

# High Quality, Certified Snap-N-Shoot® Standards of Reb A and Stevia Impurities Ensure Accuracy and Consistency in Quantitative Applications

Authors: [Mitzi Rettinger](#), Isil Dilek, PhD, Sandra Ybarra, Uma Sreenivasan, PhD, and Sherri Pogue

## Abstract

FDA requirements for the use of Rebaudioside-A (Reb-A) in food and beverage products include a purity of no less than 95%. A component of the Stevia leaf, Reb-A contains naturally occurring impurities whose levels vary depending on the cultivation process, isolation and blending techniques, and the environmental conditions during farming and harvesting.

Laboratories often encounter multiple technical challenges in the analysis of Reb-A. The material properties of Reb-A provide significant issues with hygroscopicity, making preparation of a consistent and accurate reference standard problematic for laboratories. Weighing accuracy of mg amounts of Reb-A can be influenced by balance environment and weighing technique. Method selection may yield different results due to elution order differences between methods, issues with method accuracy or repeatability, or lack of impurity resolution. The use of an accurate, quantitative and stable solution standard of Reb-A and its impurities prepared in a controlled inert environment and packaged in an ampouled format under inert atmosphere eliminates these particular issues.

Multiple factors impact accuracy and consistency of a Reb-A reference standard. This poster will discuss these factors and their influence on the design, preparation, and certification of a solution standard of Reb-A and its impurities.

## Introduction

- FDA's "no objection letters" in December 2008 opened the door to food and beverage companies to use Reb A in food products as long as the purity is no less than 95%
- Laboratories are now challenged with quantifying the percent of Reb A in their raw materials
- For food and beverage manufacturers it is not enough to receive a vendor's certificate of analysis, it's important to verify the quality and purity of the matrix
- Reb A, a component of the Stevia leaf, has naturally occurring impurities that vary depending on environmental conditions of farming, process of cultivation, harvest, and isolation/blending
- Accuracy of this quantification depends on robustness of the analysis and quality of the reference
- To accurately quantify the % Reb A (Assay) the lab must start with accurate reference standards

Results are only as accurate as the reference!

## I. Technical Challenges in Analyzing Steviol Glycosides

- Material Properties
  - Multiple sources with varying impurity profiles
  - Hygroscopicity
- Analytical methods
  - Different methods yield different results
- Accuracy of Reference Standards
  - Full characterization/Certification
  - Process controls required in preparation of solutions
  - Stability

## A. Material Properties

### Chromatographic Purity – Only the Beginning...

The COA reads 97% - is it really?

- Residual Water & Hygroscopicity
  - The hygroscopic nature of Reb A and Reb A impurities provides a significant challenge to the accurate determination of purity/potency
  - Absorption of moisture over time means water content must be re-evaluated prior to each use in quantitative applications. Sample handling/preparation may impact results
- Residual Solvent
  - A neat reference material such as Reb A may contain residual solvent from processing the plant despite high chromatographic purity
- Trace Inorganic Content
  - Due to the environmental conditions of the farmland, extraction process, or purification procedure, many materials may contain trace inorganics

Use of a Purity/Potency Factor mass balance equation is critical to properly calculate the amount of material needed to achieve accurate concentration of the reference standard

$$\text{Purity Factor} = \left[ \frac{1100 - (\text{wt \% OVI}) - (\text{wt \% H}_2\text{O}) - (\text{wt \% ROI})}{100} \right] \times \frac{\text{Chrom Purity}}{100}$$

### Purity Factor Impact

Compound	Chrom. Purity (%)	Residual Solvent Content (%)	Trace Inorganic Content (%)	Residual Water Content (%)	Purity Factor for Quantitative Use (%)	PF Difference from Chrom Purity (%)
Rebaudioside A	98.39	1.19	< 0.1	5.58	91.73	-6.66
Rebaudioside B	86.11	0.06	< 0.1	4.17	82.47	-3.64
Rebaudioside D	91.51	0.22	< 0.1	4.57	87.12	-4.39
Steviol	99.22	1.74	< 0.1	1.55	95.52	-3.70
Stevioside	92.33	0.86	< 0.1	4.44	87.44	-4.89
Steviolbioside	92.02	0.45	< 0.1	9.86	82.53	-9.49
Rubusoside	98.32	0.42	< 0.1	3.90	94.07	-4.25

Without full characterization of the neat material, significant error may be introduced into the concentration of the reference solution.

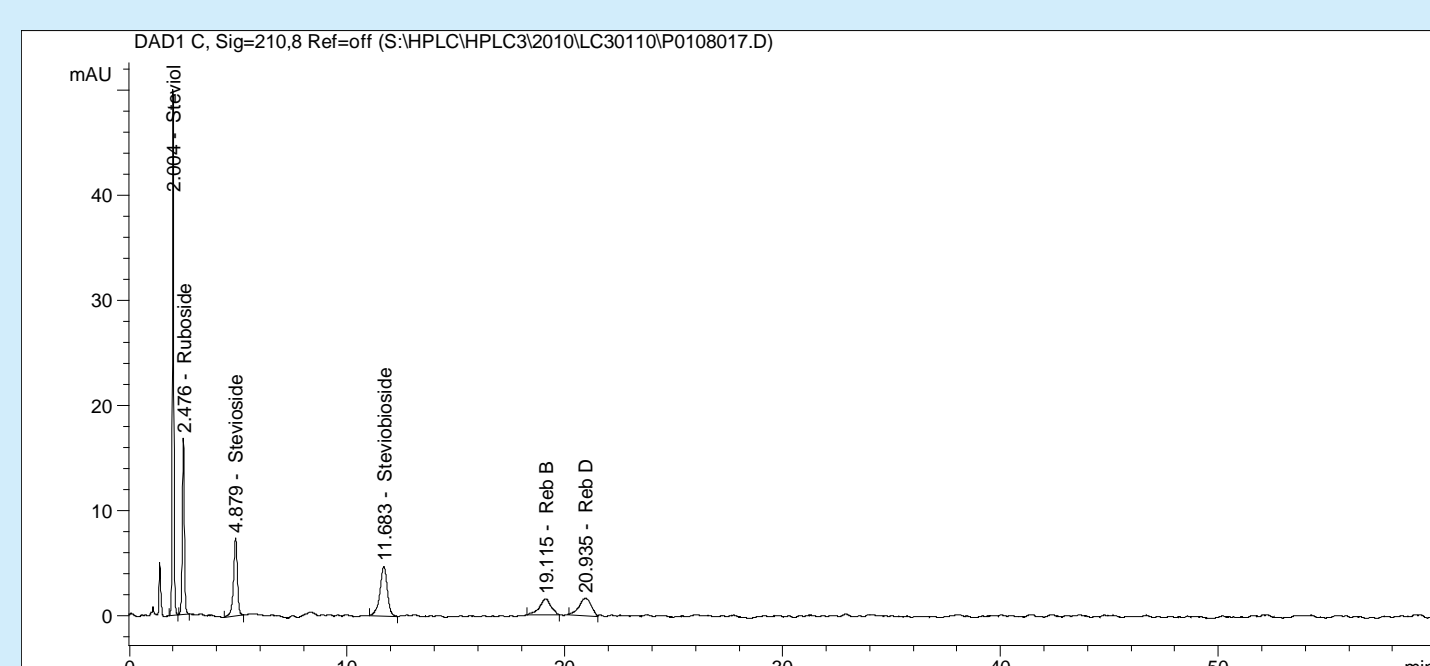
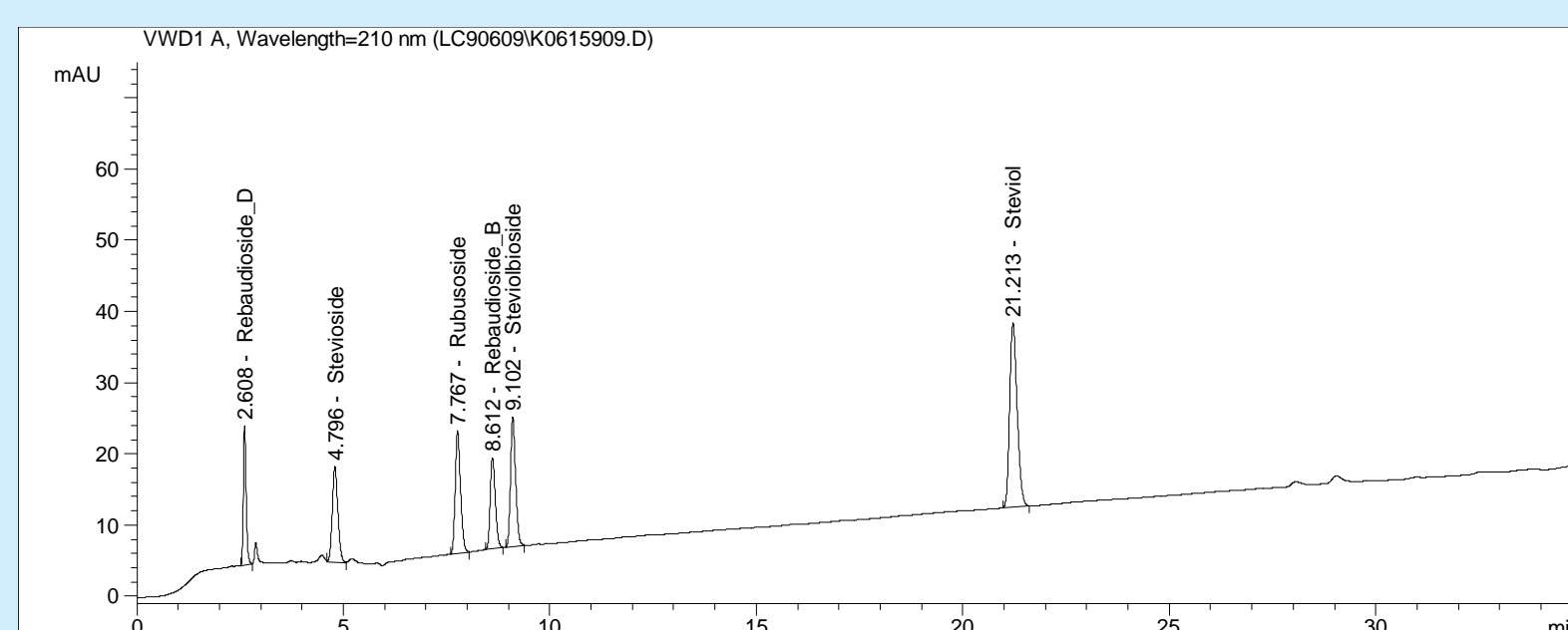
## B. Analytical Issues

The analytical method needs to be accurate, robust, repeatable and reliable, and should provide resolution of all impurities.

- Reb A impurities are related glycosides differing primarily in number and type of sugar
- It is chromatographically challenging due to similarity in structure and polarity
- Important that the method resolve the Reb A and its impurities
- Different methods can give different elution order for the impurities
- Even within method, elution order can vary over time due to column brand or instability/short life of select columns such as amino columns
- If method is not stable it is also more difficult to determine if material is adulterated or contaminated

### Method Examples for Method Variability - Reb A Impurities Standard

Cerilliant Method	
Analysis Method	HPLC/UV
Column	Prodigy ODS 3, 5µ, 4.6 x 250 mm
Mobile Phase	Acetonitrile (ACN) 0.1% H <sub>3</sub> PO <sub>4</sub> in H <sub>2</sub> O
Gradient Time	ACN
0	35
2	35
3.5	95
35.1	35
Flow Rate	1.0 mL/min
Wavelength	210 nm
Column Temp	40°C
Run Time	35 min
Post Time	5 min



USP Method	
Analysis Method	HPLC/UV
Column	Luna NH <sub>2</sub> , 5µ, 4.6 x 150 mm
Mobile Phase	Acetonitrile-Amonium Acetate (80:20)
Flow Rate	1.0 mL/minute
Wavelength	210 nm

Different Amino Columns		
Compound	Agilent Zorbax NH <sub>2</sub> Retention Time (min.)	Phenomenex Luna NH <sub>2</sub> Retention Time (min.)
Reb A	7.1	7.2
Reb B	36	11.1
Reb D	16	17.2
Stevioside	4.5	4.2
Steviolbioside	18	6.2
Rubusoside	3.0	2.2

Use of Certified Reference Standards of Reb A Impurities aids in monitoring method performance as well as quantitation

## C. Accuracy of Reference Standards Critical to Accurate Quantitation

### Certified Neat Reference Standard

- Analysts prepare volumetric solutions by weighing neat materials and diluting
- Chrom purity alone insufficient
  - Residual content must be considered and weighing adjustments made
- Storage & stability
  - Evaluate water content before each use
  - Concentration of stock solutions may change over time



### Snap-N-Shoot® Certified Solution Standard

- Premade solution for immediate use as-is or in dilutions
- Chrom purity and residual impurities are accounted for at time of preparation
- Certified value remains constant
- Ampouled format prevents changes over time due to hygroscopicity, degradation or evaporation
- Single use format for consistency; eliminates contamination issues



## II. Snap-N-Shoot® Certified Solution Standards Prepared in Inert Environment

Preparation begins with full characterization of the neat material

- Chromatographic purity through use of multiple methods/techniques
  - eliminates improper assignment due to random analytical error – results must agree within 0.5%
  - ensuring separation of impurities
- All residual impurities
  - water content by Karl Fisher
  - residual solvent by GC/FID headspace
  - Inorganic content by microassh
- Identity confirmation by multiple methods

Preparation Process Controls are in place and validated including material handling, weighing & dilution accuracy, dispensing & homogeneity.

Certification of ampouled solution concentration and ampoule to ampoule consistency is performed.

Stability is verified and traceability established.

## A. Weighing Accuracy

Balance environment & weighing technique can significantly influence reference accuracy

- Balance Selection
  - Use qualified balances – calibrations traceable to NIST
  - Minimum weighings established to achieve USP tolerances of NIST 0.1% relative error
  - 5, 6, or 7 place balance required
- 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 < 5%



Size of Weighing Influences Accuracy – larger weighings are more accurate. Can be costly with expensive impurities

Sample Mass	Mass Uncertainty	
	5place Balance	4place 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	7place	6place	5place	4place
Balance Resolution	0.0001 mg	0.001 mg	0.01 mg	0.1 mg
Minimum Weighing	1 mg	3 mg	20 mg	125 mg

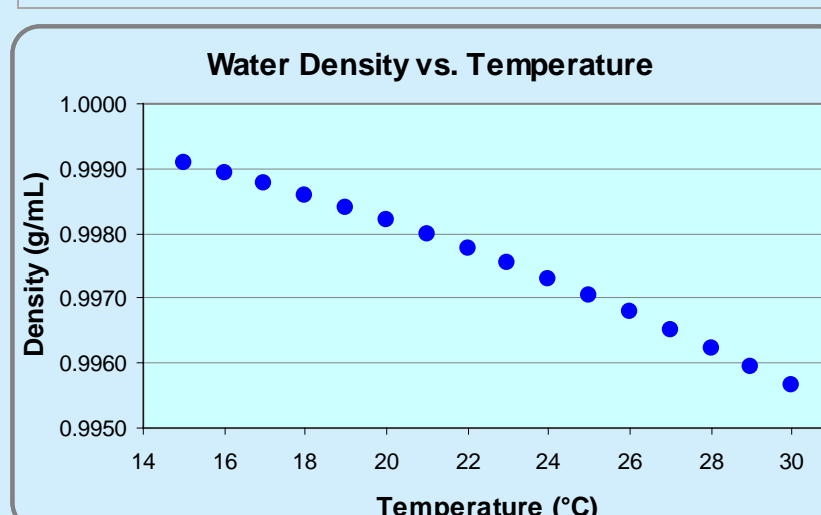
## B. Gravimetric Approach Add Solvent by Weight



Gravimetric addition of diluent provides reproducibility

- 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 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.

### Effect of Thermal Expansion on Volumetric Dilutions



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

### Comparison of Tolerances for Volumetric and Gravimetric Dilutions

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%

Use of sensitive, qualified balances and proper techniques provides a lower error than dilution to visual fill line of Class A volumetric flasks

Source: Chemical Handbook Fundamental Version, Rev. 3 (1984)

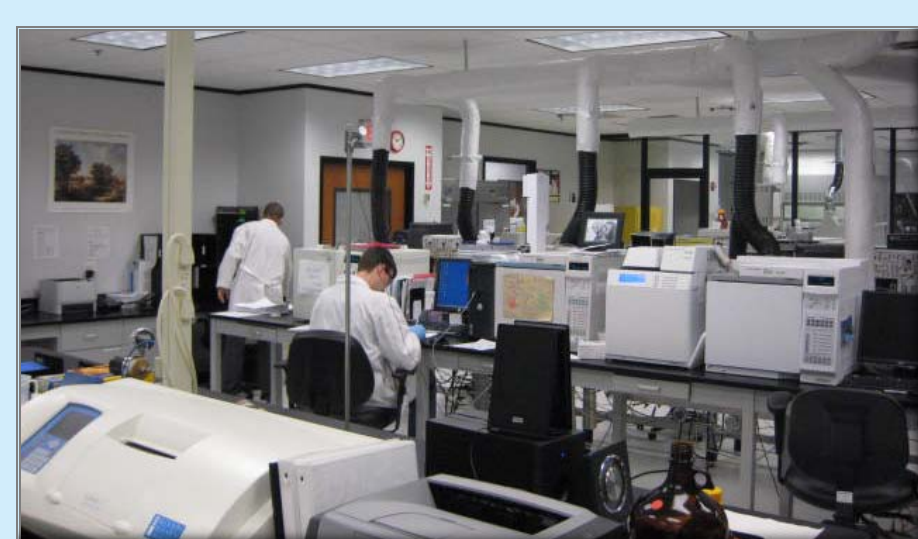
## C. Dispensing & Packaging

- Solution standards are dispensed into single use volumes and flame sealed under inert atmosphere
- Process controls ensure
  - Consistency of volume dispensed
  - Homogeneity from vial to vial and across the lot
  - No contamination
  - No degradation

Flame sealed under argon into amber ampoules  
Protection from degradation, evaporation, & contamination



## D. Analytical Verification & Certification



- Solution standard concentration is verified analytically by comparison to a multi-point independently prepared calibration curve.
- 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.
- Solution purity is verified to demonstrate no contamination or degradation has occurred during preparation.

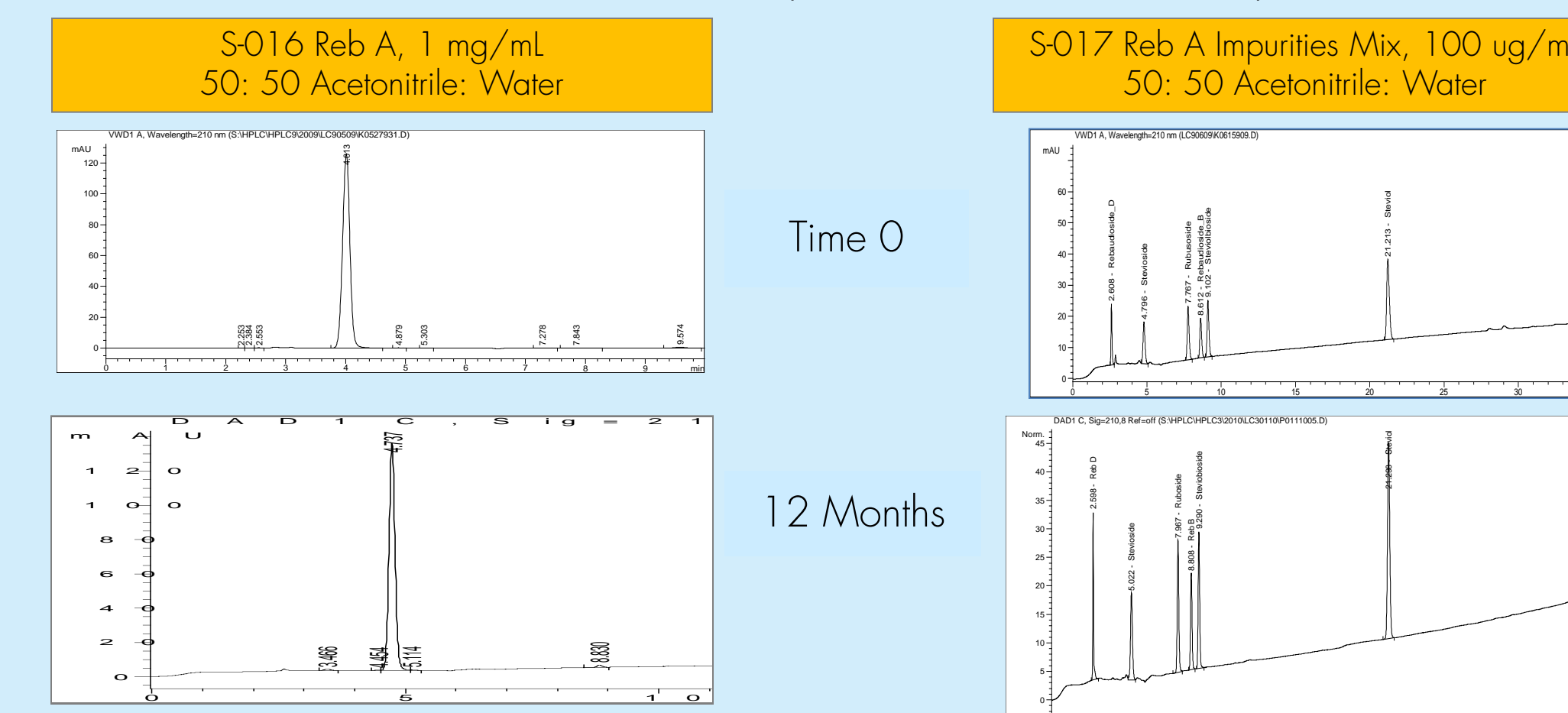
## Cerilliant's Approach

## III. Solution Stability

- Expiration (shelf life) is established through real-time stability studies
- Solution purity and concentration are re-evaluated at multiple intervals
- 12 months of shelf life has been established for Reb A and Reb A Impurities solution standards. Stability studies are ongoing.

Neat Material	98.4%
Solution Purity Time Zero	98.8%
Solution Purity 6 months	98.2%
Solution Purity 12 Months	98.1%

### Reb A & Reb A Impurities Solution Stability

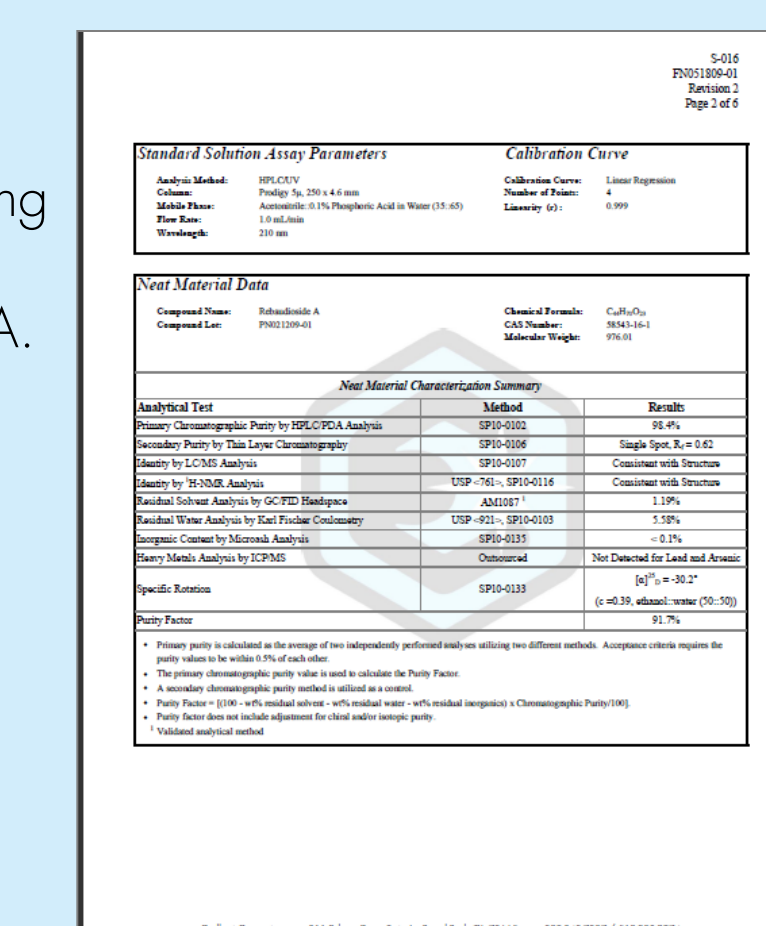
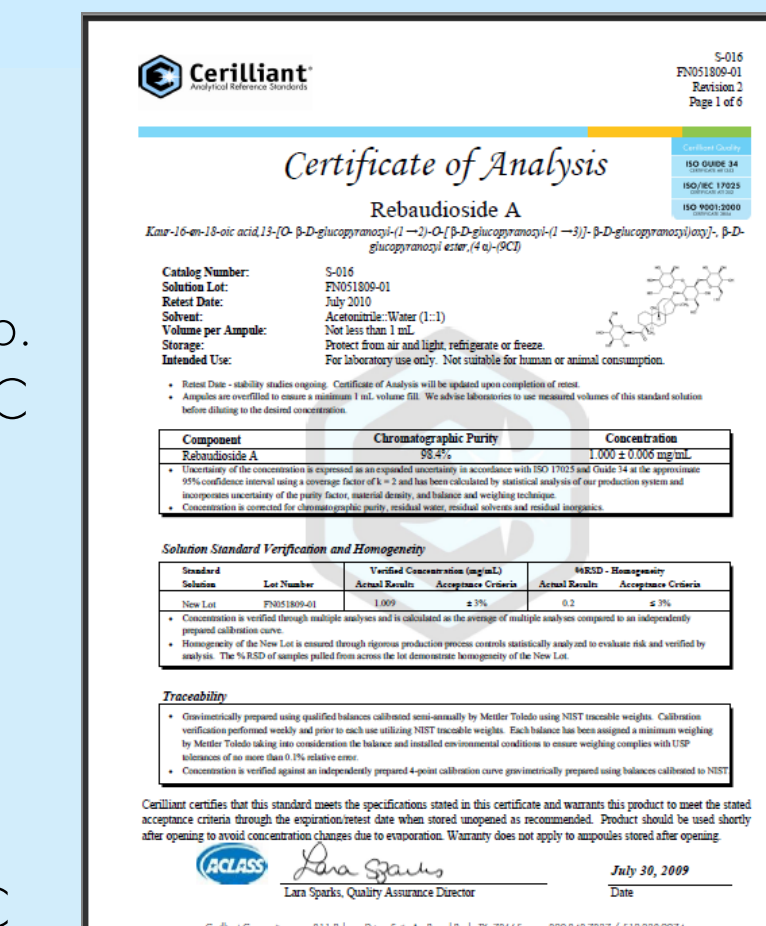
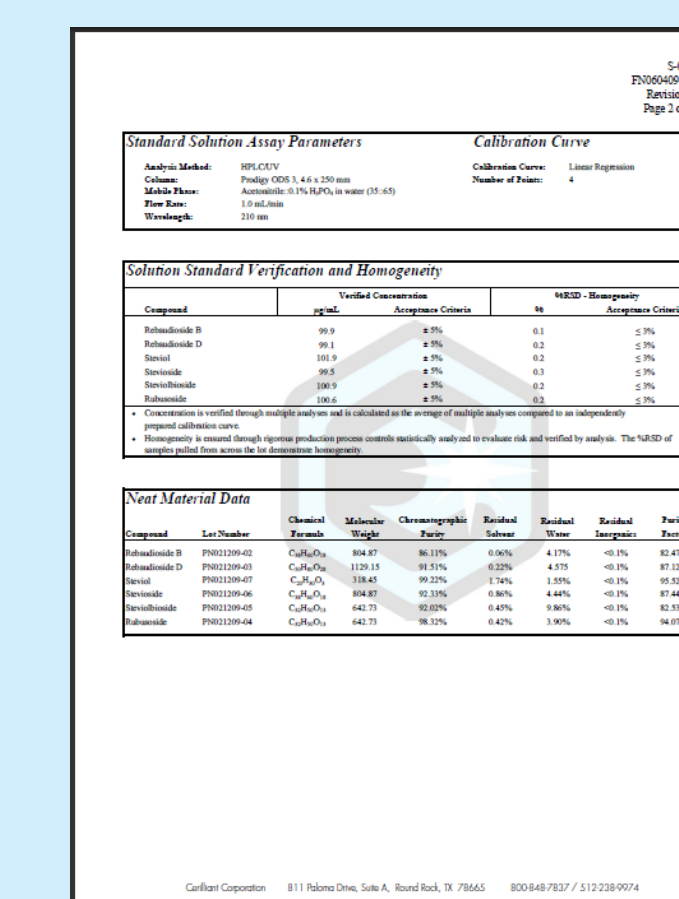


## IV. Traceability

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.

Snap-N-Shoot® Certified Solution Standards

- Prepared and certified to ISO Guide 34 and ISO/IEC 17025 standards.
- Neat material certification by ISO/IEC 17025 accredited testing lab.
- 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.
- Analytical verification of concentration and homogeneity by ISO/IEC 17025 accredited testing lab utilizing validated methods.
- Concentration is reported with uncertainty in accordance with ISO/IEC 17025 and ISO Guide 34.
- 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.



## V. Comparison of Approaches

	Category	Cerilliant Snap-N-Shoot® Certified Solution Standards	Certified Neat Reference Material (solutions from neat materials)
Accuracy & Stability	Lot to lot consistency / Reproducibility	Prepared using a validated process	More variability and labor costs & inconsistency of reference may lead to possible product batch investigation and/or rejections
	Concentration Accuracy	Consistent across lot & preserved in ampouled format	Cannot be ensured – Hygroscopicity of the neat affects concentration from weighing to weighing Stored bulk solutions can concentrate over time due to evaporation of solvent
Cost Efficiencies	Stability over time	Years	Weeks/months
	Labor	Eliminated labor required to analyze neat, weigh, and prepare stock solutions	More labor; more cost
	Materials	Reduced material costs	Increase in material usage due to frequent weighings (important on costly impurities)
	Convenience of use	Snap-N-Shoot®	Weigh, dilute, verify

## VI. CONCLUSION

Reb A and Reb A impurities are challenging to accurately analyze using a single method. Different methods produce different results. Residual impurities in and hygroscopicity of neat materials mean care must be taken in preparation of assay reference solutions.

Use of high quality certified ampouled solution standards eliminates issues of method variability and difficulties with accurately preparing assay reference solutions. Cerilliant offers Snap-N-Shoot® Certified Solution Standards of Rebaudioside A and Rebaudioside A impurities in an accurate and convenient format for quantitative & qualitative testing of Reb A ingredients.