This document outlines the process used to evaluate measurement uncertainty for the analysis of ethanol and follows the NIST 8-Step Process.

Measurement Uncertainty definition: "*a parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand*" - ISO/IEC Guide 99:2007, International Vocabulary of Metrology– Basic and General Concepts and Associated Terms

Measurement Traceability*: This has been established by

- The Certified Reference Material (CRM) calibrators used to calibrate the instrument
- The CRM aqueous controls used to monitor the performance of the analysis
- The routinely calibrated equipment used in the analysis:
    - Pipette–diluter used to sample calibrators, quality control samples and the measurand in duplicate also dilutes each with a specified amount of internal standard solution
    - Analytical Balance used to check the proper functioning of the pipette-diluter and to prepare the whole blood control

***** See *Tox-Trn-Apx6 Measurement Traceability Maps for Ethanol Analysis*

Measurement Assurance:

- A whole blood matrix control containing ethanol is prepared (see *TOX-SOP-17 Protocol for the Analysis of Ethanol*) and used to monitor the performance of the analysis. The target concentration is determined in-house by analyzing a minimum of 40 replicates on at least two different instruments and taking the average concentration of these measurements. The values of the whole blood control (WBC), which are run nine times in a full batch, are recorded in a quality control (QC) log. The laboratory has greater than 100 measurements made using each WBC. The laboratory has determined that the control must be within +/- 5% of the established target concentration to be acceptable based on the data in the QC log. Outliers are identified using the Grubb's Test or other applicable statistical calculations.
- Aqueous NIST-traceable CRM ethanol solutions, purchased from a different vendor than the CRMs used as calibrators, are used as quality controls to monitor the performance of the analysis across the concentration range, to verify the calibration curve and are used to evaluate method bias on an ongoing basis.
- Pipette-diluters used in the analysis are checked and calibrated once a year by an accredited external calibration laboratory. In addition, an intermediate check is performed for accuracy and precision in-house using a calibrated balance (see *TOX-SOP-45 Protocol for Pipette & Dispenser Diluter Maintenance*).
- The balance used in the pipette-diluter procedure to ensure its proper functioning is checked once a year by an accredited external calibration laboratory. In addition, intermediate checks are performed routinely in-house using NIST-traceable weights (see *TOX-SOP-50 Protocol for Balance Maintenance*).

NIST 8-Step Process of Estimating Measurement Uncertainty in the Analysis of Ethanol:

Step 1 – Specify the measurement process

The quantity of ethanol in a biological sample is determined by Dual Column Headspace Gas Chromatography with Flame Ionization Detection (GC-FID)* using the following:

- The measurement process can be shown by the following mathematical equation:

$$C_{measurand} = C_{calibrators} \times \frac{I_{measurand}}{I_{calibrators}} + b +/- U$$

Where: C is the concentration
I is the instrument (GC) response
b is a bias
U is the expanded uncertainty

- The concentration of ethanol in the samples and controls is determined from a 5 level (in duplicate) calibration curve generated each analytical run** using aqueous NIST-traceable CRM ethanol solutions from Cerilliant. Concentration range 0.025 g/100ml to 0.400 g/100ml
- A 100µl sample (whole blood, plasma/serum, calibrator or control) followed by 1000µl of a 0.015 g/100ml n-propanol internal standard solution is added to headspace vials using an automated pipette-diluter.
- Samples are prepared and analyzed in duplicate.
- Whole blood control (matrix) and CRM aqueous controls are placed at regular intervals throughout the batch
- The headspace vials are sealed and incubated at 60ºC for ~15 minutes before being injected into the dual column GC-FID

* *See TOX-SOP-17 Protocol for the Analysis of Ethanol (current revision)*

** *An analytical run (batch) constitutes a calibration with samples and controls prepared with the same internal standard solution and analyzed within 24 hours of that calibration.*

Step 2 – Identify uncertainty components

The uncertainty budget is developed by identifying and characterizing the sources of uncertainty as ***Type A*** (empirical data evaluated by statistical methods) or ***Type B*** (data or information in the analytical process evaluated by knowledge and sound scientific judgment).

Type A contributions will normally be based on historical quality control data. This is typically the most significant contribution to the total uncertainty. *NOTE*: QC data with reasons documented on the batch data and those which were determined to be outliers using the Grubb's Statistical Test or other applicable statistical calculations were not included.

Type B contributions must be considered as to whether or not their inclusion in the determination is significant. Information on Type B uncertainties will normally be obtained through certificates of analysis, calibration certificates and manufacturer's specifications.

Uncertainty Component	Method Evaluation
Quality Control Samples	
Whole blood control - reproducibility	Quantified in ***Type A*** evaluation of process reproducibility data – whole blood control QC data. SOP requires duplicate test agreement and includes a significant dilution of sample to eliminate matrix effect. Evaluated during method validation. Measurement assurance includes a whole blood control (WBC) to be analyzed every 5 samples in duplicate.
CRM – Aqueous control - bias	Quantified in ***Type A*** evaluation of the data from aqueous calibration range
Sampling of Measurand	
Matrix	Quantified in ***Type A*** evaluation of the data from duplicate analysis of case samples, which includes multiple matrices including whole blood and plasma/serum samples.
Homogenization – mixing and tissue grinding of visual clots	Quantified in ***Type A*** evaluation of the data from duplicate analysis of case samples.
Temperature – all calibrators, quality control samples and measurand are brought to room temperature. Variation in the time at room temperature between samples. Variation in room temperature at different times of the day throughout the year	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data) and of the data from duplicate analysis of case samples.
Calibrators	
CRM – Uncertainty stated in certificate of analysis supplied with each calibrator	Quantified in ***Type B*** evaluation of the certificate of analysis of the NIST-traceable calibrators
Matrix	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data). Evaluated during method validation
Preparation of aliquots of Calibrators, Quality Control Samples and Measurand	
Pipette-diluter: Volume of sample, volume of internal standard and dilution	Quantified in ***Type B*** evaluation of calibration certificate issued by an ISO/IEC 17025:2005 accredited external calibration laboratory. Precision is the most important quality which is captured in the ***Type A*** evaluation of process reproducibility data (WBC QC data) and of the data from duplicate analysis of case samples.
Variation in use by multiple staff – levels of experience and training	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data) and of the data from duplicate analysis of case samples
Headspace vials: Crimping Material of stopper	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data) and of the data from duplicate analysis of case samples
Time between replicate sampling of measurand	Captured in ***Type A*** evaluation of the data from duplicate analysis of case samples

Storage stability and stability from preparation to analysis	Evaluated during method validation and captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Internal Standard	
Preparation of Internal Standard	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Components: ammonium sulfate – reagent grade n-propanol – reagent grade	No influence The measurement result will only be impacted by the volume of the internal standard added to each sample
Concentration of Internal Standard	No Influence SOP requires use of the same lot of internal standard for all samples in an analytical batch The measurement result will only be impacted by variation in the volume of the internal standard added to each sample
Stability of Internal Standard	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Analysis	
Instrument parameters (such as FID gas mixture, carrier gas quality; GC temperatures-oven, injector and detector; Headspace sampler-equilibration temperature and time, sample loop temperature, injection volume etc)	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Interference from the matrix	Duplicate listing of component see Sampling of Measurand section above
Instrument precision	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data) and of the data from duplicate analysis of case samples
Data Processing	
Calibration model	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data) and through CRMs used as QC
Integration parameters	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Processing algorithms	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Staff	
Multiple analysts	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Training	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)
Experience	Captured in ***Type A*** evaluation of process reproducibility data (WBC QC data)

The following sources of uncertainty were identified and characterized for the budget:

#	Sources of Uncertainty	Type
1	Reproducibility - WBC QC data	A
2	Matrix – Duplicates of case sample data	A
3	Bias - Aqueous QC data	A
4	Pipette - Diluter	B
5	CRM - Certification of Calibrators	B

Step 3 – Quantify uncertainty components

#	Sources of Uncertainty	Relative Std Dev (or CV)
1	Reproducibility - WBC QC data	a
2	Matrix - Duplicates of case sample data	b
3	Bias - Aqueous QC data	c
4	Pipette - Diluter	d
5	CRM - Certification of Calibrators	e

a WBC QC data from the applicable date range (if multiple lots used within applicable date range then the pooled standard deviaton / weighted average will be used)
b Matrix duplicates from the applicable date range (including samples that were reanalyzed, proficiency samples, and plasma/serum samples)
c From the bias evaluation of the concentration range of aqueous controls, the concentration with the largest deviation will be used (multiple lots of the same concentration will be appropriately weighted)
d From the calibration certificate of the pipette-diluter with the largest process uncertainty (k=2 at 95% coverage probability)
e From the certificate of analysis of CRM calibrator with the largest standard deviation (k=2 at 95% confidence interval)

Step 4 – Convert quantities to standard uncertainties

#	Sources of Uncertainty	Type	Relative Std Dev (or CV)	Distribution Model	Divisor	Standard Uncertainty (1σ)
1	Reproducibility - WBC QC data	A	a	Normal	√2*	a^1
2	Matrix - Duplicates of case sample data	A	b	Normal	√2*	b^1
3	Bias - Aqueous QC data	A	c	Normal	√2*	c^1
4	Pipette - Diluter	B	d	Normal	2**	d^1
5	CRM - Certification of Calibrators	B	e	Normal	2**	e^1

**Divisor of square root of two used because the QC is not averaged, but subjects are reported as the average of two independent analyses*

***Divisor of two was used because the certificates state the uncertainty of their respective measurements at a 95% coverage probability (k=2) on the calibration certificate and certificate of analysis (unless the standard uncertainty is determined prior to entry in the budget then use divisor of 1)*

Step 5 – Calculate combined standard uncertainty (U_c)

$$\boldsymbol{U_c} = \sqrt{(a^1)^2 + (b^1)^2 + (c^1)^2 + (d^1)^2 + (e^1)^2}$$

Note: U_c is commonly known as the "root-sum-of-squares" (square root of the sum-of-the-squares) or "RSS" method of combining uncertainty components estimated as standard deviations

Step 6 – Expand the combined standard uncertainty by coverage factor (k)

Coverage factor of k=3 (from student T test table for ∞) at a 99.73% coverage probability

Expanded uncertainty = U_c x 3

#	Sources of Uncertainty	Type	Relative Std Dev (or CV)	Distribution Model	Divisor	Standard Uncertainty (1σ)
1	Reproducibility - WBC QC data	A	a	Normal	√2	a^1
2	Matrix - Duplicates of case sample data	A	b	Normal	√2	b^1
3	Bias - Aqueous QC data	A	c	Normal	√2	c^1
4	Pipette - Diluter	B	d	Normal	2	d^1
5	CRM - Certification of Calibrators	B	e	Normal	2	e^1
	Combined Uncertainty (RSS)	U_c				
	Coverage Factor k=3	99.73%				
	Expanded Uncertainty	U_c x 3				
	*Reported Uncertainty**	*5%*				

**Reported uncertainty administratively set at ±5% provided the expanded uncertainty is ≤5%. The associated coverage probability will be calculated.*

Note: All calculations performed using Excel® software. (No numbers rounded or truncated until the final calculated combined uncertainty.)

Step 7 – Evaluate the expanded uncertainty

The expanded uncertainty is appropriate for the test method and meets the laboratory requirements as well as the customer's needs. This is based on the precision and accuracy of the results of aqueous and whole blood controls as well as the certification of calibrators.

Step 8 – Report the uncertainty

The measurement result will be reported as y ± U, where y is the measured quantity value and U is the expanded uncertainty (reported uncertainty may be administratively set). The reported result will also include the coverage probability. The laboratory reports the uncertainty to the customer by converting the reported uncertainty back to the reporting units and ensuring that no more than two significant figures are reported for the uncertainty. Uncertainty will be reported to three decimal places and rounded up unless the fourth decimal place is a zero. Coverage probability will be truncated to two decimal places.

For example, an average blood alcohol measurement of 0.125 g/100ml results in an uncertainty of 0.00625 g/100ml (using an expanded uncertainty equal to 5%). To follow the rounding rules of the *Guide to Uncertainty Measurement* (Section 7.2.6 GUM BIPM-JCGM 100:2008), this is rounded up to 0.007 g/100ml and would be reported as "0.125 ± 0.007 grams of ethanol per 100 ml whole blood at a coverage probability of 99.73%."

The measurement uncertainty will be reviewed and recalculated at least once a year.

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