What Makes a Good Reference Standard?

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Abstract

To ensure accuracy of results in the analytical laboratory analytical chemists must rely on the accuracy, stability, and consistency of reference materials or spiking solutions.

There are several factors critical to production of a high quality reference standard or spiking solution including raw material handling, characterization and potency; certification and qualification of solutions; and homogeneity and stability of the solution. Certified Analytical Reference Standard Solutions prepared in a diluent that promotes stability and packaged under argon in flame sealed ampoules can be stable for many years. This allows the laboratory the convenience of fewer lot changes and more consistent data over time.

This presentation walks through the parameters important to the manufacture, analysis, uncertainty and storage of certified reference standards. Results are only as good as the reference.

Results are only as accurate as the reference!

- Accuracy and reliability of analytical results is dependent on accuracy and reliability of the method of analysis, accuracy in the preparation of samples, and accuracy of the calibrators used.
- Highly pure, well-characterized, reference standards are critical to the accuracy of the analysis
- Design, preparation, packaging, and storage of reference standards affect the traceability, accuracy of concentration, stability, and uncertainty

What makes a Good Reference Standard? One Suitable for Quantitative Applications?

- ✓ High purity thoroughly & accurately characterized components – neat material characterization
- ✓ Prepared using accurate, calibrated, and qualified balances (pipettes & glassware when needed)
- ✓ Accurate weighing operation
- ✓ Accurate solvent addition
- ✓ Traceability of all components
- ✓ High purity diluents and/or stabilizers, compatible with the compound(s)
- ✓ Analyzed to verify accuracy & consistency
- ✓ Appropriate packaging and storage
- ✓ Assessment of shelf life

Neat Material Characterization

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

Reference Standard Design & Preparation

Characterization Considerations

- Are vendor certified values complete, accurate and reliable?
- Reliability/repeatability of method?
- Is there an adjustment for salt form?
- Does the vendor provide uncertainty on the purity factor (potency)?

Characterization of neat materials should include

- Purity and impurities
- Residuals
- Verification of identity

Glycosides-hygroscopic

Opiates-hydrates

Many organic materials - residual solvents (benzodiazepines)

Neat Materials - Certification

Identity

- Multiple techniques
  - 1D and 2D NMR
    - Proton
    - Carbon-13
    - Other nuclei
  - FTIR
  - GCMS, LCMS, LCMSMS
  - Other techniques as needed: EA, Optical Rotation, DSC, Melting Point, TGA
- Comparison to literature references

Purity / Potency

- Mass Balance – Orthogonal approach
  - Multiple techniques for chrom purity and residuals
  - Based on ISO Guide 34
  - Used by NIST
  - Appropriate mass balance equation critical
- Assays – when appropriate
  - Availability of established methods with high precision
  - Availability of primary reference materials

Purity and Impurities

Chromatographic Purity

- Purity and related substances
- Method development
  - Literature methods
  - Existing methods for similar compounds
  - Base line separation
  - Resolution of known impurities
- Use at least 2 techniques and different columns
  - values must agree within 0.5% of each other

Residual Impurities

- Residual water
  - USP <921>; system suitability
- Residual solvent by GC Headspace –
  - Cerilliant validated method or USP <467>
- Residual inorganic content
  - Micro ROI method based on USP <281> - less material with comparable results
- NMR evaluation
- EA or other techniques

Assignment of Purity Factor

Mass Balance Equation

- Incorporates chromatographic purity and related substances
- Assigned on an “as-is” basis – adjustments for salts made when preparing solution
- Equation may be modified to address impurities from orthogonal chromatographic techniques, chiral purity, etc.

$$PurityFactor = \left[[100 - (wt\%Solvents) - (wt\%H_2O) - (wt\%Inorganics)] * \frac{ChromPurity}{100} \right]$$

wt%Solvents: the weight percentage of residual solvents present in the neat material

wt%H_2O: the weight percentage of water present in the neat material

wt%Inorganics: the weight percentage of inorganic content in the neat material

ChromPurity: based on the chromatographic purity of the specified primary purity method, either GC or HPLC

Complete Characterization Critical

Use of chromatographic purity alone can introduce significant error into the concentration of the reference solution

Compound	Chrom. Purity (%)	Residual Solvent Content (%)	Trace Inorganic Content (%)	Residual Water Content (%)	Purity Factor for Quantitative Use (%)
Ranitidine HCl	99.5	0.87	0.13	None Detected	98.47
Cyanidin-3-glucoside	94.7	ND	< 0.1	6.29	88.71
Cyanidin-3-galactoside	94.7	ND	< 0.1	4.83	90.08
Oxazepam Glucuronide	99.9	ND	2.37	8.96	88.58
Morphine	99.8	ND	< 0.1	3.36	96.45
Morphine-3-B-D-glucuronide 1/2007	99.6	1.38	< 0.1	3.11	95.1
Morphine-3-B-D-glucuronide 4/2009	99.6	1.38	< 0.1	7.23	91

Impact of Hygroscopicity

Compound	First Analysis Date	Second Analysis Date	First Analysis Water (%)	Second Analysis Water (%)	Months Stored Between Analyses	Change in Water Content
Morphine	10/2007	5/2009	0.66	3.36	19	409%
Vardenafil di HCl	10/2008	1/2010	0.42	5.64	15	1243%
Digoxin (e.g. of Sample Handling)	6/2/2006 Bench top	6/4/2009 Glove box	1.15%	0.56%	NA	-51%

Changes in residual water content over time during storage and handling can impact accuracy of the reference solution concentration and analytical variability

Is it practical to check moisture content before each use of a neat reference material in an analytical lab?

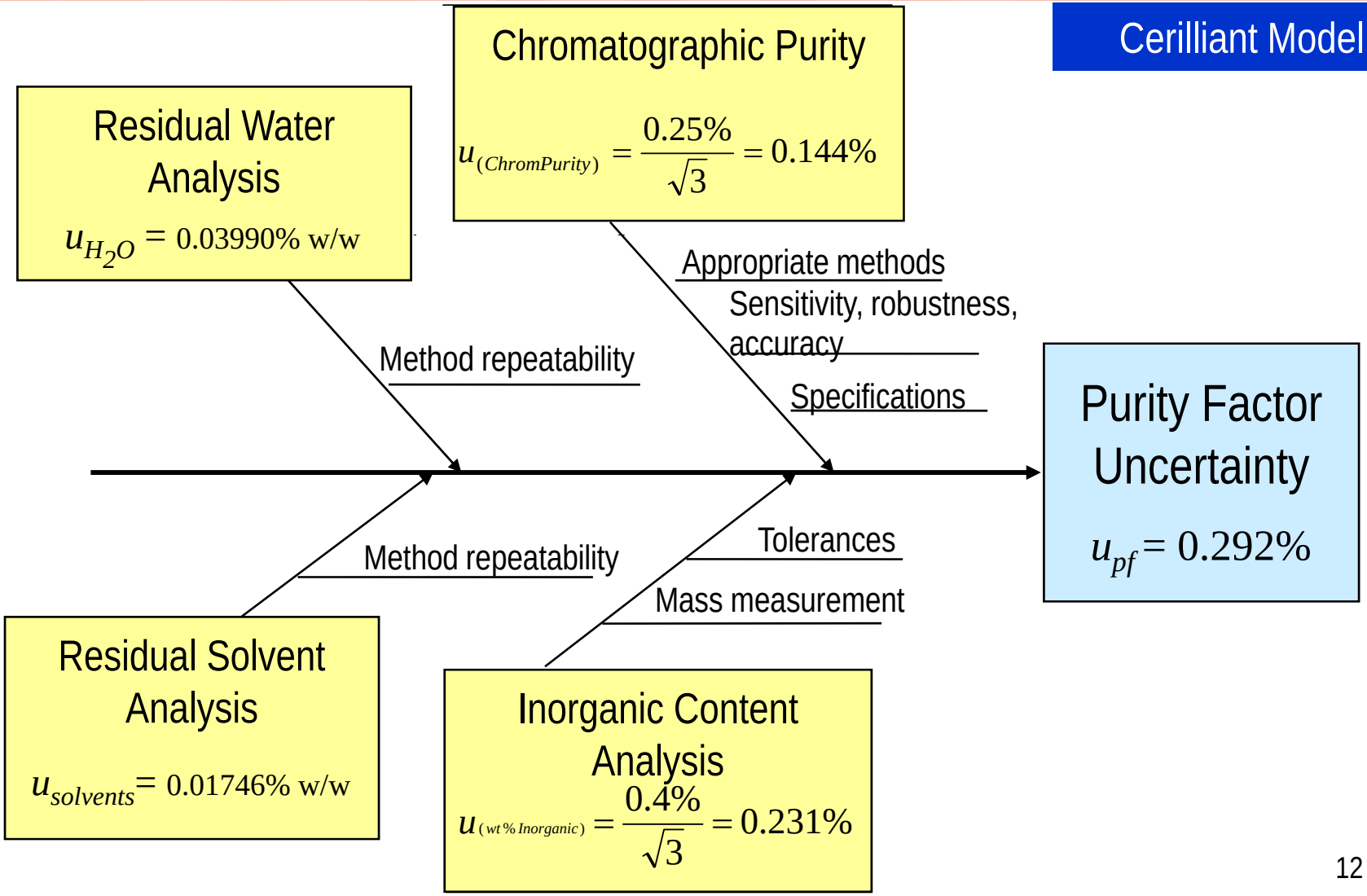
Materials were stored under normal freezer conditions in sealed, screw-cap amber vials. Water content was analyzed by Karl Fisher Coulometry based on USP method <921>.

Uncertainty of the Purity Factor

- Important to understand the uncertainty assigned to the purity factor
- Cerilliant approach
  - Followed ISO Guides 34, 35 and the Eurachem CITAC Guide in the development of uncertainty statements for the neat material purity factors
  - Combined uncertainty for the purity factor was calculated from the root sum square of the standard uncertainties of the individual components in a measurement equation
  - The process involved development of uncertainty budgets for each of the tests that contribute to the purity factor mass balance equation
- A similar approach would be required for assays and should include uncertainty of the primary reference standard certification, the standard curve preparation and the uncertainty of the analytical method

Uncertainty of the Neat Material Purity Factor

Cerilliant Model



Diluent/Solvent Considerations

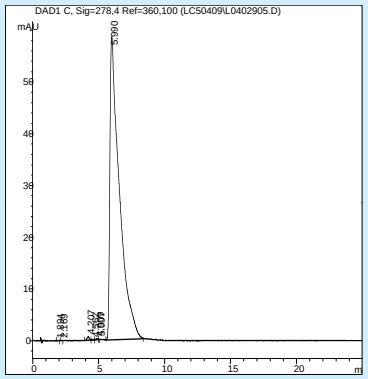
Reference Standard Design & Preparation

Solvent compatibility is critical to long term stability

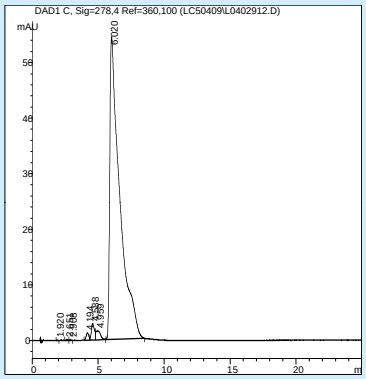
- Solubility
  - Does the target compound dissolve at the required concentration?
  - Precipitation can occur over time or at reduced storage temperatures
- Compatibility with analysis
  - Solvent interferences in the chromatogram: UV cut-off; baseline effects
  - Non-polar solvents not ideal with reverse phase HPLC
  - Water not compatible with GC
- Solvent stability
  - THF/ethers form peroxides
  - Acetonitrile oxidizes & forms acetic acid
- Compound stability in the solvent
  - Protic solvents – sirolimus (immunosuppressant) degrades in methanol over time but is stable in acetonitrile long term
- Purity, identity and traceability

Solution Development – Diluent's Impact on Stability Example: Sirolimus

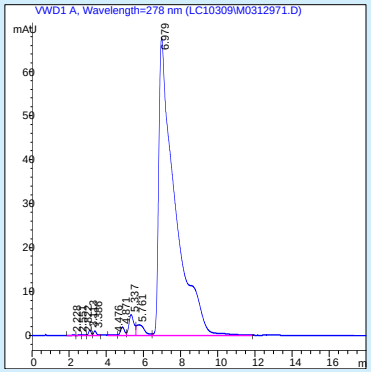
Rapid degradation in Methanol. Stable in Acetonitrile



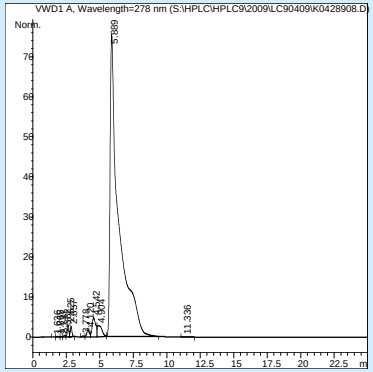
Freshly prepared solution
Time 0
Solution purity=99.6%



Ambient; 4 hours
Solution purity: 96.4%

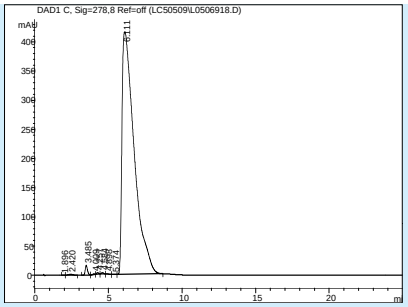


Freezer; 1 week
Solution purity: 95.0%

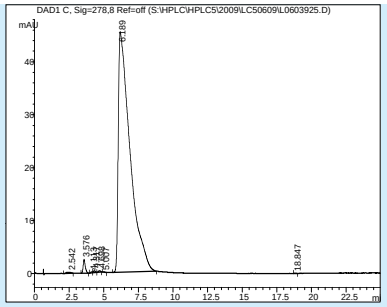


Freezer; 2 months
Solution purity: 93.5%

Methanol



Time 0
Solution Purity: 98.8%



Freezer; 4 weeks
Solution Purity: 98.5%

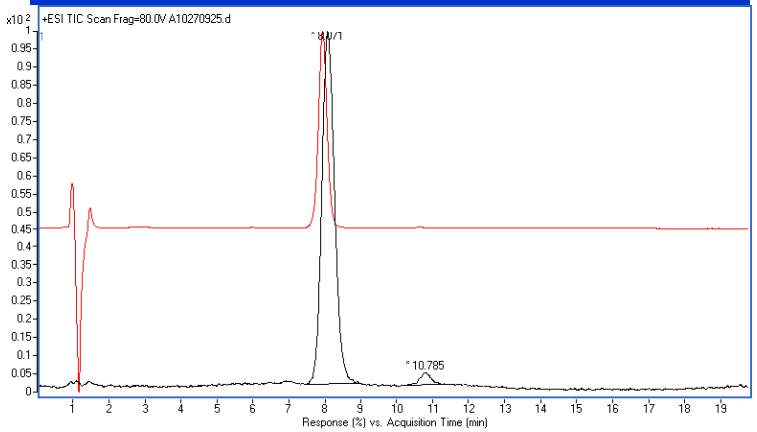
Stability Assessment: Purity of Sirolimus in Acetonitrile			
Testing Interval	Refrigerator	Freezer	Sub-freezer
Time 0	98.9		
1 Week	98.3	99.1	99.1
2 Weeks	96.6	98.7	98.2
4 Weeks	95.0	98.5	98.8
4 Months	-	-	99.0

- Stable during routine analysis under ambient conditions (3 hours).
- Degrades to 96% within 2 weeks in the refrigerator.

Acetonitrile

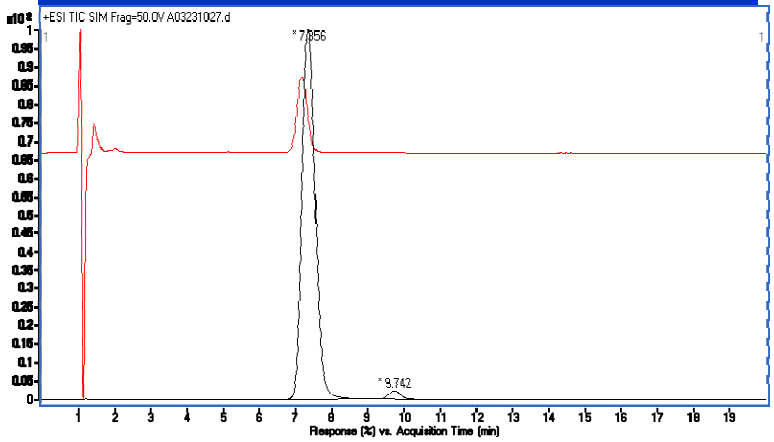
Solution Development – Evaluation of Solvent & Storage Conditions Example: 25-Hydroxyvitamin-D3

Acetonitrile



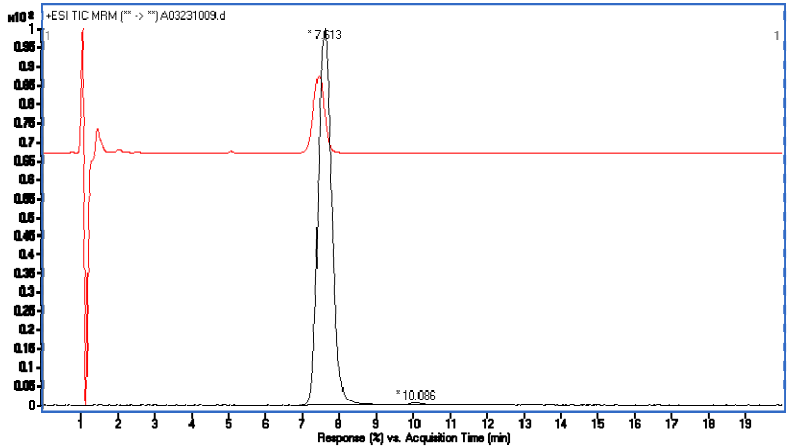
25-Hydroxyvitamin-D3, 100 ug/mL in acetonitrile. Freezer storage condition. Red is UV. Black is TIC.

Ethanol



25-Hydroxyvitamin-D3, 500 ng/mL in ethanol. Freezer 2 weeks.

HPLC and LCMS analysis of 25-Hydroxyvitamin-D3 in different solvents and storage conditions demonstrates improved performance of ethanol solution and at sub-freezer conditions



25-Hydroxyvitamin-D3, 500 ng/mL in ethanol. Sub-Freezer 2 weeks.

Manufacturing

Material / Equipment Needs

- Hygroscopicity
- Sensitivity to air or light
- Static potential
- Viscosity / volatility
- Room selection
- Environmental controls – glove box

Gravimetric Preparation

- Weight/Weight
- Higher precision vs. volumetric
- Balance selection
- Batch size flexibility vs. volumetric
- Traceability with weigh tapes
- Repeatability

Dispensing

- Equipment checks
- Line purge
- Tubing & syringes
- Sampling plans
- Segregation
- Evaporation control

Robust manufacturing practices critical to accuracy & consistency

Solution Preparation - Weighing Accuracy

Balance environment & weighing technique and can significantly influence reference accuracy

Reference Standard Design & Preparation

- Improper balance selection can lead to high level of uncertainty
  - Qualified balances – calibrations traceable to NIST
  - Minimum weighings should be determined to achieve USP tolerances of NMT 0.1% relative error
  - 5, 6, & 7 place balances may be needed
  - Calibration verification procedures – weekly & pre-use

Importance of Balance Selection and Mass Uncertainty		
Sample Mass	Mass Uncertainty	
	5-place Balance	4-place Balance
1 mg	8.0%	45.0%
10 mg	0.80%	4.5%
100 mg	0.080%	0.45%
1000 mg	0.0080%	0.045%

Cerilliant Minimum Weighing Requirements				
Balance	7-place	6-place	5-place	4-place
Balance Resolution	0.0001 mg	0.001 mg	0.01 mg	0.1 mg
Minimum Weighing	1 mg	3 mg	20 mg	125 mg

Solution Preparation - Weighing Accuracy

Reference Standard Design & Preparation

- Accuracy of weighing can be influenced by:
  - tongs vs. gloved hands
  - balance equilibration time
  - sample and solvent temperature
  - ambient temperature
  - vibrations
  - movement of air
- Hygroscopic materials handled in glove box
  - Inert atmosphere
  - Relative humidity $\leq$ 5%
- Air currents, drafts around the balance, and additional vibrational forces on the pan can significantly affect balance repeatability.

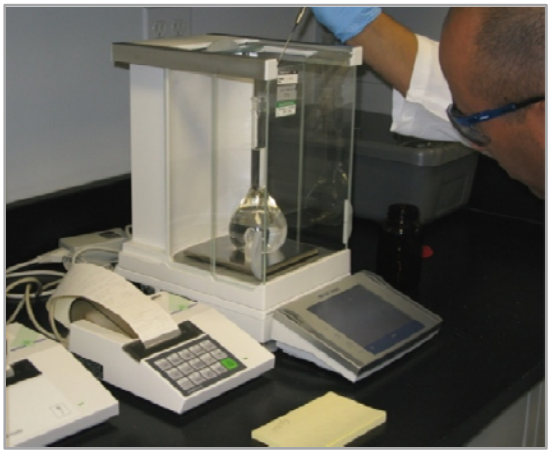
For Example:

Cerilliant studies indicate that when gloved hands are used as opposed to tongs for handling sample vials, uncertainty of mass measurement increased approximately 10 fold.

Solution Preparation - Gravimetric Approach

Reference Standard Design & Preparation

Gravimetric addition of diluent is accurate and reproducible



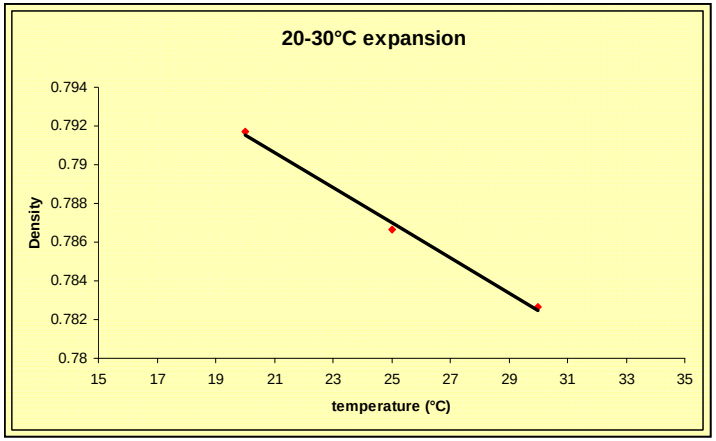
- Target solvent weight calculated from target volume by adjusting for density. Actual solvent weight can be calculated back into volume to report concentration in mg/mL
- **Ensures lot-to-lot consistency** – Measurement of volume by mass eliminates temperature dependence of flask accuracy and allows all solutions to be consistently prepared at the same chosen reference temperature.
- **Eliminates subjectivity** of the 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

Diluent Addition
Gravimetric vs. Volumetric Methods

Included for reference

Thermal expansion will affect volumetric preparation of a solution but can be controlled by gravimetric addition of solvent

Methanol



0.57% difference in concentration when prepared volumetrically at 20° vs. 25°C

Source: Handbook of Thermophysical and Thermochemical Data, CRC Press

Method	Batch Size		
	10 mL	100 mL	1000 mL
Volumetric flask standard error Source: ASTM E288-03, Standard specification for laboratory glassware, 2003	0.20%	0.08%	0.03%
Analytical balance uncertainty			
Balance Type	5 Place	5 Place	1 Place
Typical values per Mettler Toledo	0.001%	0.0001%	0.009%
Values established by Cerilliant based on typical values by Mettler and Cerilliant weighing SOPs	0.0036%	0.00125%	0.009%

Bench preparation of sample and reference on different days may create variability due to density change

Dispensing & Packaging

Reference Standard Design & Preparation

- Solution standards dispensed into single use volumes and flame sealed under inert atmosphere
- Process controls
  - Line clearance, validated cleaning procedures and new tubing to prevent contamination
  - Batch homogeneity prior to dispensing (ensured with thorough mixing - stirring or sonicating)
  - Consistency of volume dispensed verified throughout dispensing
  - Material specific controls employed as needed: continuous chilling, continuous stirring, nitrogen blanket over bulk material
  - Flame sealed under inert atmosphere

Ampouled format sealed under argon protects from hygroscopicity, degradation, evaporation, & contamination - Promotes Stability

Analytical Verification & Certification

Reference Standard Design & Preparation

Accuracy

Comparison to a primary source or certified second source – curve/calibration standard

Comparison of multiple independent preparations

Purity

Consistent with neat material

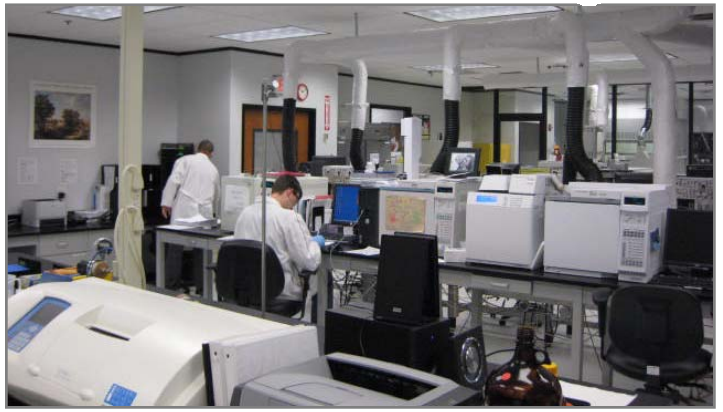
No contamination or degradation

Homogeneity

Across the batch of ampoules/vials

Consistency

Lot-to-lot consistency verified by comparing to the previous lot



Purity & Concentration are Analytically Verified

Assessment of Solution Stability

Solution Stability

Enhanced stability from properly prepared ampouled solutions

- Expiration (shelf life) is established through real-time stability studies
- Solution purity and concentration are re-evaluated at multiple intervals
- Solutions properly designed and prepared can be stable for years vs. weeks/months

Certified Solution Standards – Stability Examples

Solution Stability

Flame sealed under argon – ampouled solutions can provide long-term stability. 5+ years for many

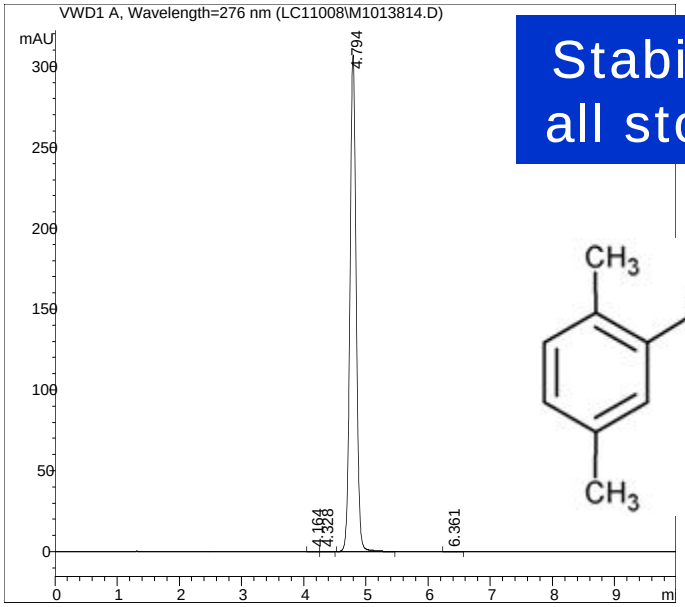
Compound/Solvent	Age of Stability Sample	Purity		Analyzed Concentration	
		Original	Stability Interval	Original	Stability Interval
Fentanyl/methanol (ug/mL)	5 years	99.1%	99.9%	97.6	98.6
6-Acetylmorphine/acetonitrile (ug/mL)	5.5 years	98.0%	99.5%	98.8	97.8
Nortriptyline HCl/methanol (mg/mL)	5 years	99.8%	99.9%	0.995	0.970
Codeine/methanol (mg/mL)	5.5 years	99.9%	99.4%	0.989	0.995
Haloperidol/methanol (mg/mL)	6 years	99.8%	99.8%	0.988	0.970

Concentration acceptance criteria for each of the examples = ± 3% and incorporates variability of the analysis.

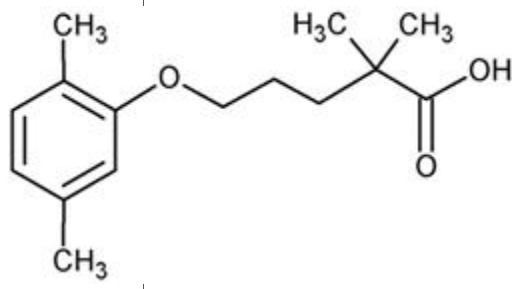
Accelerated Stability Example: Gemfibrozil

Solution Stability

Storage Condition/ Test Interval	Gemfibrozil solution purity(%)
Initial (t=0)	99.9
Freezer (-1 to -25°C)	
1 week	99.9
2 weeks	99.9
4 weeks	99.9
Refrigerate (1 to 15°C)	
1 week	99.9
2 weeks	99.9
4 weeks	99.9
Ambient (18 to 30°C)	
1 week	99.9
2 weeks	99.9
4 weeks	99.9
Elevated (40°C)	
1 week	99.9
2 weeks	99.9
4 weeks	99.9



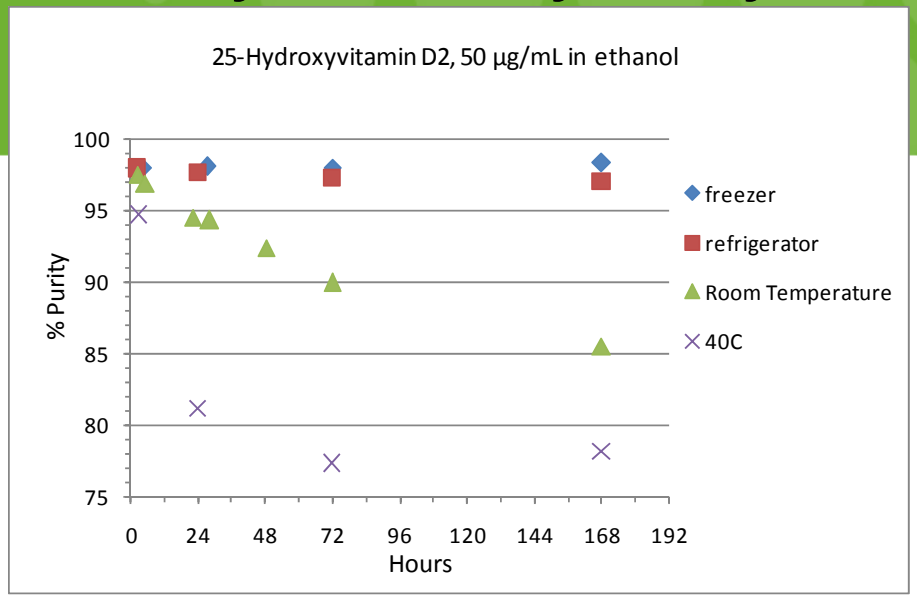
Stability exhibited at all storage conditions



Catalog Product:	G-012, 1 mg/mL in methanol
Analysis Method:	HPLC/UV
Column:	Betasil Phenyl 4.6 x 150 mm
Mobile Phase:	Acetonitrile::0.1% H_3PO_4 in Water
Flow Rate:	1.0 mL/min
Wavelength:	276 nm
Calibration Curve:	Linear Regression
Number of Points:	3
Linearity (r):	1.000

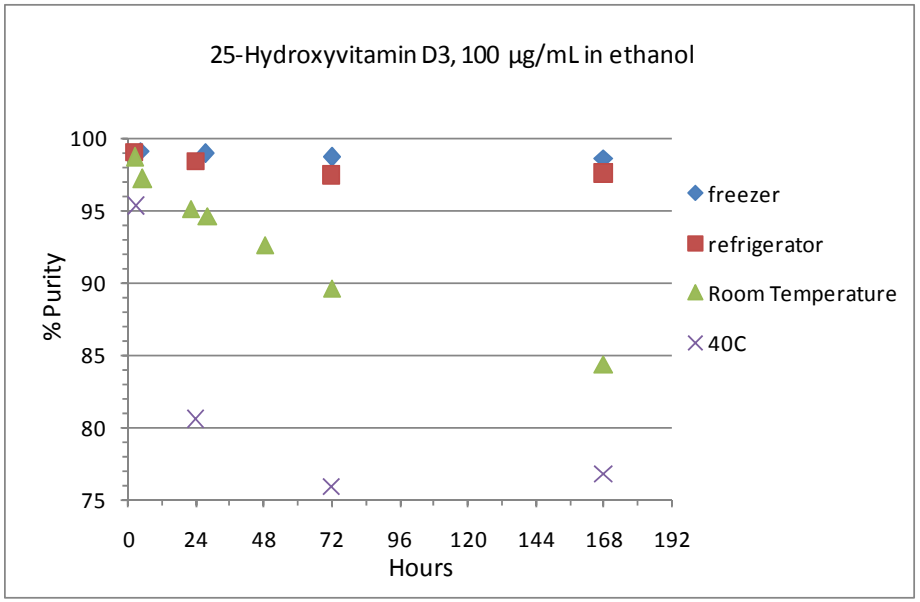
Stability of 25-Hydroxyvitamin D2 & D3

Solution Stability



Accelerated stability

- Multiple temperatures
  - Freezer, refrigerate, and 40°C
- 2 hours to 7 days



Supports handling, shipping, and short & long term storage

Stability of Anthocyanins

Solution Stability

Catalog Number	Name	Neat Material Purity	Solution Standard Purity time 0	Solution Standard Purity 4 years
C-069	Cyanidin-3-glucoside	94.6	95.2	93.1
C-070	Cyanidin-3- galactoside	94.6	93.4	82.3
P-057	Petunidin-3-glucoside	91.9	91.1	92.1
P-058	Peonidin-3-galactoside	92.4	90.2	88.6

Column:	Prodigy ODS, 4.6 x 250 mm		
Mobile Phase:	A:	50% H_2O/48.5% CH_3CN/1% CH3COOH/0.5% H3PO4	
	B:	0.5% H_3PO_4	
Gradient:	Time (mins)	%A	%B
Program:	0	10	90
	28	50	50
	32	75	25
	32.1	10	90
Flow Rate:	0.9 mL/min		
Wavelength:	520 nm		

Concentration: 500 µg/mL, 2%HCl in Methanol
Container: Amber glass ampoules under Argon
Storage: Freezer

Uncertainty of the Gravimetric Preparation

Certification includes assessment of uncertainty of the reference preparation in compliance with ISO 17025

Certification & Uncertainty

Neat Material Purity

- Uncertainty associated with all testing performed must be included
- Chromatographic purity – Residual water – Residual solvent – Residual inorganic content

Mass Measurement

- Uncertainty associated with all weighing operations
- Specific to technique, equipment, scale & environment

Solvent Addition

- Uncertainty associated with the method of solvent addition
- Consider solvent temperature, glassware or balance tolerances, solvent density

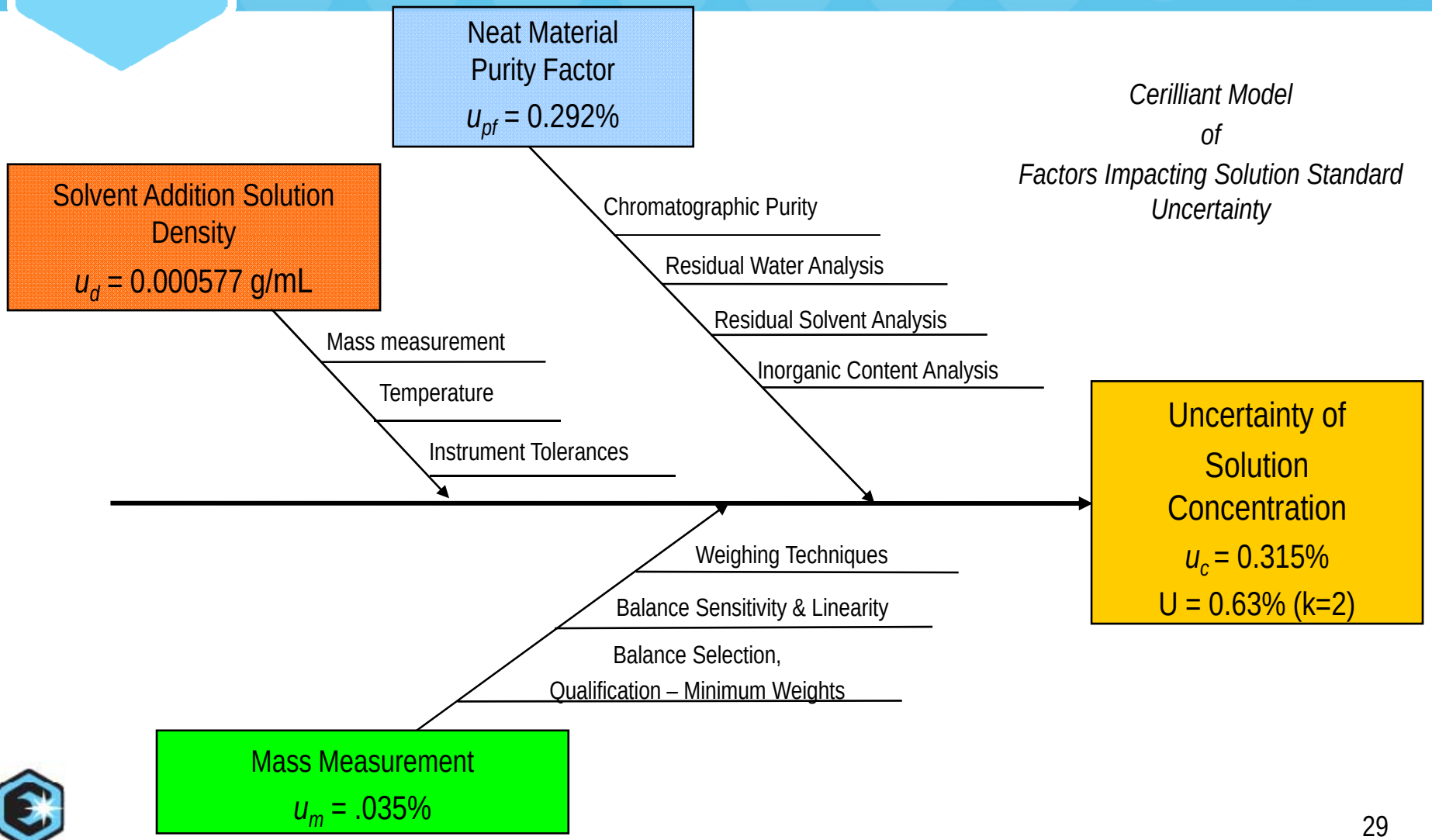
Each process was examined in detail and uncertainty determined using a combination of experimental results and instrument and process tolerances.

Certification & Uncertainty

Certification & Assessment of Uncertainty

Assessment of Uncertainty is a requirement for compliance with ISO 17025

Cerilliant Model of Factors Impacting Solution Standard Uncertainty



Cerilliant
Analytical Reference Standards

A-064
FN022009-02
Revision 0
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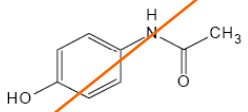
Certificate of Analysis

Cerilliant Quality
ISO GUIDE 34 ACCREDITED
ISO/IEC 17025 ACCREDITED
ISO 9001:2000 CERTIFIED

Acetaminophen

4-Acetamidophenol



Catalog Number: A-064
Solution Lot: FN022009-02
Expiration Date: February 2014
Solvent: Methanol
Volume per Ampule: Not less than 1 mL
Storage: Protect from air and light, refrigerate or freeze.
Intended Use: For laboratory use only. Not suitable for human or animal consumption.

- Expiration Date has been established through real time stability studies.
- Ampules are overfilled to ensure a minimum 1 mL volume fill. We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.

Component	Chromatographic Purity	Concentration
Acetaminophen	99.9%	1.000 ± 0.006 mg/mL

- Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and mass.
- Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

Solution Standard Verification and Homogeneity

Standard Solution	Lot Number	Verified Concentration (mg/mL) Actual Results	Verified Concentration (mg/mL) Acceptance Crtieria	%RSD - Homogeneity Actual Results	%RSD - Homogeneity Acceptance Crtieria
New Lot	FN022009-02	0.998	± 3%	0.8	≤ 3%
Previous Lot	FN092707-01	0.996	± 3%	0.4	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration curve.
- Homogeneity of the New Lot is ensured through rigorous production process controls statistically analyzed to evaluate risk and verified by analysis. The % RSD of samples pulled from across the lot demonstrate homogeneity of the New Lot.
- The % RSD of the Previous Lot represents variability of the analysis.

Traceability

- Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error.
- Concentration is verified against an independently prepared 4-point calibration curve gravimetrically prepared using balances calibrated to NIST.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date.

ACLASS

Lara Sparks, Quality Assurance Director

March 18, 2009
Date

Cerilliant Corporation 811 Paloma Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

Solution Purity is verified chromatographically post ampouling to ensure no degradation or contamination

Certification of the Solution Standard

Concentration & Uncertainty of the gravimetric preparation expressed as:

1.000 ± 0.0006 mg/mL

Description of Cerilliant's Uncertainty value & confidence interval:

"Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the purity factor, material density, and mass".

Analytical Verification of Concentration & Homogeneity

Gravimetrically prepared concentration is verified analytically. Acceptance criteria incorporates variability of the analysis. Homogeneity is verified analytically by analyzing ampoules pulled from across the lot.

Traceability Statement describing traceability to SI units

"Gravimetrically prepared using qualified balances calibrated semi-annually by Mettler Toledo using NIST traceable weights. Calibration verification performed weekly and prior to each use utilizing NIST traceable weights. Each balance has been assigned a minimum weighing by Mettler Toledo taking into consideration the balance and installed environmental conditions to ensure weighing complies with USP tolerances of no more than 0.1% relative error".

Cerilliant
Analytical Reference Standards

What Makes a Good Reference Material?

- Fully characterized high purity neat materials and high purity diluents
  - Careful assignment of chromatographic purity by multiple methods
  - Analysis of residual impurities including water, inorganics and solvent
- Validated preparation process ensuring consistency and accuracy of solution concentration, purity & stability
- Qualified balances in their installed state with minimum weighings set for <0.1% relative error
- Gravimetric approach in solvent addition
- Traceability to SI units
- Uncertainty statement encompassing all aspects of standard preparation from neat material characterization to solution preparation.
- Prepared in a stable ampouled format
- Preparation and certification by an ISO Guide 34 and ISO 17025 accredited laboratory

Pre-made Ampouled Certified Solution Standards - A significant advantage over neat reference materials

Cerilliant®
Analytical Reference Standards

For information on products and services stop by Booth 208

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Cerilliant Quality

ISO GUIDE 34
CERTIFICATE AR1353

ISO/IEC 17025
CERTIFICATE AT1352

ISO 9001:2008
CERTIFICATE 3854