D_3	M-003	Cerilliant	100 µg/mL
Oxycodone-D_3	O-005	Cerilliant	100 µg/mL
(±)-11-nor-9-Carboxy-Δ^9-THC-D_9	T-007	Cerilliant	100 µg/mL

8. Preparation of Required Reagents

A reagent preparation form, located in a binder in the reagent preparation room, must be filled out for each applicable reagent prepared, noted by an asterisk (*) below. It must include the date, preparing analyst, final volume, assigned lot number, and expiration date, as well as the weight/volume, manufacturer(s), and lot number(s) of reagents used in the preparation.

A. 1% Liquinox Cleaning Solution

1. Add 10 mL of Liquinox liquid detergent to 1L of warm tap water in a plastic bucket.

B. 6N NaOH*

1. Dissolve 60.0 g of NaOH in ~150 mL of DI water contained in a large beaker. Slight heating may be necessary. Once dissolved, allow the solution to cool to room temperature. Transfer to a 250 mL volumetric flask and bring to final volume with DI water. If a larger final volume is needed, adjust preparation weights and volumes accordingly. This reagent can be used for up to six months from the date of preparation.

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C. 0.1M Phosphate Buffer, pH 7*

1. Dissolve 10.22 g of sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7\ H_2O$) and 8.54 g of sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) in ~800 mL of DI water contained in a large beaker. While stirring, monitor the pH with a calibrated pH meter and slowly adjust to pH 7.0 with the drop-wise addition of 6N NaOH. Transfer to a 1 L volumetric flask and bring to final volume with DI water. If a larger final volume is needed, adjust preparation weights and volumes accordingly. This reagent can be used for up to one month from the date of preparation.

D. 1% HCl in MeOH

1. Add 100 µL of HCl to 10 mL of MeOH contained in an amber glass vial. Gently swirl to ensure adequate mixing. Adjust volumes accordingly for the number of samples being analyzed. This reagent must be prepared fresh for each batch.

E. HX:EtOAc:IPA (70:25:5)*

1. Mix together 700 mL of HX, 250 mL of EtOAc, and 50 mL of IPA in a glass bottle. Gently shake to ensure adequate mixing. If a larger final volume is needed, adjust preparation volumes accordingly. This reagent can be used for up to two weeks from the date of preparation.

F. 5mM AmF/0.1% FA*

1. Dissolve 0.315 g of AmF in ~500 mL of LC/MS grade water contained in a large beaker. Once dissolved, transfer to a 1 L volumetric flask. Add 1 mL of FA and gently swirl to ensure adequate mixing. Bring to final volume with LC/MS grade water. Measure the pH using a calibrated pH meter. If a larger final volume is needed, adjust preparation weights and volumes accordingly. This reagent can be used for up to one month from the date of preparation.

G. 5mM AmF/0.1% FA:MeOH (50:50)

1. Mix together 25 mL of 5mM AmF/0.1% FA with 25 mL of LC/MS grade MeOH in a 50 mL polypropylene screw-cap tube. Gently shake to ensure adequate mixing. If a larger final volume is needed, adjust preparation volumes accordingly. This reagent must be prepared fresh for each batch.

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9. Preparation of Working Stock Solutions

A. Requirements

1. Reference materials used in the preparation of the positive control and ISTD working stock solutions must be obtained from a reference material producer (RMP) that is accredited to ISO 17034 and can be used for up to one year from the date they were initially opened or until the expiration date listed on the certificate of analysis provided by the RMP, whichever is earlier.
2. If the listed reference materials used to prepare the positive control and/or ISTD working stock solutions (see Sections 7, C and 7, D) are unavailable for whatever reason, it is acceptable to use an equivalent reference material than what is listed, as long as it meets requirement **1.** listed above.
   a. If an equivalent reference material is used, a note must be added to the working stock solution preparation log (see Section 9, A, 7) with the RMP, part number, concentration, lot number, and expiration date of the equivalent reference material.
3. All working stock solutions must be prepared using gastight syringes, volumetric pipettes, and volumetric flasks, where specified.
4. All glassware used in the preparation of any working stock solution must be clean, dry, and free from any visible defects.
5. Once finished with any laboratory glassware, properly rinse out with DI water and soak in a 1% Liquinox cleaning solution.
6. Once prepared, the SCLCMSMS CTL Intermediate Solutions A and B must each be stored in appropriately labeled 15 mL amber glass vials with PTFE-lined caps below 0°C, and they can be used to prepare a new SCLCMSMS POS CTL working stock solution, as necessary, for up to one year from the date of preparation.
7. Once prepared, the SCLCMSMS POS CTL working stock solution must be evenly split between four separate kits (Kits A, B, C, and D), each stored in appropriately labeled 20 mL amber glass vials with PTFE-lined caps below 0°C. All four kits can be used for up to one year from the date of preparation of the SCLCMSMS CTL Intermediate Solutions A and B; however, Kit A should be used for months 1-3, Kit B for months 4-6, Kit C for months 7-9, and Kit D for months 10-12.
8. Once prepared, the ISTD working stock solution must be stored in an appropriately labeled 120 mL amber glass vial with a PTFE-lined cap below 0°C, and it can be used for up to one year from the date of preparation.
9. A working stock solution preparation log, located in the appropriate method folder on the (G:) toxicology network drive (\\MENAS3\TOX DATA), must be filled out with the reference material lot numbers and expiration dates and turned into the screening section supervisor or designee when new positive control and/or ISTD working stock solutions are prepared. CME lot numbers will then be assigned by the screening section supervisor or designee.

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10. The labels on each working stock solution must have the following information:
    a. Working stock solution identifier (e.g., SCLCMSMS POS CTL)
    b. Analyte group concentrations and storage solvent
    c. Preparation and expiration dates
    d. Analyst initials and CME lot number

B. Glassware

1. The following glassware items are required to prepare the positive control and ISTD working stock solutions:

Glassware	Size	Quantity
Volumetric Flask	10 mL	2
	100 mL	2
Volumetric Pipette	1 mL	2
Gastight Syringe	50 µL	1
	100 µL	1
	250 µL	1
	500 µL	1
	1 mL	1

C. Positive Control Working Stock Solutions

1. SCLCMSMS CTL Intermediate Solution A (Group 1: 2.00 mg/L; Group 2: 10.00 mg/L; Group 3: 30.00 mg/L)
    a. Using appropriately sized gastight syringes, add the following positive control reference materials into a 10 mL volumetric flask and bring to final volume with ACN:
        1) Group 1: 20 µL
        2) Group 2: 100 µL
        3) Group 3: 300 µL
2. SCLCMSMS CTL Intermediate Solution B (Group 4: 50.00 mg/L; Group 5: 100.00 mg/L)
    a. Using appropriately sized gastight syringes, add the following positive control reference materials into a 10 mL volumetric flask and bring to final volume with ACN:
        1) Group 4: 500 µL
        2) Group 5: 1 mL

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3. SCLCMSMS POS CTL (Group 1: 20.00 ng/mL, 1.00 ng/mL extracted; Group 2: 100.00 ng/mL, 5.00 ng/mL extracted; Group 3: 300.00 ng/mL, 15.00 ng/mL extracted; Group 4: 500.00 ng/mL, 25.00 ng/mL extracted; Group 5: 1,000.00 ng/mL, 50.00 ng/mL extracted)
   a. Using separate volumetric pipettes, add 1 mL of the SCLCMSMS CTL Intermediate Solutions A and B into a single 100 mL volumetric flask and bring to final volume with ACN.

D. Internal Standard Working Stock Solution

1. SCLCMSMS ISTD (Group 1: 200.00 ng/mL, 10.00 ng/mL extracted; Group 2: 1,000.00 ng/mL, 50.00 ng/mL extracted)
   a. Using appropriately sized gastight syringes, add the following isotopically-labeled ISTD reference materials into a 100 mL volumetric flask and bring to final volume with ACN:
      1) Group 1: 200 µL
      2) Group 2: 1 mL

10. Laboratory Procedure Requirements

A. Requirements

1. Prior to performing casework, the analyst must have completed the training module for this laboratory procedure, as well as have been deemed competent/proficient in the techniques used in this laboratory procedure within the previous 12 months:
   a. Instrumentation
      1) Liquid chromatography-mass spectrometry
   b. Sample preparation techniques
      1) Supported liquid extraction
   c. Screening analysis types
      1) Routine analysis
   d. Data processing
      1) Shimadzu LabSolutions
      2) Shimadzu Insight
2. Allow all reagents, working stock solutions, negative matrices, and case samples to come to room temperature before use.
3. Do not use expired chemicals, reagents, or working stock solutions.
4. Once finished with any laboratory glassware, properly rinse out with DI water and soak in a 1% Liquinox cleaning solution.
5. All negative matrices utilized in this laboratory procedure must be negative for all target analyte(s), ISTD(s), and known interferences.
6. All glassware used in this laboratory procedure must be clean, dry, and free from any visible defects.

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7. When using adjustable volume pipettes, the tips must be initially primed prior to any measurements, and they must be disposed of in an appropriate biohazard container.
8. For the routine analysis procedure, the negative and positive controls must be extracted from negative whole blood.
9. Logs and checklists
   a. All blank logs and checklists are located in the appropriate folder on the (G:) toxicology network drive (\\MENAS3\TOX DATA).
   b. An extraction log must be filled out for each analysis batch. It must include the batch number, date, and analyst, as well as the necessary lot numbers and applicable expiration dates of items used during the extraction (e.g., working stock solutions, extraction columns, reagents/solvents, etc.).
      1) Once filled out, it must be turned in to the screening section supervisor or designee as part of the physical batch pack (see Section 15, B, 1).
   c. Prior to analysis, an instrument checklist must be filled out for each analysis batch. It must include the analyst, date, batch number, instrument method, starting instrument parameters, and a vial/sequence check.
      1) The vial/sequence check must be performed by an independent analyst.
      2) Once filled out, this checklist must be saved in the appropriate folder on the (G:) toxicology network drive (\\MENAS3\TOX DATA).
10. Any deviation from the testing procedure (i.e., use of alternate specimen type, insufficient case sample volume for routine analysis, etc.) must be documented where appropriate (e.g., test note, batch note, incident/corrective action form).

11. Bench Procedure

A. Routine Analysis

1. Routine analysis can be performed on the following specimen types:
   a. Whole blood
2. Requirements
   a. All case samples assigned for routine analysis must be analyzed in single.
   b. Vortex-mix all case samples prior to aliquoting.
3. Case sample preparation
   a. Whole blood
      1) No initial preparation required.
4. Case sample aliquoting
   a. Using a calibrated 20-200 µL adjustable volume pipette, aliquot 200 µL of each case sample into an appropriately labeled 2 mL snap-cap microcentrifuge tube.
      1) If a case sample is unable to be accurately aliquoted after homogenization with a tissue grinder (e.g., too thick or clotted), 200 mg of the case sample must be weighed out into an appropriately labeled 2 mL snap-cap microcentrifuge tube using a calibrated analytical balance.
   b. Submit to the supported liquid extraction procedure (see Section 11, B).

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5. Positive control preparation
   a. Using a calibrated 20-200 µL adjustable volume pipette or a calibrated repeater pipette, aliquot 200 µL of negative whole blood into the necessary number of appropriately labeled 2 mL snap-cap microcentrifuge tubes.
      1) 1-10 case samples require at least 2 positive controls.
      2) An additional positive control is required for every additional group of 1-10 case samples.
   b. Using a calibrated 0.5-10 µL adjustable volume pipette, add the following positive control working stock solution, cap, and then vortex-mix:

Positive Control Working Stock Solution	Volume to Add (µL)	Final Concentration (ng/mL)				
		Group 1	Group 2	Group 3	Group 4	Group 5
SCLCMSMS POS CTL	10	1.00	5.00	15.00	25.00	50.00

   c. Submit to the supported liquid extraction procedure (see Section 11, B).
6. Negative control preparation
   a. Using a calibrated 20-200 µL adjustable volume pipette or a calibrated repeater pipette, aliquot 200 µL of negative whole blood into an appropriately labeled 2 mL snap-cap microcentrifuge tube.
   b. Submit to the supported liquid extraction procedure (see Section 11, B).

B. Supported Liquid Extraction Procedure

1. Using a calibrated repeater pipette, add the following ISTD working stock solution, cap, and then vortex-mix:

Internal Standard Working Stock Solution	Volume to Add (µL)	Final Concentration (ng/mL)	
		Group 1	Group 2
SCLCMSMS ISTD	10	10.00	50.00

2. Add 200 µL of 0.1M phosphate buffer, pH 7, cap, and then vortex-mix.
3. Centrifuge at 12,000 RPM for 10 minutes.

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4. Positive Pressure Manifold
   a. Set up rack with Biotage 400 µL ISOLUTE SLE+ columns.
   b. Use 16x100 mm culture tubes containing 100 µL of 1% HCl in MeOH to collect the elution solvents.

Step	Action	Reagent	Source	Volume (mL)	Flow Rate (psi)
1	Load samples by aliquoting 300 µL of the sample supernatant into SLE columns				2
2	Wait 10 min; clean and dry inside columns				2
3	Collect	HX:EtOAc:IPA (70:25:5)	Labeled Tube	2	2
4	Collect	HX:EtOAc:IPA (70:25:5)	Labeled Tube	2	2
5	Collect	HX	Labeled Tube	2	2
6	Collect	MTBE	Labeled Tube	2	2

C. Completion of Supported Liquid Extraction Procedure

1. Evaporate elution solvents to dryness under nitrogen at ≤40°C at 5 psi (~20 minutes).
   a. Note: Be careful not to over-dry!
2. Reconstitute the dried sample extracts with 50 µL of 5mM AmF/0.1% FA:MeOH (50:50), vortex-mix, and centrifuge at 3500 RPM for 30 seconds.
3. Transfer the reconstituted samples to appropriately labeled clear or amber ALS vials with conical inserts and then cap.
   a. Note: Ensure there are no bubbles at the bottom of any of the conical inserts.
4. Inject 3 µL for analysis by LC-MS/MS.

D. Storage of Extracted Samples

1. If, for any reason, an analysis batch cannot be analyzed on the same day as its extraction (e.g., instrument failure, air/water leak, end of day, etc.), it is acceptable to cap and store the dried sample extracts immediately following the elution solvent drying step of the Completion of Supported Liquid Extraction Procedure (see Section 11, C, 1). They must be stored between 2-8°C for no more than 24 hours, and a batch note indicating the reason for storage must be attached to the batch in CME.

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12. Instrument Procedure

A. Shimadzu Nexera 40 Series LC Pumps, Autosampler, Column Oven, Degassing Units, and System Controller with a Shimadzu LCMS-8060NX Mass Spectrometer

1. Instrument method and conditions
- **a.** SCLCMSMS_Rev1_MMDDYY.lcm
  - **1)** The MMDDYY added to the end of the method is to notate the most recent version of the method with the most up-to-date retention times, ion ratios, etc.
  - **2)** See Appendices 1 and 2 for detailed instrument conditions.
- **b.** Tray name
  - **1)** 1, 2, or 3 (from front to back)
- **c.** Sequence
  - **1)** Template available for new batch “SCREEN”
- **d.** Sample type
  - **1)** Unknown
- **e.** Analysis type (check boxes)
  - **1)** MS Integration for Quantitative (MIT)
  - **2)** MS Quantitative Calculation (MQT)
  - **3)** MS Integration for Qualitative
    - **a)** TIC (ILT)
  - **4)** MS Make Spectrum Process Table
    - **a)** Auto MS/MS (STA)
      - **i.** Merge Product Ion Scan Spectrum (MPI)
  - **5)** MS Library Search (LS)
- **f.** Injection volume
  - **1)** 3 µL
- **g.** Autopurge and column conditioning
  - **1)** Perform an Autopurge using settings that are saved in the method and/or analyze two or three “null” injections at the beginning of the batch to ensure acceptable chromatography and reproducible retention times.

2. Shutdown
- **a.** Sequence
  - **1)** Template available for new batch “SHUTDOWN”
    - **a)** Includes column wash, source clean, and instrument shutdown
  - **2)** Queue as separate batch

3. Important information
- **a.** Method files must be unique to each batch. Make a copy of the most current SCLCMSMS Revision 1 method file (SCLCMSMS_Rev1_MMDDYY) and put it in the data folder where the batch file is saved. Make sure the sequence list is directed to the newly created method file.

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13. Quality Control

A. Validation Summary

1. The following is a brief summary of the method validation results for whole blood. See the SCLCMSMS Method Validation Summary for the complete results.
   a. Limit of detection (LOD)
      1) Group 1: 0.5 ng/mL
      2) Group 2: 2.5 ng/mL
      3) Group 3: 5 ng/mL
      4) Group 4: 10 ng/mL
      5) Group 5: 25 ng/mL

B. Specimen Analysis

1. As a general precaution when analyzing postmortem specimens, unforeseen matrix effects, interference, and signal suppression may occur. If this is observed, it will be addressed on a case-by-case basis, possibly requiring the sample to be failed as unsuitable for analysis.

C. Routine Analysis

1. Batch analysis
   a. A negative control extracted from negative whole blood must be analyzed prior to the first positive control.
      1) The negative control is labeled in CME as *SCLCMSMS NEG CTL*.
   b. A positive control extracted from negative whole blood must be analyzed before and after each group of no more than ten case samples analyzed in single.
      1) The positive control is labeled in CME as *SCLCMSMS POS CTL*.
   c. A solvent blank (reconstitution solvent) must be analyzed prior to the negative control and before and after all positive controls (only after if immediately following a negative control).
   d. Example routine analysis batch:
      1) Solvent blank
      2) Negative control (blood)
      3) Positive control (blood)
      4) Solvent blank
      5) Case samples 1-10 (blood)
      6) Solvent blank
      7) Positive control (blood)
      8) Solvent blank
2. Quality control (QC) requirements
   a. Controls
      1) Negative control
         a) The negative control must be negative for all target analytes and detect all ISTDs.

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i. If a target analyte is detected in the negative control, all case samples that are positive for that analyte must be failed, reassigned, and reanalyzed for the failed analyte.

ii. If an ISTD is not detected in the negative control, all case samples must be failed, reassigned, and reanalyzed.

2) Positive controls

a) All analytes and ISTDs in each positive control must be properly detected.

i. If an analyte or ISTD is not detected in any of the positive controls, all case samples that were bracketed by the failed positive control must be evaluated on a case-by-case basis. It is at the discretion of the screening section supervisor or laboratory director to reassign the affected case samples for repeat analysis and/or additional testing.

b. Case samples

1) If an ISTD is not detected in a case sample, it must be failed, reassigned, and reanalyzed if no other analytes are detected. If analytes are detected, a test note must be added into CME regarding the cause of the missing ISTD. It is at the discretion of the screening section supervisor or laboratory director to reassign the case sample for repeat analysis.

c. Solvent blanks

1) All solvent blanks must be negative for each target analyte and the ISTD.

a) If an analyte or ISTD is detected in a solvent blank immediately prior to a case sample, as well as detected in that case sample, the case sample following that solvent blank must re-injected along with freshly prepared solvent blanks bracketing it to confirm the absence or presence of the analyte that carried over into the solvent blank.

b) If an analyte or the ISTD is detected in a solvent blank immediately prior to a positive control, the positive control following that solvent blank must re-injected along with freshly prepared solvent blanks bracketing it to confirm the presence of the positive control analyte that carried over into the solvent blank.

D. Corrective Action

1. If any aspect of a batch's quality control fails, there was a deviation from the testing procedure, or a non-routine incident occurred during the extraction or instrument setup (i.e., missing analyte in a positive control, analyte carryover in solvent blank, etc.), an Incident/Corrective Action Report, located in the appropriate folder on the (G:) toxicology network drive (\\MENAS3\TOX DATA), must be filled out and signed by the analyst, submitted to the screening section supervisor or designee for review, and a batch note indicating that an Incident/Corrective Action Report was submitted must accompany the batch in CME. The report documentation should describe the issue as well as the remedial action carried out to address the problem.

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14. Data Evaluation and Reporting of Results

A. Data Processing

1. Upon completion of the batch, open Insight Explore.
2. Open the *.lcb* batch file and load the *Flags.damlp* file.
   a. The location of the *Flags.damlp* file is in C://LabSolutions/Data
3. Highlight all samples in the sample list, right click, and click "Search library for highlighted samples."
4. Save the method file and the sample list *.damlp* file in the data folder.
   a. The batch is now loaded, processed, and saved.
5. To load a data set that has already been processed, click "Shortcuts" > "Open". Change drop-down filter from *.lcb* to *.damlp*, and then load the Insight *.damlp* data file.

B. Data Review

1. Select sample in "SAMPLE LIST" window on top right of Insight.
2. Move to "COMPOUND RESULTS" window on top left of Insight.
3. If necessary, left click the first column, "#", to organize by compound list.
   a. This will organize the analytes in RT order with ISTDs on top.
4. Then click Flag result to pull any compounds with library matches to the top
   a. Green is "Match"
   b. Red is "No Library" and do not need to be checked (no product ion scan present)
5. Use the checkboxes to mark compounds for reporting. Checkboxes are shared between each case and are not saved. Use the checkbox in the filter row to check/uncheck all between cases. The analyst must print both files after each case.
   a. Note: The report will always print in the order displayed on the "COMPOUND RESULTS" window and the report will only print checked compounds.

C. Analyte Identification

1. The identification of targeted compounds is based on the following:
   a. Library score index
      1) This factor may vary. It is usually strong for clear matches. Compounds with low ion breakdowns (e.g., Tramadol, MDA, Methamphetamine, etc.) can have high scores even when compared to background noise.
   b. Quality of MRM chromatograms
      1) There are 2 precursor>product ion breakdowns for each compound; both must be present with the same retention time to make an identification. Only 1 out of 2 MRMs present with a decent library match should be highly scrutinized before attempting to make an identification. Peak shape should be normal/gaussian.
   c. Ion abundance
      1) This is the abundance of the MRM transitions. Product scans generally do not occur with a signal less than $1.0e^4$. Low abundances may produce poor quality product ion scans, making it more difficult to interpret library matches.

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d. Quality of product ion scan chromatograms
  1) There are 3 product ion scans for each compound at different collision energies. The peak shape will not be as normal/gaussian as the MRM transitions; however, they should still contain multiple scans across the peak. Any product ion scans fewer than 3 points across the peak (i.e., product scan will appear invisible: 1 points, or as a line: 2 points) should be highly scrutinized before making an identification. There are likely not enough data points across the peaks for a reliable library match in low abundance product ion scans.

e. Visual quality of match
  1) The analyst should not solely rely on the library score index (LSI) for an identification. There should be a visual comparison of the product ion scans to the library match. Occasionally, there may be co-elution of analytes in the scans. Re-integration of a partial peak may be able to generate a cleaner scan and a better match.

f. Retention time
  1) The retention time of identified compounds in case samples should be within ±0.1 minutes of the compounds in one of the positive controls. Any shifting of a compound in question by more than ±0.2 minutes may be indicative of an interfering compound.

g. Special rules for THC and THC-COOH
  1) These two compounds do not have product ion scans and are identified by MRM transitions only. Qualifier ion ratios are set in the method and should be updated on a regular basis for optimal identification.
  2) There is an increased chance of interference with these two compounds causing ion ratios to be skewed or out of acceptable range. Any case samples with questionable identification of these two compounds may be sent for confirmatory testing at the discretion of the screening section supervisor or designee.

h. Analyte identification examples:

Match Type	Description
Good	≥1.0e^5 abundance, 2 MRMs with good quality, product ion scans present with multiple points, high LSI and easy visual comparison to library match, RT within ±0.1 minutes of same analyte in positive control
Good	High e^4 abundance, 2 MRMs with good quality, product ion scans present with multiple points, low LSI and visual comparison to library match contains diagnostic ions but additional ions from background, RT within ±0.1 minutes of same analyte in positive control
Poor	e^5 abundance, 1 MRM with good quality, product ion scans present with multiple points, mid LSI but visual comparison to library match not good, RT more than ±0.2 minutes of same analyte in positive control
Poor	High e^3 abundance, 2 MRMs but poor peak shape, product ion scans not present, mid LSI and poor visual comparison to library match

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2. Manual changes to identification criteria
   a. Ion ratios
      1) "Edit Method" > "Compounds" tab > "Ref Ions" column > "…" button > Set Number
   b. Library matches
      1) "Edit Tables" > Library Hits Window > Change Checkbox > Press "Edit Tables" again to lock
   c. Peak integration
      1) Right-click "Compound Details" > "Manual Peak Integrate" > "Horizontal", then left-click and drag from the start to finish across the desired peak.
      2) Right-click the analyte in the compounds results table, and then click "Search Library for Result".
3. The analytes identified by the software should only be used as a guide, and close consideration of these detected analytes should be evaluated objectively while considering the above criteria, as well as relevant case history and any completed CME results.

D. Printing of Results

1. Ensure the data file path for PDFCreator is pointed to the proper destination.
2. Click on "Screening Reports" > "MTS Summary Report". Check "Checked Compounds" and then click the print button.
3. Click on "Screening Reports" > "MTS Detail Report". Check "Highlights Samples" and "Checked Compounds" and then click the print button.
4. All bound and finalized PDF case sample data reports should be saved with the following identifiers in the file name: case number, specimen type and storage container, and batch number (e.g., 2023-XXXX_IVBGT_ZZZZ) on the (G:) toxicology network drive (\\MENAS3\TOX DATA) in the appropriate batch number and case number folders under "PDF DATA". All PDF data reports for QC samples (negative and positive controls) should be saved on the (G:) toxicology network drive (\\MENAS3\TOX DATA) in the appropriate batch number folder under "PDF DATA". These folders are automatically created when an analysis batch is created in CME.
   a. All unbound batch pack PDF reports associated with the negative and positive controls must be bound into one PDF in the order they were analyzed, along with the instrument checklist and sequence list, and saved with the following identifiers in the file name: CME SOP, date, and batch number.
      1) SCLCMSMS_MMDDYY_ZZZZ
   b. It is recommended, but not required, that any necessary digital annotations (e.g., text strikethroughs, highlighting, analyst signatures, etc.) be made to each individual PDF prior to binding.

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E. Reporting of Results

1. All analytes deemed identified by the analyst after the final data review must be reported in CME as "Detected". If the identification of an analyte is questionable, the analyte should remain in the PDF report and a test note indicating the uncertainty of the identification should be added into CME under the appropriate case number.

15. Submission of Results

A. eData

1. All bound case sample and batch pack PDF reports, as well as any supplemental documents for each case, must be linked appropriately in CME.
2. Case sample data linking
   a. When entering batch results, each bound case sample PDF report must be linked to their respective cases using the "Link Document to Case/Sequence" button after the results have been entered into CME for each case sample. A notification will popup indicating that the PDF document was correctly linked, and the total number of files linked to a case will be updated in the analysis batch.
   b. Should a document be incorrectly linked, or changes need to be made to a correctly linked document, the new linked document should be renamed with a new revision number added to the end of the file name.
   c. If, for any reason, any of the PDF reports were not linked to their respective case prior to submitting the batch for review, they must be linked through the Tox Case Status page in CME before a supervisor can review the case.
      1) Type in the specific case number in CME.
      2) Click on the toxicology request, scroll down to the assigned tests, and click on the test in which the PDF report was not linked.
      3) Click the "Link Document to Case/Sequence" button and link the missing PDF report. A notification will popup indicating that the PDF document was correctly linked.
3. Batch pack data linking
   a. The bound batch pack PDF report must be linked to its respective batch using the "Link Batch Documents" section in CME. The batch number and automatically populated test name must correlate, and the document description/type must be "Tox Batch RAW Data" in order to properly link the batch pack PDF report.
4. Once a batch is submitted for review, all associated Test Analysis Reports will automatically be digitally signed by the analyst who submitted the batch results for review.

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B. Batch Packs

1. Physical batch pack

a. Completed physical batch packs must be turned into the screening section supervisor or designee and include the following printed, filled out, and signed (where applicable) documents in this order:

1) Batch Review Report signed by the analyst (generated by CME)

a) Batch notes (if applicable)

2) Test Batch Summary Report (generated by CME)

3) Test Batch (generated when initial test batch number is created in CME)

4) SCLCMSMS Extraction Log

5) QC Control Report (generated by CME)

6) Incident/Corrective Action Report (if applicable)

2. Digital batch pack

a. Completed digital batch packs must be bound and linked to the batch in CME (see Section 15, A, 3) and include the following filled out and signed (where applicable) documents in this order:

1) Raw data for the negative and positive controls

2) Instrument checklist signed by an independent analyst who performed the vial/sequence check

3) Sequence list

16. Responsibilities

The analyst is responsible for performing the assay, processing the data, performing an initial review of the batch pack and case sample data to ensure that all applicable quality control aspects are acceptable or documented otherwise (see Sections 13, C through 13, D), printing the processed data to PDF, reporting the results in CME, linking the PDF case sample and digital batch pack data in CME, and ensuring all applicable worksheets, logs, and/or checklists are properly filled out and turned into the screening section supervisor or designee. The screening section supervisor or designee is responsible for performing a technical review of all batch pack and case sample data, filling out the batch pack summary, and scanning and linking the reviewed physical batch pack in CME.

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17. Definitions

ACN = Acetonitrile
AmF = Ammonium formate
DI = De-ionized
EtOAc = Ethyl acetate
FA = Formic acid
HCl = Hydrochloric acid
HX= Hexane
IPA = Isopropanol
ISTD = Internal standard
LC-MS/MS = Liquid chromatography-tandem mass spectrometry
LSI = Library score index
LOD = Limit of detection
MeOH = Methanol
MTBE = Methyl tert-butyl ether
$Na_2HPO_4 \cdot 7\ H_2O$ = Sodium phosphate dibasic heptahydrate
$NaH_2PO_4 \cdot H_2O$ = Sodium phosphate monobasic monohydrate
NaOH = Ammonium hydroxide
RMP = Reference material producer
RT = Retention time
QC = Quality control
SLE = Supported liquid extraction

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18. References

- ANSI/ASB Standard 017, *Standard Practices for Measurement Traceability in Forensic Toxicology*.
- ANSI/ASB Standard 036, *Standard Practices for Method Validation in Forensic Toxicology*.
- ANSI/ASB Standard 053, *Standard for Report Content in Forensic Toxicology*.
- ANSI/ASB Standard 054, *Standard for a Quality Control Program in Forensic Toxicology Laboratories*.
- ANSI/ASB Standard 098, *Standard for Mass Spectral Data Acceptance in Forensic Toxicology*.
- ANSI/ASB Standard 113, *Standard for Identification Criteria in Forensic Toxicology*.
- ANSI/ASB Standard 119, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Medicolegal Death Investigations*.
- ANSI/ASB Standard 152, *Standard for the Minimum Content Requirements of Forensic Toxicology Procedures*.
- ANSI/ASB Best Practice Recommendation 156, *Guidelines for Specimen Collection and Preservation for Forensic Toxicology*.
- Baselt, R.C. (2020). *Disposition of Toxic Drugs and Chemicals in Man* (12th ed.). Biomedical Publications.
- Flanagan, R.J., Cuypers, E., Maurer, H.H., Whelpton, R. (2020). *Fundamentals of Analytical Toxicology: Clinical and Forensic* (2nd ed.). John Wiley & Sons, Ltd.
- Lappas, N.T., Lappas, C.M. (2022). *Forensic Toxicology: Principles and Concepts* (2nd ed.). Elsevier.
- Levine, B.S., Kerrigan, S. (Eds.). (2020). *Principles of Forensic Toxicology* (5th ed.). Springer Nature Switzerland AG.

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19. Revisions

Date	Revision #	Type	Reason
11/28/2022	1	Major	Implementation of testing procedure into laboratory testing workflow.
09/01/2023	1	Minor	Reduced the number of analytes in the positive control, updated the preparation of the working stock solutions to include only those representative analytes, and added in the requirement of splitting the positive controls into four separate aliquots to extend the shelf life and stability of the positive control working stock solution.

20. Supplemental Documents

A. Testing Procedure Forms

1. SCLCMSMS Revision 1 Working Stock Solution Preparation Log
2. SCLCMSMS Revision 1 Extraction Log
3. Appendix 1: Insight Acquisition Report
4. Appendix 2: Insight Processing Method Report

B. Laboratory Forms

1. Incident/Corrective Action Report

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