Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Research Section Supervisor
CC: Alka Lohmann, Technical Services Director
Date June 16, 2025
RE: Validation Plan
Validation of Volatiles Quantitation and Confirmation by Headspace Dual Column Gas Chromatography Flame Ionization Detection (GC-FID)

8/6/25

Validation Plan- Volatiles Quantitation and Confirmation by Headspace Dual Column Gas Chromatography Flame Ionization Detection (GC-FID)

It is proposed to validate a method for acetone, ethanol, isopropanol, and methanol identification and quantitation in biological matrices using headspace dual column gas chromatography flame ionization detection (GC-FID). An Agilent Technologies 8697 Headspace Sampler XL and an Agilent Technologies 8890 GC will be employed during the validation. The instrument will be equipped with hydrogen as the carrier gas and nitrogen for vial pressurization. This validation will encompass four different variables that will be independently evaluated with all experiments delineated in the validation plan. This includes the assessment of 20 mL and 10 mL headspace vials as well as a 1:10 and 1:5 dilution for sample preparation. Given the independent evaluation of these variables, the validation experiments set forth within the validation plan will be performed a total of four times.

Ethanol will be evaluated using seven calibration levels while acetone, isopropanol, and methanol will be quantitatively assessed using six calibration levels. The working range of ethanol will be 0.010% w/v to 0.500% w/v and the working range for all other target compounds will be 0.010% w/v to 0.400% w/v. The calibrator and control concentrations for each compound are delineated in Table 1.

Table 1 Calibrator and control concentration for the analysis of volatiles

Target Compound	Calibration Levels (% w/v)	Controls (% w/v)
Acetone	0.010, 0.025, 0.050, 0.100, 0.200, 0.400	0.050, 0.100, 0.200
Ethanol	0.010, 0.025, 0.050, 0.100, 0.200, 0.400, 0.500	0.050, 0.100, 0.200, 0.400
Isopropanol	0.010, 0.025, 0.050, 0.100, 0.200, 0.400	0.050, 0.100, 0.200
Methanol	0.010, 0.025, 0.050, 0.100, 0.200, 0.400	0.050, 0.100, 0.200

The lower limit of quantitation and limit of detection are administratively established at 0.010% w/v for all target compounds. During method development the autosampler parameters were optimized and are listed in Table 2.

Table 2 Headspace autosampler parameters

Parameter	Setting
Vial Equilibration	4 minutes
Injection Time	1 minute
Vial Shaking	5
Heating	70°C
Vial Pressurization	On (15 psi)
Loop Temperature	100°C
Transfer Line Temperature	110°C
Vial Size	20 mL or 10 mL

The gas chromatography flame ionization detection (GC-FID) parameters are listed in Table 3.

Table 3 GC-FID parameters

Parameter	Setting
Inlet Temperature	110°C
Gas Flow	27.9 mL/min
Run Time	4.5 minutes
Column 1	Agilent Technologies DB-ALC1
Column 2	Agilent Technologies DB-ALC2
Carrier Gas	Hydrogen
Temperature	45°C
FID Heater	250°C

During the validation, at a minimum, a calibration curve, positive controls, and a negative control shall be included with each analytical run. Additionally, a positive control shall be analyzed at least every 10 injections. Metrological traceability shall be established through the use of certified reference materials for each calibration level. All calibrators, controls, and samples shall be sequentially aliquoted (in order of injection sequence).

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 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. Linearity and Calibration Model
4. Carryover
5. Interferences
    a. Endogenous Compounds
    b. Internal Standard
    c. Commonly Encountered Analytes
6. Dilution Integrity
7. Comparison of Authentic Specimens
8. References

1. Bias and Precision

The method is intended for the quantitative analysis of multiple matrices (e.g., blank blood, antemortem blood, postmortem blood, urine, and vitreous), 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 evaluated is 0.010% w/v to 0.500% for ethanol and 0.010% w/v to 0.400% w/v for methanol, isopropanol, and acetone. 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 0.030% w/v, 0.160% w/v, and 0.350% w/v.

The bias of the fortified pooled blood 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 ±6% or 0.0040% w/v (ethanol) and ±10% or 0.0050% w/v (methanol, isopropanol, acetone) 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 0.030% w/v, 0.160% w/v, and 0.350% w/v. 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 ±6% (ethanol) and ±10% (methanol, isopropanol, acetone) 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 ±6% (ethanol) and ±10% (methanol, isopropanol, acetone) 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 will equal the lower limit of quantitation (LLOQ). The lower limit of quantitation was established to be 0.010% w/v for methanol, ethanol, isopropanol, and acetone. Since the LOD and LLOQ are equal, the experimental design for both experiments is the same allowing the use of the same data for LOD and LLOQ. This is evaluated by fortifying, at minimum, three different blank matrix sources per matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and vitreous). The three different blank matrix sources shall be analyzed over a minimum of three analyses to demonstrate that all predetermined detection and identification criteria are met in at least 95% of the replicate results.

Predetermined identification criteria:

Retention Time: ±3%

Instrumental Response: Both analytical columns shall have a detectable peak for the analyte of interest that is appropriately labeled using the same integration parameters throughout the validation.

b. Lower Limit of Quantitation (LLOQ)

The lower limit of quantitation for this validation shall be established by evaluating the lowest non-zero calibrator for the method. For each matrix type (i.e., blank blood, antemortem blood, postmortem blood, urine, and vitreous), a minimum of three different blank matrix sources shall be fortified at the lowest calibrator concentration and analyzed over a minimum of three analytical runs. The replicate analyses will be utilized to demonstrate that all detection, identification, bias, and precision criteria are met in at least 95% of the replicate results.

Predetermined acceptance criteria:

Retention Time: ±3%

Instrumental Response: Both analytical columns shall have a detectable peak for the analyte of interest that is appropriately labeled using the same integration parameters throughout the validation.

Back Calculated Concentration: ±6% or 0.0040 %w/v (ethanol) and ±10% or 0.0050% w/v (methanol, isopropanol, acetone)

3. Linearity and Calibration Model

The calibration model shall be established by determining the working range of analyte concentration over which the method shall be used. The working range to be evaluated shall be 0.010% w/v to 0.500% for ethanol and 0.010% w/v to 0.400% w/v for methanol, isopropanol, and acetone. A total of seven non-zero calibrators will be evaluated as shown in Table 2. 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 1 for each target compound. A blank sample and a minimum of six 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 ≥0.995, and the back calculated calibrator concentrations must be within ±6% or 0.0040 %w/v (ethanol) and ±10% or 0.0050% w/v (methanol, isopropanol, acetone) of the target.

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 accuracy and precision, limit of quantitation, and dilution integrity within the validation.

4. Carryover

Carryover will be evaluated by analyzing blank matrix samples immediately following the highest concentration calibrator (0.400% w/v) for methanol, isopropanol, and acetone within the injection sequence. For ethanol, progressively higher concentrations of fortified matrix, up to 1.00% w/v, will be assessed 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, 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.

a. Endogenous Compounds

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, and vitreous) 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, urine, and vitreous) 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 (concentration delivered within method) 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, urine, and vitreous) 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 other volatile compounds including, but not limited to, acetaldehyde, chloroethane, 1,1-difluoroethane, sevoflurane, toluene, and other commonly used solvents within the laboratory (e.g., acetonitrile, toluene, n-butylchloride). In addition to commonly encountered analytes, each volatile compound (including internal standard) within the method will be evaluated individually.

6. Dilution Integrity

The dilution integrity will be assessed for scenarios including concentrations above the ULOQ. Common dilution ratios (1:5, 1:10, 1:20) will be evaluated for bias and precision per matrix type utilizing the experiments delineated in Section 1. To assess the impact of sample dilution ratio, at minimum three, matrix sources per matrix type will be evaluated for bias and precision at each dilution ratio. The concentration will be adjusted depending upon the dilution factor and the adjusted concentration must be within the predetermined acceptance criteria for both bias and precision.

7. Comparison of Authentic Specimens

To evaluate the robustness of the method, approximately 100 authentic specimens of different matrix types will be evaluated on the current helium carrier gas method and this hydrogen carrier gas method. No more than 20% of the samples shall be negative for any target. If more than 20% of initially analyzed samples are negative, more specimens shall be evaluated to reach the 20% threshold.

To minimize variability from the evaporation of volatiles within a specimen, the quantitative analysis shall be performed on each method no greater than five days from the date the specimen was opened. The replicate tolerance criteria in the Toxicology Procedures Manual, Section 7.8.8 (Qualtrax Revision 32) will be followed which includes replicates shall be within ±5% or within ±0.0040% w/v of the mean, whichever is greater. The average concentration and ±5% range will be calculated to evaluate this criterion.

8. References

Herdzik, L. and Wagner, R. In-house method development for the analysis of volatiles using hydrogen as the carrier gas. **2025**.

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

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

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