Preparation, Uncertainty, & Certification of Ethanol Standards

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Introduction

Ethanol Reference Standards are Critical to the Accurate Quantitation of Blood Alcohol in Forensic Analysis

Ethanol standards are widely used in forensic and toxicology applications for determination of blood alcohol content. Results of blood alcohol testing have significant legal implications and are frequently used as evidence in courts of law. The blood alcohol analysis process must therefore be reliable and defensible.

A critical component of blood alcohol analysis is the calibrator used for quantitation of results. Ethanol reference standards are widely available for this purpose and are sold in many formats – bottled and ampouled. The accuracy and uncertainty associated with these standards are important contributors to the accuracy and associated uncertainty of the blood alcohol test result. It is imperative that the uncertainty of the reference standards be within the margins of the blood alcohol testing uncertainty and that the certified concentration is accurate and completely traceable to international units of measure.

Critical Elements

- Proper certification of the neat material
- Accuracy of mass measurement
- Accuracy of diluent addition
- Dispensing, packaging and stability
- Analytical verification
- Traceability to SI units of measure & traceability to NIST SRM
- Certification & Uncertainty

Cerilliant Certified Ethanol Reference Standards are manufactured and certified to ISO Guide 34 and ISO/IEC 17025 standards and are traceable to SI units and to NIST SRM ethanol standards. The preparation, certification and uncertainty of these standards is presented in this poster.

Certification of the Neat Ethanol

Complete & accurate characterization of the neat ethanol is essential to accuracy of the solution

Certification Considerations

- Ethanol is widely available in high purity and is stable for many years when stored appropriately
  - What is the grade of ethanol used?
  - Is the ethanol vendor accredited?
  - What are the specifications of the ethanol procured for use in the standard?
  - How is the ethanol certified?

Cerilliant Practice

- Ethanol procured for standards meets ACS/USP specifications
- Vendor COA provides complete testing information, vendor is certified to ISO9001:2000
- The ethanol is tested for identity, purity and water content and then certified by Cerilliant's ISO/IEC 17025 accredited testing lab
- Certification ensures traceability through certification by an accredited testing lab
- The neat ethanol is stored in 5 mL ampoules, flame sealed under argon to protect from moisture absorption during storage

Characterization of neat ethanol

- Determination of purity
  - Chromatographic purity by GC/FID using 2 different columns
- Verification of identity
  - By GC/MS
- Determination of residual water content
  - Karl Fischer Coulometry <USP921>
  - Ethanol is hygroscopic. Residual water content must be determined and included in purity factor calculations for use of ethanol in quantitative applications
- Assignment of a mass balance purity factor value for use in preparation of the solution standard

Purity Factor Calculation

- The purity factor (PF) mass balance measurement equation is used to calculate the amount of ethanol required to achieve an accurate concentration of the solution standard, accounting for both purity and residual water content.

$$PurityFactor = (100 - (wt\%H_2O)) \left(\frac{ChromPurity}{100}\right) \pm U$$

- U represents the combined uncertainty of the purity factor at ~95% confidence and includes uncertainty of both the purity determination and the residual water analysis.

Uncertainty of the Purity Factor

- Uncertainty of the neat ethanol purity factor was achieved by evaluating the uncertainty of the analytical tests used in the Purity Factor equation.
- Uncertainty of chromatographic purity is based on specifications for chromatographic purity by two different methods to be within 0.5%.

$$u_{(ChromPurity)} = \frac{0.25\ \%}{\sqrt{3}} = 0.144\ \%$$

- Uncertainty of residual water content is based on repeatability experiments on the Karl Fischer Coulometric method (USP<921>).

$$u_{H_2O} = 0.03990\%\ w/w$$

All instruments are fully qualified and calibrated
Requalification is performed annually and system suitability is performed daily
Balances are qualified and calibrated.
All weighings are traceable to SI units

Results were combined in a Kratgen Spreadsheet[1] to determine uncertainty of the neat ethanol purity factor

Kratgen Spreadsheet for Uncertainty of the Purity Factor

Variable name, symbol	Input Value	Units	Uncertainty source description	Reported uncer.	Type	Distribution	Factor to normalize	Standard uncer., u_i
Water Content, $wt\%H_2O$	0.0745	%w/w	QC Specification	0.0399	B	comb. std., $k = 1$	1	3.99E-02
ChromPurity	99.997	%	Test Specification	0.250	B	Uniform	0.57735027	1.44E-01

Sequential Perturbation		u(wt%H2O)	u(ChromPurity)
		0.03990	0.14434
	df	99999	99999
Measurement Equation Inputs	**Value**		
$wt\%H_2O$	0.0745	0.11440	0.07450
ChromPurity	99.9970	99.99700	100.14134
Result	**99.922502**	**99.8826**	**100.0667**
difference		-0.03990	0.14423
u_c	0.14965	1.59191E-03	2.08023E-02
df		0.07109	0.92891
k	2	-0.99997	0.99926
U	0.29929	**0.300%**	$U_{relative}$, %
		0.150%	$U_{relative}$, %

$$PurityFactor = (100 - (wt\%H_2O)) \left(\frac{ChromPurity}{100}\right) \pm U$$

RESULTS	
PF (wt%)	99.9225
k	2
U (wt%)	0.29929

Solution Standard Preparation and Uncertainty

Mass Measurement

Mass Measurement Accuracy / Traceability

- Cerilliant requires minimum sample masses (specified for each balance) to limit relative uncertainty to ≤0.1% as prescribed by USP NF.
- Balance selection and minimum weighings are outlined in standard operating procedures and were determined through the combination of manufacturer tolerances and repeatability experiments performed.
- Improper weighing technique can increase uncertainty. Proper weighing techniques are outlined in standard operating procedures.

Qualification and Traceability

- Each balance has been fully qualified in its installed state, is calibrated semi-annually to manufacturer tolerances and adjusted weekly with NIST traceable weights. Calibration verified prior to each use using NIST traceable weights.

Mass Measurement Uncertainty

Process Scale	250-500 mL	500-750 mL
Approx. Gross Mass	500 grams	1 kg
Tare Container	none	none
Ref./Net Mass (g)	500	1000
Uncertainty Components (grams)		
s_p (from repeatability)	0.0017	0.0012
u_{Buoy}	0.0006	0.0012
u_{lin}	0.0002	0.0003
Measurement Equation:	$u_{bp} = \sqrt{s_p^2 + u_{lin}^2 + u_{buoy}^2}$	
Combined Standard Uncertainty		
u_{bp} (grams)	**0.0018**	**0.0017**
u_{bp} Relative to Net Mass Weighed	**0.0004%**	**0.00017%**
Expanded Uncertainty (k=2)		
U (grams)	**0.0036**	**0.0035**
U_{bal}	**0.0007%**	**0.00035%**

Mass measurement uncertainty was determined from a combination of balance manufacturer specified tolerances for sensitivity and linearity and repeatability experiments following specified weighing procedures. Balance manufacturer tolerances alone are insufficient. Values are proportional to the net mass being measured and are specific to the balance utilized.

u_{sens}: Uncertainty due to the balances sensitivity tolerance
- Includes the uncertainty of the balances built-in reference weight used for internal calibrations
- Balance manufacturer calibrations incorporate traceability to NIST SI units and their associated uncertainty in the sensitivity component

u_{lin}: Uncertainty due to non-linearity of the characteristic curve
- From the balance manufacturer

u_{rep}: Repeatability
- Includes effects from readability, drift, static, ambient drafts, thermal drafts, vibration, gross/net weight, eccentric loading, temperature stability, electromagnetic interferences/radio frequency interferences, weighing procedure, installation, tare container geometry, adsorption/absorption, balance settings, and operator technique
- Determined by tests of 20 replicate weighings conducted by multiple operators at various test loads and net weights on all balances used to prepare solution standards

Diluent Addition - Gravimetric vs. Volumetric Methods

Cerilliant Process is Gravimetric

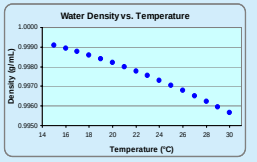
- Target solvent mass calculated from target volume by adjusting for density
- Actual solution mass calculated back into volume to report concentration as mg/dL

Advantages of Gravimetric Approach

- Ensures lot-to-lot consistency – Measurement of volume by mass eliminates temperature dependence of flask accuracy and allows all solutions to be consistently prepared at the same chosen reference temperature.
- Eliminates the subjectivity of visual fill line in volumetric addition
- Mass measurements provide traceability to SI units of measure
- Weigh tapes provide an audit trail
- Allows accurate formulation of batch volumes well beyond the capacity of Class-A flasks

Uncertainty of Diluent Addition

- Uncertainty related to diluent addition arises from uncertainty in the density value used for the solution.
- Based on instrument tolerances for density measurement (Type B)



Thermal expansion will affect volumetric accuracy of calibrated flasks

0.21% difference in concentration of aqueous solutions when prepared volumetrically at 15° vs. 25°C

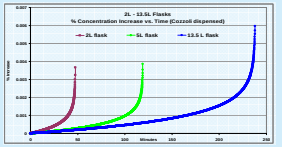
$$u_d = \frac{0.001}{\sqrt{3}} = 0.000577\ g/mL$$

Dispensing Process

Identification and Control of Critical Parameters

The Dispensing Process was Analyzed:

- In a test case, every ampoule, from the beginning to the end of a run, was tested analytically for concentration homogeneity.
- The study identified potential for dilution/fewer fill volumes in the early ampoules and potential for evaporation induced concentration in late ampoules.
  - Dilution is eliminated and consistency of volume ensured by purging the lines with product prior to filling.
  - Evaporative losses are controlled through protection of the bulk container during dispensing and through speed of the dispensing process.
- Process speed: Typical Cozzoli speed is fast, 50 containers per minute (1L in 17 minutes), minimizing degradation and potential for evaporative losses.
- Evaporative losses were further evaluated in evaporative studies where the evaporation of solvent from open containers was measured gravimetrically.
- Evaporative loss of solvent during ampouling on the Cozzoli dispenser/sealer was modeled and determined to be <0.006% over 4 hrs. Not a significant contributor to solution standard uncertainty.



Process Controls Ensure Consistency of Fill Volume & Lot Homogeneity

- Cerilliant ethanol solution standards are dispensed into non-silanized amber ampoules using a Cozzoli dispensing/sealing system.
- Ampoules are purged with argon prior to flame sealing.
- Sealing effectively is verified daily and weekly using dye tests.
- New tubing is used for each product to eliminate risk of contamination.
- 316 stainless steel syringes are cleaned before and after each dispensing using a validated cleaning process.
- Lines are purged with product prior to ampouling to eliminate dead volumes and ensure consistency of fill volume.
- Filling is verified gravimetrically using a stratified random sampling plan on balances calibrated semi-annually and verified before use to NIST traceable weights.
- Concentration and homogeneity are verified analytically using a stratified random sampling plan developed from an analysis of critical points in the filling/sealing process.

The dispensing process is sufficiently controlled as to not be a significant contributor to uncertainty calculations and is, therefore, excluded.

Cerilliant Ethanol Solution Standard Stability

- The ampouled ethanol solution standards are autoclaved to control microbial growth.
- Expiration is established through real-time stability studies.
- Solution purity and concentration are reevaluated at multiple intervals. Stability is established as long as purity and concentration continue to meet original release criteria.
- Five Years of shelf-life has been established.
- Stability is not a significant contributor to uncertainty and is, therefore, excluded.

Assessment and Reporting of Uncertainty of the Certified Concentration

Solution Standard Certification & Uncertainty

Cerilliant evaluated every step involved in the preparation of its Certified Ethanol Solution Standards and determined that the primary contributing factors impacting uncertainty were: uncertainty of the Purity Factor; Mass Measurement uncertainty and Diluent Addition uncertainty.

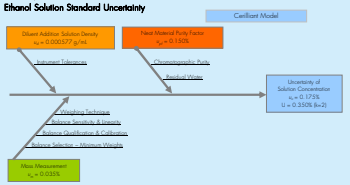
Measurement Equation for Concentration Uncertainty

$$C = \frac{(m_{s+a} - m_v)\, d}{(m_{f+s} - m_f)\, p} \pm U$$

Where: C = Concentration of solution (mass/volume)
m_{s+a} = mass of analyte + vial
m_v = mass of empty vial
m_{f+s} = mass of flask + solvent
m_f = mass of empty flask
d = density of solution
p = purity adjustment factor for the neat material
U = the assigned combined expanded measurement uncertainty

- The gravimetrically prepared concentration is the "True Value" and is reported on the certificate of analysis with stated uncertainty as ±mg/dL.
- Verification of the solution standard concentration is performed post ampouling demonstrating accuracy and ampoule to ampoule consistency (batch homogeneity).
  - Validated Headspace GC/FID method with known uncertainty is used
  - Concentration verified against NIST SRM and Cerilliant Control
  - Control is prepared from a different lot of ethanol and qualified against NIST SRM
  - Solution purity is verified to demonstrate no contamination or degradation has occurred during preparation
  - Samples are pulled from across the batch to demonstrate homogeneity. %RSD of results is reported on the COA

Ethanol Solution Standard Uncertainty



The Kratgen Spreadsheet shows the calculation of uncertainty and contributions of each uncertainty component

Kratgen Spreadsheet – Uncertainty of the Certified Concentration

standard uncertainty of mass measurements:	0.00035 *Net Mass (g)
standard uncertainty of diluent addition:	0.0015
standard uncertainty of density	0.000577 g/mL

$$C = \frac{(m_{s+a} - m_v)\, d}{(m_{f+s} - m_f)\, p} \pm U$$

Input descriptions	Symbol	Value	Units	Reported uncer.	Type	Distribution	Factor to normalize	Standard uncer., u_i	Rel. u_i (%)
mass of vial	m_v	0	g	0.038529876	A	comb. Std.	1	**0.038530**	0.7721%
mass of vial+analyte	m_{s+a}	**115.08536**	g	0.038529876	A	comb. Std.	1	**0.038530**	0.033%
mass of flask	m_f	**100**	g	9.625245	A	comb. Std.	1	**9.625245**	9.625%
mass of flask+solvent	m_{f+s}	**27600.7**	g	9.625245	A	comb. Std.	1	**9.625245**	0.035%
adjustment factor	p	**1.00078**	g/g	0.0015	B	comb. Std.	1	**0.001500**	0.150%
solution density	d	1	g/mL	0.001	B	uniform	1.732050808	**0.000577**	0.058%

		u(mv)	u(mv+a)	u(mf)	u(mf+s)	u(p)	u(d)
Sequential Perturbation		0.03853	0.03853	9.62525	9.62525	0.00150	0.00058
	df						
ME Inputs	**Value**						
m_v	5.00000	5.03853	5.00000	5.00000	5.00000	5.00000	
m_{s+a}	115.08536	115.08536	115.12389	115.08536	115.08536	115.08536	
m_f	100.00000	100.00000	100.00000	109.62525	100.00000	100.00000	100.00000
m_{f+s}	27600.70000	27600.70000	27600.70000	27600.70000	27610.32525	27600.70000	27600.70000
p	1.00078	1.00078	1.00078	1.00078	1.00078	1.00228	1.00078
d	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00058
Result g/mL	0.00400	0.00400	0.00400	0.00400	0.00400	0.00399	0.00400
	0.00000	0.00000	0.00000	0.00000	0.00000	-0.00001	0.00000
u_c	0.00000700	0.00000000002	0.00000000002	0.00000000002	0.00000000002	0.00000000004	0.00000000001
Contribution to U		**3.999%**	**3.999%**	**4.002%**	**3.996%**	**73.121%**	**10.882%**
k	2.000	-0.00004	0.00004	0.00000015	-0.00000015	-0.00398	0.00400
U	0.000014	g/mL	0.350%	$U_{relative}$, %			
U	1.40	mg/dL	0.175%	u_c relative			

Results		
Conc.	±	Expanded Uncertainty (k=2)
399.9882	±	**1.400098** mg/dL

Analytical Verification & Method Validation

A Validated Analytical Method is used to Verify Solution Concentration and Ampoule to Ampoule Consistency

- Solution standard concentration is verified analytically by comparison to an appropriate NIST SRM.
- A calibration control is used in the analysis. Control is made from a different lot of neat ethanol which has been certified. Control is qualified to NIST SRM.
- Homogeneity across the lot is verified by testing samples pulled from across the lot. A stratified random sampling plan is utilized and includes samples of the first and last ten ampoules plus one per every 400 ampoules dispensed.
- Concentration and homogeneity are verified using a validated Headspace GC/FID method.

Validation ensures the analytical method is accurate, robust, repeatable and reliable

Linearity

- Linearity of the method was determined by plotting measured signals (peak area) as a function of analyte concentration (mg/mL) across the range.
- The linear relationship was evaluated by calculating a regression line by the method of least squares.
- The method is linear from .5 to 600 mg/dL Ethanol in Water.

Method Validation – Linearity			
Low Range (.5 - 100 mg/dL)		High Range (100 - 600 mg/dL)	
Linear Equation	y = 9.9648x - 8.4537	Linear Equation	y = 10.056x - 17.671
r^2	1.000	r^2	1.000

Linearity ensures the analytical method is reliable for quantitation across a range of concentrations

Accuracy

- Accuracy was assessed using a minimum of nine determinations over at least three concentration levels covering the specified range.
- Each sample was prepared in triplicate and analyzed once.
- %RSD values represent the reproducibility of the method.

Method Validation – Accuracy			
Theoretical conc.	Prepared conc.	%RSD	%Difference to Prepared Conc.
5.0	4.99985	0.528	2.94311
10.0	10.0002	1.036	1.09008
25.0	25.00025	1.533	2.03911
50.0	50.0005	0.753	.61788
100.0	100.03689	0.873	0.20825
200.0	200.0000	1.074	0.27578
300.0	300.00999	0.918	0.58511
400.0	397.82216	0.917	0.28336
500.0	499.99501	1.047	-0.27432
600.0	600.00199	0.968	-1.66216

Accuracy demonstrates consistency and reproducibility

Validation Summary

- The validated GC/MS method can adequately detect and quantitate ethanol concentrations ranging from .5 to 600 mg/dL.
- The method is robust to slight modifications in temperature ramp, injection time, and vial incubation time, but is sensitive to changes in flow.
- When all analyses were evaluated from precision, intermediate precision and linearity, the overall %RSD was 1.145%, representative of the uncertainty of the instrument response including day to day, operator, instrument and sample preparation variability.

Uncertainty of the Analytical Verification

Uncertainty assessment for verification includes uncertainty related to the analytical method and instrument response and uncertainty reported on the value assigned to the NIST SRM.

Measurement equation for uncertainty of analytical concentration verification

$$C_{ver} = \frac{Area_{ver} * C_{NIST}}{Area_{NIST}} \pm U$$

Where: $Area_{ver}$ = area response of the standard
$Area_{NIST}$ = area response of the NIST SRM
C_{NIST} = conc of the NIST SRM with stated uncertainty

NIST SRM Calibrator	Conc.	± (expanded uncert)	std uncert.	Units	k	Conv Fact
SRM2891	0.01951	0.00018	0.00009	wt %	2	99800
	19.47098	0.17964	0.08982	mg/dL		
standard uncertainty of area:		1.145	response %			
standard uncertainty of NIST SRM conc			0.08982	mg/dL		

$$C_{ver} = \frac{Area_{ver} * C_{NIST}}{Area_{NIST}} \pm U$$

Input Description	Symbol	Value	Units	Reported uncer.	Type	Distribution	Factor to normalize	Standard uncer., u_i	Rel. u_i (%)
Area of std	$Area_{ver}$	**268.11486**	response	3.069915147	A	comb. Std.	1	**3.069915**	1.145%
Area of NIST	$Area_{NIST}$	**205.94708**	response	2.358094368	A	comb. Std.	1	**2.358094**	1.145%
Conc of NIST	C_{NIST}	**19.47098**	mg/dL	0.08982	A	comb. Std.	1	**0.089820**	0.461%

Factors Impacting Uncertainty of the Analytical Verification

- Uncertainty is specific to the analytical technique (GC/FID, GC/Headspace FID, titration etc) and within technique to the specific laboratory method.
- GC Headspace methods can vary in precision depending on the specific instrument (vendor) and parameters used.
- Variables includes sample preparation, analyst training, instrument response, instrument parameters (incubation time, split ratio, etc.).
- Uncertainty of the calibrator concentration must also be included.
- If a curve is run, analytical uncertainty applies to each curve point analyzed and must be factored into determination of the overall uncertainty.
- The biggest contributor to uncertainty in our study is the GC Headspace analytical method, representing approximately 90% of the uncertainty.

Sequential Perturbation		u(Area std)	u(Area NIST)	u(C NIST)
		3.06992	2.35809	0.08982
ME Inputs	**Value**			
$Area_{ver}$	268.11486	271.18478	268.11486	268.11486
$Area_{NIST}$	205.94708	205.94708	208.30518	205.94708
C_{NIST}	19.47098	19.47098	19.47098	19.56080
Result (mg/dL)	**25.34855**	25.63879	25.06159	25.46548
		0.29024	-0.28696	0.11693
u_c	**0.424556619**	0.082240	0.082343	0.013673
Contribution to U		**46.723%**	**45.681%**	**7.595%**
k	2.000	0.09454	-0.12169	1.30186
U	**0.84913**		**3.350%**	$U_{relative}$, %
			1.675%	

Results		
Conc.	±	Expanded Uncertainty (k=2)
25.3485	±	**0.849132** mg/dL

Traceability is Provided from Beginning to End

Traceability is the property of a measurement result whereby it can be related to stated references usually through national or international standards through an unbroken chain of comparisons all having stated uncertainties.

- Preparation and certification by ISO Guide 34 and ISO/IEC 17025 accredited company.
- Neat material certification by ISO/IEC 17025 accredited testing lab.
- The purity of the neat material is included in the uncertainty of the standard preparation.
- Balances installed, qualified and calibrated semiannually by ISO/IEC 17025 accredited testing lab utilizing NIST traceable weights.
- Weekly and pre-use calibration verifications performed using NIST traceable weights – pre-use verification weigh tapes included in solution standard batch record.
- Gravimetric preparation for analyte and diluent – weigh tapes included in solution standard batch record – traceability to SI units of measure.
- Balance tolerances experimentally verified for the manufacturing process and included in uncertainty calculation.
- Fill volume is gravimetrically verified during the dispensing process.
- Analytical verification of concentration and homogeneity by ISO/IEC 17025 accredited testing lab utilizing validated methods.
- The concentration is reported with uncertainty in accordance with ISO/IEC 17025 and ISO Guide 34.
- The uncertainty value is reported with a coverage factor, k=2, representing an approximately 95% confidence for the stated concentration.
- The neat material traceability and test data are provided on the COA.

Conclusions

- The accuracy and traceability of calibrators used in the determination of blood alcohol content is critical to the outcome and defensibility of the analysis.
- An understanding of vendor preparation and certification practices as well as factors included in the determination of uncertainty are necessary to ensure compliance with regulatory requirements and to supporting analytical results in courts of law.
- Cerilliant Certified Ethanol Reference Standards are suitable for use in forensic investigations. Cerilliant standards are manufactured and certified to the highest industry standards to ensure accuracy and precision including ISO Guide 34 and ISO/IEC 17025 requirements and are traceable to SI units and to NIST SRM ethanol standards.

Cerilliant®
Analytical Reference Standards

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