

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Theresa Starpoli, Director of Technical Services
Date: September 22, 2026
RE: Validation Plan
 Addition to the Miscellaneous Basic Drug Quantitation and Confirmation by LC-MS/MS

Validation Plan-Q Addition to the Miscellaneous Basic Drug Quantitation and Confirmation by LC-MS/MS

It is proposed to validate a method for the quantitative analysis of miscellaneous basic drug compounds in biological matrices using liquid chromatography tandem mass spectrometry (LC-MS/MS). The target compounds and associated internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Compound	Internal Standard
Amitriptyline	Nortriptyline-D ₃
Citalopram	Citalopram-D ₆
Cyclobenzaprine	Cyclobenzaprine-D ₃
Dextromethorphan	Dextromethorphan-D ₃
Diphenhydramine	Diphenhydramine-D ₃
Fluoxetine	Fluoxetine-D ₆
Hydroxyzine	Hydroxyzine- ¹³ C ₆
Ketamine	Ketamine-D ₄
Nortriptyline	Nortriptyline-D ₃
Paroxetine	Paroxetine-D ₆
Phencyclidine	Phencyclidine-D ₅
Quetiapine	Quetiapine-D ₈
Sertraline	Sertraline-D ₃
Trazodone	Trazodone-D ₆

The current method within the Toxicology Procedures Manual (Qualtrax Revision 35) was slightly modified during method development. Additionally, the newly added target compounds and internal standards were optimized using the Agilent Technologies Optimizer software. The liquid chromatography parameters are listed in Table 2 while the ionization parameters are listed in Table 3.

Table 2 Liquid chromatography parameters

Parameter	Setting															
Column	Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 μm particle size (PN: 697775-902)															
Column Thermostat	40°C															
Mobile Phase A	0.1% Formic Acid in Water															
Mobile Phase B	0.1% Formic Acid in Acetonitrile															
Flow Rate	0.5 mL/min															
Injection Volume	5.0 μL															
Gradient	<table><tr><th>Time (min)</th><th>% Solvent A</th><th>% Solvent B</th></tr><tr><td>0.00</td><td>90</td><td>10</td></tr><tr><td>5.00</td><td>50</td><td>50</td></tr><tr><td>5.01</td><td>10</td><td>90</td></tr><tr><td>6.00</td><td>10</td><td>90</td></tr></table>	Time (min)	% Solvent A	% Solvent B	0.00	90	10	5.00	50	50	5.01	10	90	6.00	10	90
Time (min)	% Solvent A	% Solvent B														
0.00	90	10														
5.00	50	50														
5.01	10	90														
6.00	10	90														
Post Time	2.00 min															

Table 3 Instrumental source conditions

Parameter	Setting
Gas Temperature	350°C
Gas Flow	12 L/min
Nebulizer Pressure	40 psi
Capillary Voltage	2500 V
Delta EMV	200 V

The tandem mass spectrometer was utilized in Dynamic MRM acquisition. The parameters for all compounds within the method (including previously validated) are listed in Table 4.

Table 4 Dynamic MRM settings

Compound	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Fragmentor (V)	Collision Energy (V)	Retention Time (min)	Cell Accelerator (V)
Amitriptyline	278.2	233 91	105	12 24	4.71	7
Citalopram	325.2	262 109	145	16 24	4.00	7
Citalopram-D ₆	331.2	262 109	140	16 28	4.00	7
Cyclobenzaprine	276.2	215 202.1	126	47 55	4.20	4
Cyclobenzaprine-D ₃	279.2	215.1 205.1	132	52 24	4.20	4
Dextromethorphan	272.2	171 147	164	44 36	3.37	4
Dextromethorphan-D ₃	275.2	215.1 147	140	28 36	3.37	4
Diphenhydramine	256.2	167 152	80	4 40	3.87	7

Diphenhydramine-D ₃	259.2	167 152	80	8 40	3.87	7
Fluoxetine	310.1	148.1 44.1	80	4 9	4.50	4
Fluoxetine-D ₆	316.2	154.1 44	94	8 12	4.50	4
Hydroxyzine	375.2	201.1 165.1	110	17 77	4.10	4
Hydroxyzine- ¹³ C ₆	381.2	207 171	105	20 50	4.10	4
Ketamine	238.1	125 115	92	36 72	1.60	4
Ketamine-D ₄	242.1	129 92.1	100	40 76	1.60	4
Nortriptyline	264.2	233 91	105	8 20	4.61	7
Nortriptyline-D ₃	267.2	233 91	110	8 20	4.61	7
Paroxetine	330.2	192.1 70.1	110	21 33	3.90	4
Paroxetine-D ₆	336.2	198.2 76.1	140	21 37	3.90	4
Phencyclidine	244.2	159 86.1	92	12 12	3.10	4
Phencyclidine-D ₅	249.2	164.1 96.1	80	12 48	3.10	4
Quetiapine	384.2	279.1 253.1	140	25 21	3.10	4
Quetiapine-D ₈	392.2	286.1 258.1	140	29 25	3.10	4
Sertraline	306.1	275 159	94	8 28	4.70	4
Sertraline-D ₃	309.1	275 159	85	8 32	4.70	4
Trazodone	372.2	176 148	140	24 36	3.34	7
Trazodone-D ₆	378.2	182 150	140	24 36	3.34	7

The quantifier ions for each compound are bold in Table 4 and the listed retention times are approximate. In addition to the sample acquisition method, a blank solvent method will also be evaluated between each sample in a worklist to mitigate carryover. The chromatographic conditions for the solvent method are listed in Table 5.

Table 5 Instrumental parameters for solvent method

Parameter	Setting
Column	Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size (PN: 697775-902)
Column Thermostat	40°C
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.5 mL/min
Injection Volume	1.0 µL
Gradient	Time (min)
	% Solvent A
	% Solvent B
	0.00
	90
	10
	0.10
	10
	90
	2.10
	10
	90
Post Time	1.00 min

The sample preparation procedure within the Toxicology Procedures Manual was modified during method development to remove the utilization of methylene chloride as well as decrease sample and solvent volumes. The UCT Clean Screen DAU 50 mg bed mass column will be employed for validation. The established sample preparation procedure is a solid phase extraction utilizing 250 µL of biological matrix. The sample preparation procedure is listed in Table 6.

Table 6 Solid phase extraction procedure

Solid Phase Extraction Procedure
Prepare calibrators and controls Add 250 µL of case specimens/blank blood to appropriately labeled tubes Add 25 µL of internal standard solution and vortex briefly Add 2.0 mL 0.1 M phosphate buffer (pH 6) to each tube. Vortex briefly and let stand for 5 minutes Centrifuge at approximately 3500 rpm for 15 minutes to achieve separation Solid phase extraction (unless otherwise instructed, column flow rate is approximately 1 mL/min) Add 800 µL of hexane to each column and aspirate Add 800 µL of methanol to each column and aspirate Add 800 µL of deionized water to each column and aspirate Add 300 µL of 0.1 M phosphate buffer (pH 6) to each column and aspirate Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with approximately 1-2 mL/minute flow Add 1.6 mL of deionized water to each column and aspirate Add 500 µL of 1.0 M acetic acid to each column and aspirate Add 800 µL of methanol to each column and aspirate Dry column for approximately 2 minutes at maximum pressure or flow Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube Elute column with 3.0 mL of freshly prepared ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3) elution solvent by gravity flow into a screw-cap conical centrifuge tube Evaporate eluate to dryness at approximately 50°C under nitrogen Reconstitute with 100 µL of mobile phase A Vortex briefly

The working range for the quantitative compounds will be 10 ng/mL to 1000 ng/mL which includes a total of eight non-zero calibrators. For amitriptyline the working range will be from 5 ng/mL to 500 ng/mL. Nortriptyline will have a working range from 50 ng/mL to 5000 ng/mL. The matrices to be assessed quantitatively during the validation include blank blood, antemortem blood, and postmortem blood. Urine and liver will be assessed qualitatively.

During the validation, three control concentrations will be utilized as batch controls for the quantitative compounds. The calibrator and control concentrations are listed in Table 7. Calibrator and control standards and working solutions will be prepared in methanol.

Table 7 Calibrator and control concentrations

	Amitriptyline (ng/mL)	Nortriptyline (ng/mL)	All Other Analytes(ng/mL)
Calibrator 1	5	50	10
Calibrator 2	10	100	20
Calibrator 3	25	250	50
Calibrator 4	50	500	100
Calibrator 5	100	1000	200
Calibrator 6	250	2500	500
Calibrator 7	375	3750	750
Calibrator 8	500	5000	1000
Control 1	15	150	30
Control 2	200	2000	400
Control 3	400	4000	800

An Agilent Technologies 1260 Infinity II liquid chromatograph coupled to an Agilent Technologies 6470 tandem mass spectrometer and an Agilent Technologies 1290 Infinity multisampler coupled to an Agilent Technologies 6460 tandem mass spectrometer will both be used for the validation.

A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 37) and the Toxicology Procedures manual (Qualtrax Revision 35). The validation plan is in accordance with ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019).

1. Bias and Precision
 - a. Bias
 - b. Within-run Precision
 - c. Between-run Precision
2. Sensitivity
 - a. Estimated Limit of Detection (LOD)
 - b. Lower Limit of Quantitation (LLOQ)
3. Linearity and Calibration Model
4. Carryover
5. Interferences
 - a. Endogenous Compounds
 - b. Internal Standard
 - c. Commonly Encountered Analytes
6. Dilution Integrity
7. Stability
8. Robustness
9. References

1. Bias and Precision

The method is intended for the quantitative analysis of multiple matrices (i.e., blank blood, antemortem blood, postmortem blood, and urine). Therefore, bias and precision experiments shall be conducted for all matrix types and include only the quantitative compounds within the method. The presence of ionization suppression was assumed and therefore the number of matrix sources per matrix type will be increased from one to three.

a. Bias

Bias shall be measured using fortified matrix samples. To evaluate bias, a minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The working range to be evaluated is 10 ng/mL to 1000 ng/mL for all targets apart from amitriptyline and nortriptyline. Amitriptyline and nortriptyline will have working ranges of 5 ng/mL to 500 ng/mL and 50 ng/mL to 5000 ng/mL, respectively. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and the high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for bias include 30/15/150 ng/mL, 400/200/2000 ng/mL, and 800/400/4000 ng/mL (all targets/amitriptyline/nortriptyline).

The bias of the fortified pooled samples will be assessed using Equation 1.

Equation 1

Bias should be as low as possible but shall not exceed $\pm 20\%$ at each concentration level when analyzing common biological fluids. The same data used in the evaluation of bias shall also be used for the determination of within-run and between-run precision.

b. Within-run Precision

Precision will be expressed as the percent coefficient of variation (%CV). During method validation, within-run precision is measured using fortified matrix samples. A minimum of triplicate determinations per concentration (low, medium, and high) over a total of five batch analyses shall be evaluated. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and the high concentration shall be within approximately 80% of the highest end of the working range. The low, medium, and high concentrations to be evaluated for bias include 30/15/150 ng/mL, 400/200/2000 ng/mL, and 800/400/4000 ng/mL (all targets/amitriptyline/nortriptyline). The within-run precision shall be calculated using Equation 2.

Equation 2

$$\text{Within – run Precision (\%CV)} = \left(\frac{\text{Standard Deviation of Batch Mean}}{\text{Calculated Mean of Batch}} \right) \times 100$$

The within-run precision for each analytical run will be calculated. The analytical run with the largest within-run precision shall be utilized for the overall within-run precision of the process. Within-run precision shall not exceed $\pm 20\%$ at each concentration level when analyzing each matrix type.

c. Between-run Precision

Between-run precision will be measured using fortified matrix samples. A minimum of triplicate determinations per concentration (as delineated above) over a total of five batch analyses shall be evaluated. The between-run precision shall be calculated using Equation 3.

Equation 3

$$\text{Between – run Precision (\%CV)} = \left(\frac{\text{Standard deviation of combined means}}{\text{Calculated grand mean}} \right) \times 100$$

The between-run precision will be calculated using the combined data from the multiple analyses over the minimum of five batches. The standard deviation and mean will be calculated to determine the between-run precision. Between-run precision shall not exceed $\pm 20\%$ at each concentration level when analyzing each matrix type.

2. Sensitivity

a. Estimated Limit of Detection (LOD)

The estimated limit of detection for this validation shall be defined as an administratively defined decision point (threshold concentration). The administratively defined decision point for the quantitative compounds will be half of the lower limit of quantitation (2.5 ng/mL amitriptyline, 25 ng/mL nortriptyline, 5 ng/mL all other compounds).

The decision point shall be evaluated by fortifying, at minimum, nine different blank matrix sources per matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver). The number of matrix sources per matrix type were tripled based on the assumption of ionization suppression within the method. Additionally, urine and liver will be analyzed for estimated limit of detection as part of the qualitative validation for these matrices.

The nine different blank matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met. The predetermined identification criteria include Gaussian peak shape, retention time within $\pm 3\%$, signal-to-noise greater than or equal to 3.3. Additionally, the qualifier ratio acceptance criteria shall be in accordance with Table 1 of ANSI/ASB Standard 098, Standard for Mass Spectral Analysis in Forensic Toxicology (First Edition 2023). Table 8 describes the acceptance criteria requirement.

Table 8 Qualifier ratio acceptance criteria

Relative Intensity (% of target ion)	Tolerance (%)
Greater Than 50%	± 20
20 to 50%	± 25
10 to 20%	± 30
Less Than 10%	± 50

The predetermined acceptance criteria shall be met for $\geq 95\%$ of the samples to establish the estimated lower limit of detection. If the administratively defined decision point does not meet the predetermined acceptance criteria for $\geq 95\%$ of the samples, more samples shall be evaluated to establish the criteria.

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator (5/50/10 ng/mL amitriptyline/nortriptyline/all other compounds) for the method. For each matrix type (i.e., blank blood, antemortem blood, and postmortem blood), a minimum of nine different blank matrix sources shall be fortified at the lowest calibrator concentration and analyzed over a minimum of three analyses. The number of matrix sources per matrix type were tripled based on the assumption of ionization suppression within the method. The replicates per matrix source will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met. The predetermined identification criteria include Gaussian peak shape, retention time within $\pm 3\%$, signal-to-noise greater than or equal to 10, and a back calculated concentration within $\pm 20\%$. Additionally, the qualifier ratio acceptance criteria shall be in accordance with Table 1 of ANSI/ASB Standard 098, Standard for Mass Spectral Analysis in Forensic Toxicology (First Edition 2023).

The predetermined acceptance criteria shall be met for $\geq 95\%$ of the samples to establish the lower limit of quantitation. If the administratively defined decision point does not meet the predetermined acceptance criteria for $\geq 95\%$ of the samples, more samples shall be evaluated to establish the criteria.

3. Linearity and Calibration Model

The calibration model for the quantitative compounds within the method shall be established by determining the working range of analyte concentrations over which the method shall be used. The working range to be evaluated shall be 5 ng/mL to 500 ng/mL for amitriptyline, 50 ng/mL to 5000 ng/mL for nortriptyline, and 10 ng/mL to 1000 ng/mL for all other compounds. A total of eight non-zero calibrators (Table 9) will be evaluated. Within the working calibration range, there will be a correlation between peak area ratio of analyte and internal standard and the analyte concentration in the sample. The determined calibration model is the mathematical equation that describes this correlation.

To establish the calibration model, a minimum of five replicate determinations from different batches will be utilized. The calibration samples shall include the concentrations delineated in Table 9 for each target compound. A blank sample and the eight different non-zero concentration levels shall be used to establish the calibration model. Although the least squares model for regression is preferred, the best and simplest model (e.g., weighted, unweighted, linear, quadratic) that best fits the data will be chosen. The origin shall be ignored in each calibration model, the correlation coefficient shall be ≥ 0.990 , and the back calculated calibrator concentrations must be within $\pm 20\%$ of the target.

Table 9 Working range calibration sample concentrations

Amount of 1.25/12.5/2.50 mg/L solution (μ L)	Amount of 0.125/1.25/0.250 mg/L solution (μ L)	Final concentration (ng/mL)
	10	5/50/10
	20	10/100/20
	50	25/250/50
10		50/500/100
20		100/1000/200
50		250/2500/500
75		375/3750/750
100		500/5000/1000

NOTE: The concentrations are in order of amitriptyline, nortriptyline, and all other compounds.

The model will be established by residual analysis and statistical comparisons (ANOVA) between model fits. A plot of the residual values for each calibration type shall be generated to evaluate the effectiveness of the calibration model. The plot(s) will be visually evaluated to determine the model with homoscedasticity over the working range. Once established, the calibration model shall be utilized to obtain data regarding bias and precision, limit of quantitation, and dilution integrity within the validation.

4. Carryover

Carryover will be evaluated by analyzing blank matrix samples (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver) immediately following progressively higher concentrations (high calibrator concentration) of fortified matrix within the injection sequence. The highest analyte concentration at which no analyte carryover is observed, in the blank matrix,

is determined to be the concentration at which the method is free from carryover. Analyte carryover is indicated by a response greater than 10% of the LLOQ. This concentration shall be confirmed using triplicate analysis with a minimum of three sources per matrix type.

5. Interferences

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method shall be monitored. If present, the impact on identification and quantitation shall be evaluated. If the instrumental response is less than 10% of the LLOQ response for the qualifier or quantifier ions, the impact is deemed insignificant.

a. Endogenous Compounds

Where possible, negative matrix samples from different sources without the addition of an internal standard shall be analyzed for possible endogenous interferences. A minimum of ten matrix samples for each matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver) within the validation should be evaluated, whenever possible.

b. Internal Standard

To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified with the highest calibrator concentration without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine) sample, per matrix type, shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard (20 ng/mL) without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine) sample, per matrix type, shall be evaluated.

c. Commonly Encountered Analytes

Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current method).

Potential interferents to be evaluated:

- Barbiturates (30 mg/L)
- Amphetamines (2.0 mg/L)
- Benzodiazepines (2.0 mg/L)

Carisoprodol and meprobamate (100 mg/L)
Anti-epileptic drugs (40 mg/L)
Basic drugs from previously made mixes (6.0 mg/L)
Acid/neutral drugs from previously made mixes (6.0 mg/L)
Opioids and cocaine (0.2/2.0/1.0 mg/L)
Fentanyl derivatives (0.05/0.1 mg/L)
Novel psychoactive substance (1.0 mg/L)
Cannabinoids (0.1/0.2/0.5 mg/L)

In addition to commonly encountered analytes, each compound and internal standard within the method will be evaluated individually to evaluate the potential impact of structural similarity or degradation. Metabolites including N-desmethyl-diphenhydramine, desmethylocitalopram, and m-cPP will also be evaluated for interferences.

6. Dilution Integrity

The dilution integrity will be assessed for concentrations above the upper limit of quantitation (ULOQ) with sufficient sample volume (large volume). The large volume dilution will be evaluated using 250 µL of matrix and diluting with blank matrix. The matrices included in the evaluation will be blank blood, antemortem blood, and postmortem blood. Common dilution ratios (1:2 and 1:10) will be evaluated for bias and precision per matrix type utilizing the experiments delineated in Section 1. The concentration will be adjusted depending upon the dilution factor and the adjusted concentration must be within the predetermined acceptance criteria ($\pm 20\%$ of the undiluted target concentration) for both bias and precision.

7. Stability

During the validation period, the stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. This enables the simulation of an abrupt abortion, delay, or interruption during instrumental analysis.

At minimum, a single blank matrix source, per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), will be extracted at two concentrations (30/15/150 ng/mL [all targets/ amitriptyline/ nortriptyline], 800/400/4000 ng/mL [all targets/ amitriptyline/ nortriptyline]) and analyzed at minimum every twenty-four hours for a seven-day period (or until no sample remains) with triplicate injections at each time point.

For day one, the samples will be extracted and immediately analyzed. The ratioed responses (analyte to internal standard) will be averaged and all other ratioed responses from subsequent time points will be evaluated against the average ratioed response. The average instrumental ratioed responses for each time point will be compared to the day one instrumental ratioed response and plotted. Compounds are considered stable if the average ratioed response of the triplicate injections for a time point falls within the method's predefined acceptable bias (i.e.,

±20%). For example, if the peak area increases above 120% or decreases below 80% of the original response the compound is no longer deemed stable.

Stability should be carried out by injecting samples from the same autosampler vial throughout the stability experiments.

8. Robustness

Robustness will be determined by performing the validation on multiple instruments within the laboratory.

9. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 37, 2026.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 35, 2026.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. 2019.

ANSI/ASB Standard 098, Standard for Mass Spectral Analysis in Forensic Toxicology, 1st Edition. 2023.

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Theresa Starpoli, Director of Technical Services
Date: September 22, 2026
RE: Method Development Summary
Method Development for the Addition to the Miscellaneous Basic Drug
Quantitation and Confirmation by LC-MS/MS

Method Development Summary for the Addition to the Miscellaneous Basic Drug Quantitation and Confirmation by LC-MS/MS

The method development for the addition of compounds to the miscellaneous basic drug quantitation and confirmation has been a multifaceted project. Initial development was performed by the Western Toxicology Section to add phencyclidine, ketamine, and dextromethorphan to the Miscellaneous Basic Drug Quantitation and Confirmation by LC-MS/MS method in the Toxicology Procedures Manual (Qualtrax Revision 35). Additional compounds were subsequently evaluated by the Chemistry Research Section including amitriptyline, nortriptyline, citalopram, diphenhydramine, and trazodone. Validation plans were previously drafted for both projects and approved. The compounds from the Western Toxicology Section and Chemistry Research Section were subsequently combined during validation to be performed by the Chemistry Research Section. During method validation, regulations on the utilization of methylene chloride in the laboratory were established. The solid phase extraction method utilizes methylene chloride as part of the elution solvent. Therefore, additional method development was required to eliminate methylene chloride from the sample preparation procedure.

In addition to the removal of methylene chloride from the sample preparation procedure, evaluations were conducted to reduce biological matrix volume and solvent volumes. Furthermore, additional internal standards were evaluated.

Target Compounds

The compounds evaluated were the compounds within the published Toxicology Procedures Manual method, Western Toxicology Section validation, and Chemistry Research Section validation. The target compounds and internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Compound	Internal Standard
Amitriptyline	Nortriptyline-D ₃
Citalopram	Citalopram-D ₆
Cyclobenzaprine	Cyclobenzaprine-D ₃
Dextromethorphan	Dextromethorphan-D ₃
Diphenhydramine	Diphenhydramine-D ₃
Fluoxetine	Fluoxetine-D ₆
Hydroxyzine	Hydroxyzine- ¹³ C ₆
Ketamine	Ketamine-D ₄
Nortriptyline	Nortriptyline-D ₃
Paroxetine	Paroxetine-D ₆
Phencyclidine	Phencyclidine-D ₅
Quetiapine	Quetiapine-D ₈
Sertraline	Sertraline-D ₃
Trazodone	Trazodone-D ₆

During method development, different internal standards were evaluated for amitriptyline and hydroxyzine. During previous validation experiments, amitriptyline was paired with amitriptyline-D₃ and hydroxyzine was paired with quetiapine-D₈. When evaluating amitriptyline-D₃ without the presence of target compounds, a contribution was noted with nortriptyline. The instrumental response for nortriptyline when analyzing amitriptyline-D₃ was greater than 10% of the lower limit of quantitation instrumental response and therefore was deemed significant. To assess the quantitative impact of removing amitriptyline-D₃, the amitriptyline calibration curves were re-processed with nortriptyline-D₃ internal standard. With accuracies within $\pm 20\%$ of the known concentration, the change in internal standard was deemed appropriate.

During the initial method development and validation of hydroxyzine, a deuterated matched internal standard was not commercially available. Therefore, hydroxyzine was paired with quetiapine-D₈. Hydroxyzine-¹³C₆ is now available from Cerilliant and was evaluated as an internal standard for hydroxyzine. The internal standard was optimized, and hydroxyzine will be evaluated using both internal standards during validation.

Instrumental Method Development

The instrumental parameters within the Toxicology Procedures Manual (Section 31.5.10) were maintained during method development. The liquid chromatography parameters are listed in Table 2.

Table 2 Liquid chromatography parameters

Parameter	Setting
Column	Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size (PN: 697775-902)
Column Thermostat	40°C
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.5 mL/min
Injection Volume	5.0 µL
Gradient	Time (min)
	% Solvent A
	% Solvent B
	0.00
	90
	5.00
	50
	5.01
	10
	6.00
	10
Post Time	2.00 min

It should be noted that the injection volume was increased from 1 µL to 5µL. This change was due to the change in the sample preparation procedure. The current method requires 1.0 mL of biological matrix, while the newly developed method requires 250 µL of biological matrix. The source conditions are listed in Table 3.

Table 3 Instrumental source conditions

Parameter	Setting
Gas Temperature	350°C
Gas Flow	12 L/min
Nebulizer Pressure	40 psi
Capillary Voltage	2500 V
Delta EMV	200 V

The source conditions were evaluated using source optimizer when the newly established targets were added to the method. The gas flow changed from 10 L/min to 12 L/min and the capillary voltage changed from 4000 volts to 2500 volts. Additionally, the delta EMV was adjusted from 400 volts to 200 volts. The method utilizes Dynamic MRM acquisition using the settings listed in Table 4.

Table 4 Dynamic MRM settings

Compound	Precursor Ion (<i>m/z</i>)	Product Ion (<i>m/z</i>)	Fragmentor (V)	Collision Energy (V)	Retention Time (min)	Cell Accelerator (V)
Amitriptyline	278.2	233	105	12	4.71	7
		91		24		
Citalopram	325.2	262	145	16	4.00	7
		109		24		
Citalopram-D ₆	331.2	262	140	16	4.00	7
		109		28		

Method Development Summary: Misc Base LC-MS/MS

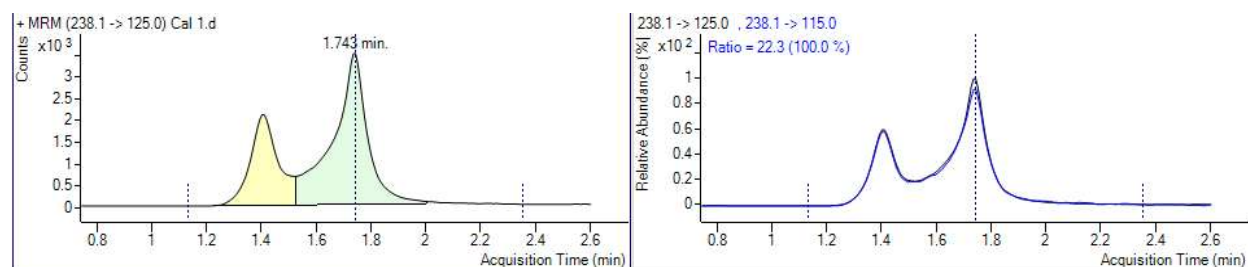
Cyclobenzaprine	276.2	215	126	47	4.20	4
		202.1		55		
Cyclobenzaprine-D ₃	279.2	215.1	132	52	4.20	4
		205.1		24		
Dextromethorphan	272.2	171	164	44	3.37	4
		147		36		
Dextromethorphan-D ₃	275.2	215.1	140	28	3.37	4
		147		36		
Diphenhydramine	256.2	167	80	4	3.87	7
		152		40		
Diphenhydramine-D ₃	259.2	167	80	8	3.87	7
		152		40		
Fluoxetine	310.1	148.1	80	4	4.50	4
		44.1		9		
Fluoxetine-D ₆	316.2	154.1	94	8	4.50	4
		44		12		
Hydroxyzine	375.2	201.1	110	17	4.10	4
		165.1		77		
Hydroxyzine- ¹³ C ₆	381.2	207	105	20	4.10	4
		171		50		
Ketamine	238.1	125	92	36	1.60	4
		115		72		
Ketamine-D ₄	242.1	129	100	40	1.60	4
		92.1		76		
Nortriptyline	264.2	233	105	8	4.61	7
		91		20		
Nortriptyline-D ₃	267.2	233	110	8	4.61	7
		91		20		
Paroxetine	330.2	192.1	110	21	3.90	4
		70.1		33		
Paroxetine-D ₆	336.2	198.2	140	21	3.90	4
		76.1		37		
Phencyclidine	244.2	159	92	12	3.10	4
		86.1		12		
Phencyclidine-D ₅	249.2	164.1	80	12	3.10	4
		96.1		48		
Quetiapine	384.2	279.1	140	25	3.10	4
		253.1		21		
Quetiapine-D ₈	392.2	286.1	140	29	3.10	4
		258.1		25		
Sertraline	306.1	275	94	8	4.70	4
		159		28		
Sertraline-D ₃	309.1	275	85	8	4.70	4
		159		32		
Trazodone	372.2	176	140	24	3.34	7
		148		36		
Trazodone-D ₆	378.2	182	140	24	3.34	7
		150		36		

The quantifier ions for each compound are bold in Table 4 and the listed retention times are approximate. It should also be noted that the phencyclidine-D₅ fragmentor voltages were different for each transition. After development, the voltage for the 249.2 *m/z* to 164.1 *m/z* was

changed from 92 volts to 80 volts. Several injections were evaluated after the change to ensure no adverse impact. The method will be validated using a fragmentor voltage of 80 volts for phencyclidine-D₅.

Using the updated acquisition method, instrumental carryover was evaluated. During the original method development and validation, significant carryover was noted for fluoxetine and sertraline. The remediation of carryover includes as solvent blank between all calibrators, controls, and samples. During method development, carryover mitigation was evaluated using the multi-wash feature on the Agilent Technologies 6470 instrument. Initially, carryover was mitigated using isopropyl alcohol and 0.1% formic acid in water followed by solvent injections between calibrators, controls, and samples. When additional compounds were added to the method, the multi-wash method created split peaks for ketamine. An example of the split peak is shown in Figure 1.

Figure 1 Ketamine split peaks observed with multi-wash



Upon further investigation, the split peak for ketamine was from residual isopropanol within the chromatographic system. Therefore, isopropyl alcohol was replaced with methanol as the needle wash solvent.

During the development of the sample preparation procedure, carryover was re-assessed with the finalized method. Given the dramatic reduction in biological matrix required for extraction, carryover was subsequently mitigated using a standard wash procedure and rapid solvent method. The original sample preparation required 1.0 mL of biological matrix with final reconstitution in 100 μ L of mobile phase A. The optimized sample preparation utilizes 250 μ L of biological matrix with reconstitution in 100 μ L mobile phase A.

The standard wash procedure in conjunction with a rapid solvent run was found to be the most effective at alleviating the potential for carryover. A standard wash for 30 seconds with methanol followed by a 3.10 minute blank solvent injection demonstrated no carryover while maintaining appropriate peak shape. The instrumental parameters for the solvent method are listed in Table 5.

Table 5 Instrumental parameters for solvent method

Parameter	Setting
Column	Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm particle size (PN: 697775-902)
Column Thermostat	40°C
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.5 mL/min
Injection Volume	1.0 µL
Gradient	Time (min)
	% Solvent A
	% Solvent B
	0.00
	90
	10
	0.10
	10
	90
	2.10
	10
	90
Post Time	1.00 min

The source of carryover was determined to be from the needle and needle seat. Given the source of carryover, the rapid solvent method was determined to be appropriate.

Sample Preparation Method Development

Given the regulations associated with the use of methylene chloride in the laboratory, the existing solid phase extraction procedure was changed to remove the use of the solvent. The current method in the Toxicology Procedures Manual is described below.

- Prepare calibrators and controls
- Add 1.0 mL of case specimens/blank blood to appropriately labeled tubes
- Add 50 µL of internal standard solution and vortex briefly
- Add 2.0 mL deionized water to each tube. Vortex briefly and let stand for 5 minutes
- Centrifuge at approximately 3500 rpm for 15 minutes to achieve separation. Transfer supernatant to clean tubes and discard the tube with the remaining pellet
- Add 2.0 mL of 0.1 M phosphate buffer (pH 6) and vortex
- Solid phase extraction (unless otherwise instructed, column flow rate is approximately 1 mL/min)
 - Add 3.0 mL of hexane to each column and aspirate
 - Add 3.0 mL of methanol to each column and aspirate
 - Add 3.0 mL of deionized water to each column and aspirate
 - Add 1.0 mL of 0.1 M phosphate buffer (pH 6) to each column and aspirate
 - Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with approximately 1-2 mL/minute flow
 - Add 3.0 mL of deionized water to each column and aspirate
 - Add 3.0 mL of deionized water to each column and aspirate
 - Add 2.0 mL of 1.0 M acetic acid to each column and aspirate
 - Add 3.0 mL of methanol to each column and aspirate

- Dry column for approximately 2 minutes at maximum pressure or flow
- Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube
- Elute column with 3.0 mL of freshly prepared methylene chloride: isopropanol: ammonium hydroxide (78:20:2) elution solvent by gravity flow into a screw-cap conical centrifuge tube
- Evaporate eluate to dryness at approximately 50°C under nitrogen
- Reconstitute with 100 µL of mobile phase A
- Vortex briefly
- If necessary, centrifuge the tubes for 15 minutes at approximately 2800 rpm
- Transfer to an autosampler vial fitted with a glass insert

Several elution solvents were evaluated and compared to determine the optimal elution solvent without the use of methylene chloride. The following solvent combinations were evaluated.

- Methanol: ammonium hydroxide (100:5)
- Ethyl acetate: acetonitrile: ammonium hydroxide (80:20:2)
- Ethyl acetate: ammonium hydroxide (100:2)
- Hexane: isopropanol: ammonium hydroxide (80:20:2)

When evaluating these solvents, the ethyl acetate: ammonium hydroxide (100:2) produced the highest instrumental response among most analytes within the method. This was particularly observed with the later eluting compounds including sertraline and fluoxetine. Three additional solvent combinations with varying amounts of ammonium hydroxide were also evaluated.

- Toluene: isopropanol (80:20) with 2 mL, 3 mL, and 4 mL ammonium hydroxide
- Acetone: isopropanol (80:20) with 2 mL, 3 mL, and 4 mL ammonium hydroxide
- Ethyl acetate: isopropanol (80:20) with 2 mL, 3 mL, and 4 mL ammonium hydroxide

During the evaluation, it was observed that solvent combinations containing acetone produced an overall lower instrumental response. It was noted that ethyl acetate produced an overall higher instrumental response when compared to toluene based solvent combinations. This was particularly true with later eluting compounds. This was not observed with quetiapine which had an instrumental response three times higher with toluene as compared to ethyl acetate based solvent combinations. It should be noted that when toluene was used, the elution solvent often appeared cloudy and not fully mixed.

To further evaluate the elution solvents, the previously evaluated solvents were paired down and the following were compared to the performance of the current elution solvent of methylene chloride: isopropanol: ammonium hydroxide (80:20:2).

- Toluene: isopropanol: ammonium hydroxide (80:20:2)
- Ethyl acetate: acetonitrile: ammonium hydroxide (80:20:2)

- Ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3)
- Ethyl acetate: acetonitrile: ammonium hydroxide (80:20:4)

Both ethyl acetate: acetonitrile: ammonium hydroxide (80:20:2) and ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3) produced the highest instrumental response when solvents were compared. This was particularly true for the later eluting compounds that typically produce lower instrumental responses. Therefore, ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3) was selected as the optimal elution solvent.

In addition to the evaluation of different elution solvents, an acetonitrile crash was also evaluated but this extraction did not produce significantly better results.

Sample Volume and Solvent Volume Reduction

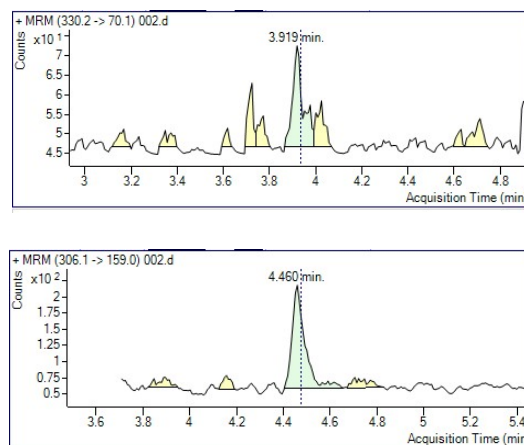
In addition to changing the elution solvent, an investigation was performed to evaluate the potential for reducing sample volume and solvent volumes. Table 6 lists the different solvent volumes that were initially evaluated.

Table 6 Sample and solvent volumes

Sample Preparation	Current Volume	1:2 Volume	1:4 Volume	1:5 Volume
Blood	1000 µL	500 µL	250 µL	200 µL
Deionized Water	2000 µL	1000 µL	500 µL	400 µL
0.1 M Phosphate Buffer	2000 µL	1000 µL	500 µL	400 µL
Solid Phase Extraction	Current Volume	1:2 Volume	1:4 Volume	1:5 Volume
Hexane	3000 µL	1500 µL	750 µL	600 µL
Methanol	3000 µL	1500 µL	750 µL	600 µL
Deionized Water	3000 µL	1500 µL	750 µL	600 µL
0.1 M Phosphate Buffer	1000 µL	500 µL	250 µL	200 µL
Deionized Water	6000 µL	3000 µL	1500 µL	1200 µL
1 M Acetic Acid	2000 µL	1000 µL	500 µL	400 µL
Methanol	3000 µL	1500 µL	750 µL	600 µL
Elution Solvent	3000 µL	1500 µL	750 µL	600 µL
Reconstitution Solution	100 µL	100 µL	100 µL	100 µL
Injection Volume	1 µL	2 µL	4 µL	5 µL

The 1:5 volume was initially evaluated and produced low instrumental responses with sertraline and paroxetine producing the lowest responses. These low instrumental responses subsequently produced poor peak shape. The chromatography for these two compounds at the 1:5 sample/solvent volume is shown in Figure 2.

Figure 2 Paroxetine (top) and sertraline (bottom) at 1:5 volume



When comparing the three sample/solvent reduction volumes, it was noted that with decreased sample volume a decrease in instrumental response was also observed. This was hypothesized to be due to the reduction of sample volume while maintaining the solid phase extraction sorbent bed mass of 200 mg. Therefore, a 1:5 solvent reduction was evaluated while maintaining the original sample preparation volumes. This still produced a reduced instrumental response.

To elucidate the source of the analyte loss, recovery studies were performed and it was determined that the 1:5 samples had a lower recovery than the current methods recovery. Given the differences in recovery, a smaller sorbent bed mass was evaluated. To evaluate the solid phase extraction sorbent bed mass, the 200 mg sorbent mass was compared to a 130 mg and 50 mg bed mass (UCT Clean Screen ZSDAU-013 and ZSDAU-005). The 130 mg bed mass was evaluated using the 1:2 volumes while the 50 mg bed mass was evaluated with the 1:5 volumes.

When assessing the two different sorbent bed masses, it was noted that the 1:2 sample/solvent volume extraction with a 130 mg sorbent bed mass produced the highest instrumental responses. When additional solvent was used for elution, the 1:5 sample/solvent volume produced significantly higher instrumental responses and therefore demonstrated the greatest potential.

Upon further investigation, the 1:5 sample/solvent volume with a 3 mL elution solvent was compared to a 1:4 sample/solvent volume 3 mL elution solvent. The 1:5 sample/solvent volume produced lower instrumental responses with some instances of poor chromatography when compared to the 1:4 sample/solvent volume. Therefore, the 1:4 sample/solvent volume with a 50 mg sorbent bed mass was determined to be most appropriate.

Additional studies were performed to evaluate the utilization of phosphate buffer and water in the sample preparation procedure or solely phosphate buffer. The instrumental responses were unchanged during the evaluation and therefore, 250 μ L of biological matrix and 2.0 mL of

phosphate buffer was utilized as the final sample preparation volumes. The finalized sample preparation volumes are listed in Table 7.

Table 7 Optimized sample and solid phase extraction volumes

Sample Preparation	1:4 Volume
Blood	250 µL
0.1 M Phosphate Buffer	2000 µL
Solid Phase Extraction	1:4 Volume
Hexane	800 µL
Methanol	800 µL
Deionized Water	800 µL
0.1 M Phosphate Buffer	300 µL
Deionized Water	1600 µL
1 M Acetic Acid	500 µL
Methanol	800 µL
Elution Solvent	3000 µL
Reconstitution Solution	100 µL
Injection Volume	5 µL

Based on the method development experiments the optimized sample preparation procedure is as follows.

- Prepare calibrators and controls
- Add 250 µL of case specimens/blank blood to appropriately labeled tubes
- Add 25 µL of internal standard solution and vortex briefly
- Add 2.0 mL 0.1 M phosphate buffer (pH 6) to each tube. Vortex briefly and let stand for 5 minutes
- Centrifuge at approximately 3500 rpm for 15 minutes to achieve separation
- Solid phase extraction (unless otherwise instructed, column flow rate is approximately 1 mL/min)
 - Add 800 µL of hexane to each column and aspirate
 - Add 800 µL of methanol to each column and aspirate
 - Add 800 µL of deionized water to each column and aspirate
 - Add 300 µL of 0.1 M phosphate buffer (pH 6) to each column and aspirate
 - Without delay, pour specimens into appropriate SPE columns. Elute from cartridges with approximately 1-2 mL/minute flow
 - Add 1.6 mL of deionized water to each column and aspirate
 - Add 500 µL of 1.0 M acetic acid to each column and aspirate
 - Add 800 µL of methanol to each column and aspirate
 - Dry column for approximately 2 minutes at maximum pressure or flow
 - Wipe the SPE column tips with Kimwipes®. Place labeled conical test tubes in the manifold test tube rack. Be sure SPE column tips are in the designated conical tube

- Elute column with 3.0 mL of freshly prepared ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3) elution solvent by gravity flow into a screw-cap conical centrifuge tube
- Evaporate eluate to dryness at approximately 50°C under nitrogen
- Reconstitute with 100 µL of mobile phase A
- Vortex briefly
- If necessary, centrifuge the tubes for 15 minutes at approximately 2800 rpm
- Transfer to an autosampler vial fitted with a glass insert

Amitriptyline Conversion to Nortriptyline

During method development, it was noted that extracted samples containing amitriptyline would produce an instrumental response for nortriptyline. When analyzing a 750 ng/mL sample containing amitriptyline, the instrumental response produced for nortriptyline was greater than 10% of the instrumental response for the 50 ng/mL calibrator (lower limit of quantitation). To better characterize the conversion, pre-extraction fortified samples, post-extraction fortified samples, and neat samples of amitriptyline at high concentrations were prepared and analyzed. Both the pre-extraction fortified, and post-extraction fortified samples demonstrated an instrumental response for nortriptyline that was approximately 2.8% and 1.7% of the amitriptyline response respectively. This experiment demonstrated the conversion of amitriptyline to nortriptyline is occurring both during sample extraction and during the sample dry down process.

A neat sample of amitriptyline was prepared by adding certified reference material directly to the 100 µL of mobile phase A. The analytical results showed minimal conversion with a response of nortriptyline being 0.01% of the amitriptyline response. This not only demonstrated the purity of the certified reference material but also demonstrated that the conversion was not occurring within the instrumentation.

To assess the impact of sample preparation steps on the conversion of amitriptyline to nortriptyline, different variables were evaluated including the utilization of amber vials, addition of isopropanol with acid for dry down, and reconstitution solvent composition. The utilization of different vials and the addition of 20 µL of 0.1% hydrochloric acid in isopropanol prior to dry down did not improve the conversion. When evaluating a reconstitution volume of 50:50 mobile phase A to mobile phase B, there was no improvement of the conversion but a noted improvement on the instrumental response was noted. Although the instrumental response improved, the utilization of the mixed reconstitution was inhibited by solvent evaporation.

The impact on conversion was also investigated by assessing ethyl acetate: acetonitrile: ammonium hydroxide (80:20:3) and ethyl acetate: acetonitrile: ammonium hydroxide (80:20:2) elution solvents. It was noted that 80:20:3 produced slightly lower conversion rates (0.5%) compared to 80:20:2 (0.8%).

Based on the lack of improvement on the rate of conversion of amitriptyline to nortriptyline, it was determined that a change in calibration range would enable the two compounds to be within the same method without potential impact on the quantitative results. The initial concentration range for both amitriptyline and nortriptyline were established to be 50 ng/mL to 750 ng/mL. After calibration range adjustment, amitriptyline will be evaluated from 5 ng/mL to 500 ng/mL while nortriptyline will be evaluated from 50 ng/mL to 5000 ng/mL. The newly established calibration ranges are listed in Table 8.

Table 8 Calibrator and control concentrations

	Amitriptyline (ng/mL)	Nortriptyline (ng/mL)	All Other Analytes(ng/mL)
Calibrator 1	5	50	10
Calibrator 2	10	100	20
Calibrator 3	25	250	50
Calibrator 4	50	500	100
Calibrator 5	100	1000	200
Calibrator 6	250	2500	500
Calibrator 7	375	3750	750
Calibrator 8	500	5000	1000
Control 1	15	150	30
Control 2	200	2000	400
Control 3	400	4000	800

Upon evaluation of the new calibration range for each compound, it was noted that the nortriptyline response was still above the 10% threshold when 500 ng/mL of amitriptyline was analyzed. It was subsequently determined that the amitriptyline-D₃ internal standard was also converting to nortriptyline. The amitriptyline data was then evaluated using nortriptyline-D₃ as the internal standard and demonstrated promise. Therefore, amitriptyline-D₃ was removed from the analytical method.

During the method development, the utilization of 48-well plates for sample analysis were investigated. Although this showed promise with all other analytes, it was determined that the utilization of 48-well plates contributed to the conversion of amitriptyline to nortriptyline. Therefore, 48-well plates cannot be used for analysis.

References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 37, 2026.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 35, 2026.