Memo To: James Hutchings, Ph.D., Toxicology Program Manager 8/6/25
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date June 16, 2025
RE: Validation Plan
Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

Validation Plan – Utilization of a Circulating Bath in the Amphetamines, Phentermine, and Designer Stimulants Quantitation and Confirmation by LCMSMS Method

It is proposed to validate the utilization of a recirculating bath within the liquid-liquid extraction procedure for the existing quantitative analysis method for amphetamines, phentermine, and designer stimulants using LCMSMS. The existing method is delineated in the Toxicology Procedures Manual Section 26, Amphetamines, Phentermine and Designer Stimulants Quantitation and Confirmation by LCMSMS method (Qualtrax Revision 32). The validation will be performed using the liquid-liquid extraction delineated in Section 26.6 of the Toxicology Procedures Manual. After centrifugation at approximately 2500 rpm for 15 minutes to achieve separation (Section 26.6.8), the samples will be placed into a PolyScience Refrigerated Circulator (PN AD15R-40A-11B) containing reagent alcohol (Fisher A962R-4). The samples will be incubated in the circulator for 3 minutes at -25°C. After incubation, the supernatant will be poured into clean, screw-cap tubes. Following the transfer, the remainder of the procedure will be followed as published (Section 26.6.10 – 26.6.12). The target analytes and associated internal standards are listed in Table 1.

Table 1 Target compounds and internal standards

Quantitative Targets	Internal Standard
Methcathinone	Mephedrone-D_3
Pseudoephedrine	Pseudoephedrine-D_3
Methylone	Methylone-D_3
Amphetamine	Amphetamine-D_{11}
Methamphetamine	Methamphetamine-D_{11}
MDA	MDA-D_5
Methedrone	Mephedrone-D_3
MDMA	MDMA-D_5
Phentermine	Methamphetamine-D_{11}
Mephedrone	Mephedrone-D_3
Alpha-PVP	Mephedrone-D_3
MDPV	Mephedrone-D_3
Bupropion	Mephedrone-D_3

The instrumental parameters delineated in the Toxicology Procedures Manual will remain unchanged for the validation. The impact of the circulating bath will be evaluated in blank blood,

antemortem blood, postmortem blood, urine, and liver. Based on the modification to the sample preparation procedure, bias, precision, estimated limit of detection, lower limit of quantitation, and endogenous interferences will be evaluated for their potential impact. Other validation parameters including regression analysis, ionization suppression/enhancement, carryover, interference from internal standard, interference from commonly encountered analytes, dilution integrity, and stability will not be assessed. In addition to the aforementioned parameters, an evaluation of the length of time the bottom layer remains frozen will be assessed in all matrix types.

A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 32) and Toxicology Procedures Manual (Qualtrax Revision 32). The validation plan is in accordance with the 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. Intermediate Precision
2. Sensitivity
    a. Estimated Limit of Detection (LOD)
    b. Lower Limit of Quantitation (LLOQ)
3. Interferences (Endogenous Compounds)
4. Frozen Sample Stability
5. References

1. Bias and Precision

The method is intended for the quantitative analysis of multiple matrices (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver). Bias and precision experiments shall be conducted for all matrix types.

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 delineated within the method is 0.01 mg/L to 2.0 mg/L. The low concentration shall be no more than approximately three times the lowest end of the working range of the method and 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 will be 0.03 mg/L, 0.8 mg/L, and 1.6 mg/L.

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

Equation 1

$$Bias\ (\%)\ Concentration_x = \left(\frac{Mean\ of\ Calculated\ Concentration_x - Expected\ Concentration_x}{Expected\ Concentration_x}\right) \times 100$$

Bias should be as low as possible but shall not exceed ±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 pooled 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 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 within-run precision will be 0.03 mg/L, 0.8 mg/L, and 1.6 mg/L. The within-run precision shall be calculated using Equation 2.

Equation 2

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

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 ±20% at each concentration level when analyzing common biological fluids.

c. Intermediate Precision

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

Equation 3

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

The intermediate 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 intermediate precision. Intermediate precision shall not exceed ±20% at each concentration level when analyzing common biological fluids.

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 was established to be 0.005 mg/L within the Toxicology Procedures Manual. Therefore 0.005 mg/L will be maintained as the decision point for reporting analytes within this method although a lower estimated LOD may be analytically achievable.

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). 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.

Predetermined identification criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥3.3

NOTE: Based on previous validation experiments for ionization suppression/enhancement. The laboratory assumed significant ionization suppression. Therefore, the number of matrix sources of the estimated limit of detection was tripled from three to nine.

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator (0.01 mg/L) for the method. For each matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and liver), 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 replicate results will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met.

Predetermined acceptance criteria:

Retention Time: ±3%
Qualifier Ratio: ±20%
Signal-to-Noise: ≥10
Back Calculated Concentration: ±20%

NOTE: Based on previous validation experiments for ionization suppression/enhancement. The laboratory assumed significant ionization suppression. Therefore, the number of matrix sources of the lower limit of quantitation was tripled from three to nine.

3. Interferences (Endogenous Compounds)

To assess for interference, the qualifier and quantifier ions for each analyte and internal standard within the method shall be monitored. Interferences below the limit of detection for the method may be deemed insignificant. 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.

Where possible, a minimum of ten 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, liver) within the validation should be evaluated, whenever possible.

4. Frozen Sample Stability

The length of time a sample remains frozen will be assessed during the validation. This is to ensure that the incubation time and temperature are appropriate to maintain the frozen conditions while transferring several samples consecutively. Samples will be placed into the -25°C circulating bath for 3 minutes. After incubation, samples will be removed and samples will be poured into clean test tubes at 1, 10, 20, 40, and 60 minute intervals. To assess the conditions in multiple matrices, ten matrix sources of blank blood, antemortem blood, postmortem blood, urine, and liver will be evaluated.

Each matrix source will be evaluated in duplicate at each timepoint. After the designated timepoint, the samples will be transferred by pouring into a clean test tube to ensure the integrity of the frozen sample. An appropriate time interval is one in which all matrix sources for all matrix types remains frozen for the designated internal.

5. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 32, **2025.**

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 32, **2025**.