Memo To: Robyn Weimer, Chemistry Program Manager RBW 9/24/25
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date September 24, 2025
RE: Validation Plan
Quantitative Analysis of Cocaine, Heroin, and Methamphetamine using a Multipoint Calibration Curve

Validation Plan – Quantitative Analysis of Cocaine, Heroin, and Methamphetamine using a Multipoint Calibration Curve

It is proposed to validate the utilization of a multipoint calibration curve for the quantitative analysis of cocaine, heroin, and methamphetamine. This validation is a modification of existing methodologies within the Controlled Substances Procedures Manual (Qualtrax Revision 23). Section 11.4 Gas Chromatography Quantitation delineates the general requirements for quantitative analysis. Further instruction is provided in Section 18.11 Amphetamine/Methamphetamine Quantitation, Section 19.8 Cocaine Quantitation, and Section 21.8 Heroin Quantitation. The quantitative analysis procedures require the utilization of a historical calibration curve employing a 1.0 mg/mL check standard and a 2.5 mg/mL calibration standard.

This validation will assess the applicability of a multipoint calibration curve. A total of six non-zero calibrators (0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, and 5.0 mg/mL) will be used during the validation. In addition to calibrators, three controls at concentrations (low, mid, and high) across the working range will be used. The low control concentration will be approximately three times the lower limit of quantitation. The high control concentration will be approximately 10% below the upper limit of quantitation. The mid control concentration will be the midpoint between the low and high control concentrations. All other method requirements for sample preparation and analysis will be in accordance with the Controlled Substances Procedures Manual.

Initial work on this project was started in 2021 and performed in the Central Laboratory Controlled Substances Section. Additional validation experiments will be performed within the Chemistry Laboratory Research Section.

An Agilent Technologies 8890 gas chromatograph coupled to an Agilent Technologies 5977C mass spectrometer will be employed during the validation. A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 33) and the Controlled Substances Procedures Manual.

1. Bias and Precision
    a. Bias
    b. Precision
2. Lower Limit of Quantitation (LLOQ)
3. Linearity and Calibration Model
4. Recovery
5. Interferences
6. Stability
7. References

1. Bias and Precision

Bias and precision will be assessed in two different ways during the validation. Initial validation studies included the evaluation of three different concentrations of controls prepared using a different vendor or lot number from the standards used to prepare the calibration curve. Additional studies will include the analysis of authentic powder material. The concentration of the authentic material will be obtained using the existing historical calibration curve and compared to the multipoint calibration curve data.

a. Bias

Bias was assessed alongside each calibration curve prepared during the initial validation studies. Bias was assessed over five different analytical runs. Within each analytical run, an independently prepared calibration curve was analyzed. Each calibrator was injected in triplicate alongside a single low, mid, and high concentration control. The bias will be calculated using Equation 1.

Equation 1

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

The bias should be as low as possible but shall not exceed ±10% at each concentration level when evaluating neat preparations. The existing acceptance criteria for bias within the Controlled Substances Procedures Manual for a single calibrator is ±5%. If the bias exceeds ±5% an assessment shall be performed to determine the bias limits within the method.

The bias of authentic samples will be assessed during the validation. When possible, authentic specimens will be obtained and the concentration determined using the current historical calibration curve method. The authentic specimens will then be prepared and analyzed with six replicate extractions and evaluated against the multipoint calibration curve method. The average concentration obtained from the six replicates using the multipoint calibration curve will be compared to the calculated concentration and associated measurement uncertainty of the historical calibration curve method. The concentration shall be within the associated range for the authentic specimen.

b. Precision

The data obtained from the bias experiments will be used to calculate the precision of the method. The precision of the neat standards and authentic specimens will be calculated using the percent coefficient of variation (%CV), Equation 2.

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

The standard deviation and the mean will be calculated to determine the precision of the method. The precision shall not exceed a %CV of 10% for all concentrations and specimen types.

2. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator (0.5 mg/mL) for the method. The lowest calibrator concentration shall be within ±10%. Each calibration curve evaluated during the validation will be assessed for the lower limit of quantitation. In addition to the back calculated concentration, the retention time shall be within ±2 seconds or 0.033 minutes, have a signal-to-noise ratio greater than 10:1, and have acceptable peak shape. All LLOQ samples shall pass the acceptance criteria and meet the requirements of the LLOQ evaluation.

3. Linearity and Calibration Model

The calibration model 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 0.5 mg/mL to 5.0 mg/mL. A total of six non-zero calibrators (0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 3.0 mg/mL, 4.0 mg/mL, and 5.0 mg/mL) 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. All data obtained from historical evaluations and current validation studies will be utilized in the determination of the best fit 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 ≥0.995, and the back calculated calibrator concentrations must be within ±10% of the target.

The model will be established by residual analysis and statistical comparisons (Analysis of Variance [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 and lower limit of quantitation within the validation.

4. Recovery

Recovery will be assessed using solid reference material and certified reference material. Two sets of control samples will be prepared using each source of material. The response ratio between the two preparations will be compared to determine the recovery of the extraction. The recovery will be assessed at one concentration within the calibration range. The evaluation of

recovery is an assessment to gain a better understanding of the performance of a method, therefore, there are no requirements for the percentage of recovery to deem the method fit for purpose.

5. Interferences

Interferences will be assessed by analyzing neat standards of commonly evaluated drugs and bulking agents. To evaluate interferences, neat reference materials will be prepared and analyzed in a single injection using the instrumental method for each quantitative compound. The retention time of each compound will be noted to assist in the creation of a database for each analytical method. If an interferent is present (e.g., within ±2 seconds or 0.033 minutes), the impact on identification and quantitation shall be evaluated. If the instrumental response is less than 10% of the LLOQ response the impact is deemed insignificant.

6. Stability

The stability of the prepared calibrators was initially assessed for the method in 2021. The stability data obtained will be verified with this validation for all quantitative compounds. To assess the stability of the calibrator solutions, a single calibration curve will be prepared on Day 1 and analyzed. This calibration curve will be subsequently evaluated weekly for three months alongside a freshly prepared control to assess bias and instability. If the concentration of the prepared control deviates greater than ±10%, it will be deemed unstable after that designated timepoint. The stability will be verified during the validation experiments.

7. References

Virginia Department of Forensic Science Quality Manual, (Qualtrax Revision 33), 2025.

Virginia Department of Forensic Science Controlled Substances Procedure Manual (Qualtrax Revision 23), 2025.