



High Quality, Certified Snap-N-Shoot® Standards of  
Reb A and Stevia Impurities ensure accuracy and  
consistency in Quantitative Applications

science, **smarter.**®

Cerilliant Quality

ISO GUIDE 34

ISO/IEC 17025

ISO 9001:2008

GMP/GLP

# 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 the plant based material
- 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 product
- Reb A, a component of the Stevia leaf, has naturally occurring impurities that are variable 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



# 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
  - Certification
  - Process controls in preparation of solutions
  - Stability



# 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
- 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

$$PurityFactor = \left[ [100 - (wt\% OVI) - (wt\% H_2O) - (wt\% ROI)] * \frac{ChromPurity}{100} \right] \pm U$$



# 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.



# Analysis of Rebaudioside A and its Impurities

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

## Cerilliant Method

Analysis Method	HPLC/UV
Column	Prodigy ODS 3, 5 $\mu$ , 4.6 x 250 mm
Mobile Phase	Acetonitrile:0.1% H <sub>3</sub> PO <sub>4</sub> (35:65)
Flow Rate	1.0 mL/minute
Wavelength	210 nm

## USP Method

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

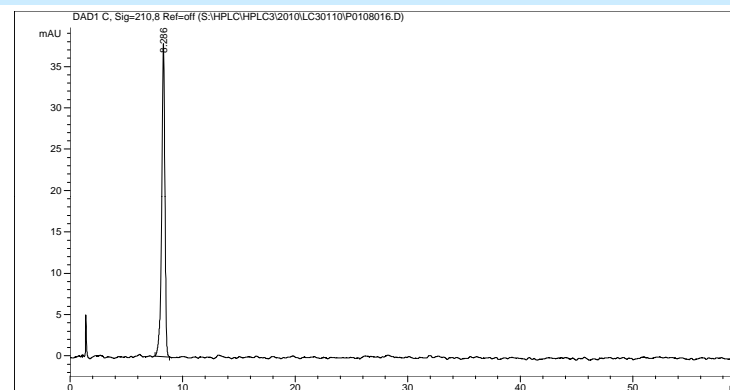
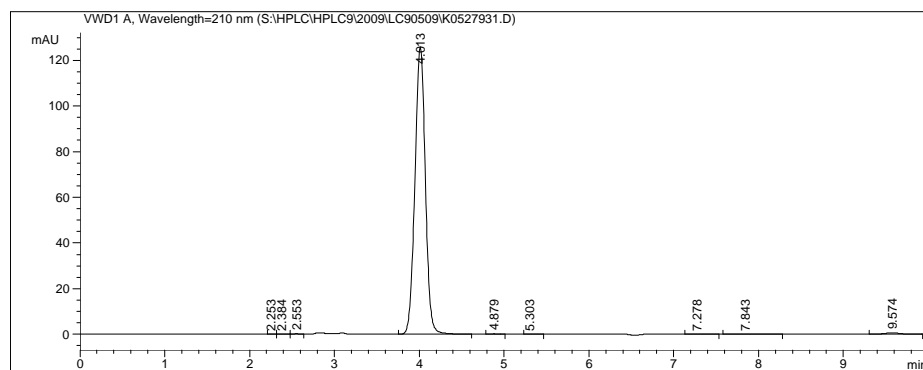


# Reb A & Reb A Impurities Chromatograms

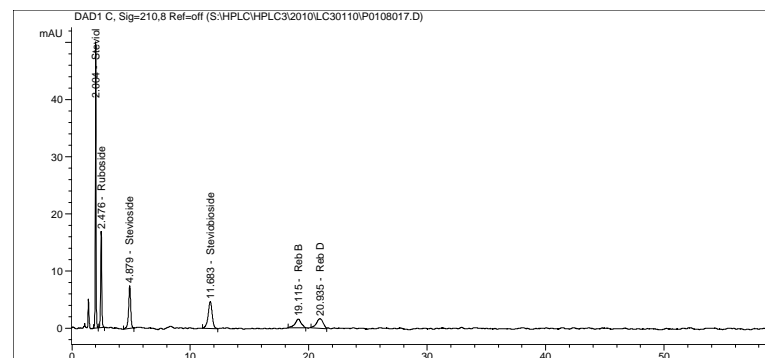
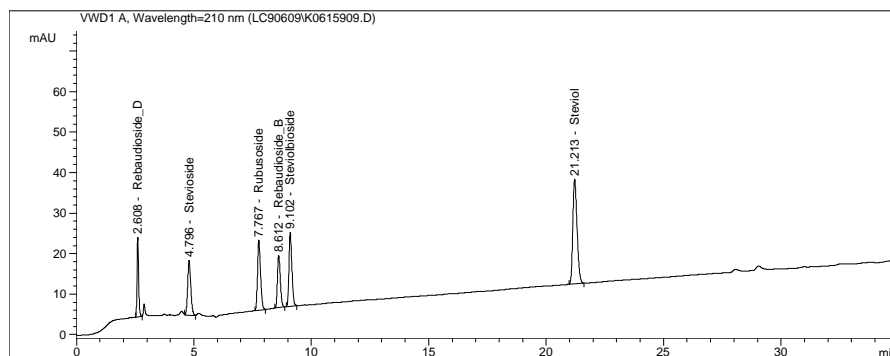
Cerilliant Method

USP Method

## Reb A Standard



## Reb A Impurities Standard



# Additional Observations USP Monograph Method

## Use of "Equivalent" Amino Columns in Analysis of Reb A Impurities by USP Monograph

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

- While the suggested column in the USP Monograph is a Zorbax NH<sub>2</sub>, use of an equivalent Luna NH<sub>2</sub> results in elution order differences of the impurities.
- Elution order differences represent a major challenge for the monograph since it calls for impurity identification by retention time (and not relative retention time).

**Use of Certified Reference Standards of Reb A Impurities  
eliminates this issue**





# 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



# Snap-N-Shoot® Certified Solution Standards

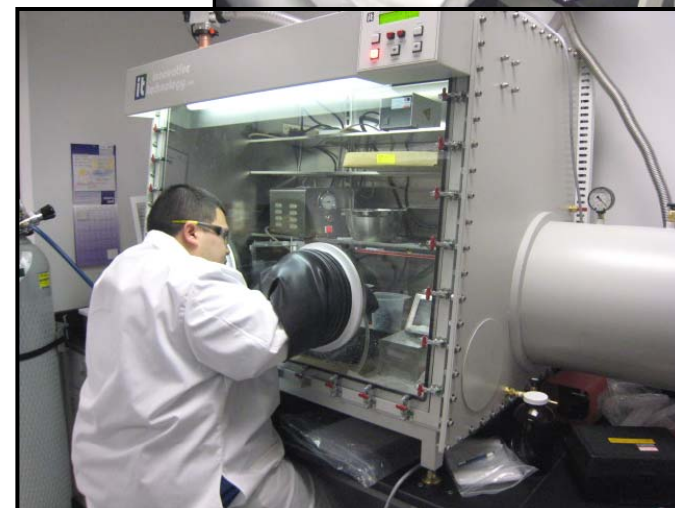
- Preparation begins with full characterization of the neat material
  - Chrom purity through use of multiple methods/techniques
    - eliminates improper assignment due to random analytical error – results must agree within 0.5%
    - ensuring separation of impurities
  - All residual impurities
    - water content by Karl Fisher
    - residual solvent by GC/FID headspace
    - Inorganic content by microash
  - Identity confirmation by multiple methods
- Preparation Process controls
  - Material handling, weighing & dilution accuracy, dispensing & homogeneity controls, and traceability
- Certification of ampouled solution concentration and ampoule to ampoule consistency
- Stability



# Weighing Accuracy

Balance environment & weighing technique and can significantly influence reference accuracy

- Balance Selection
  - Qualified balances – calibrations traceable to NIST
  - Minimum weighings established to achieve USP tolerances of NMT 0.1% relative error
  - 5, 6, & 7 place balances
- 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  $\leq 5\%$



# Size of Weighing Influences Accuracy

Larger weighings are more accurate.  
Can be costly with expensive impurities

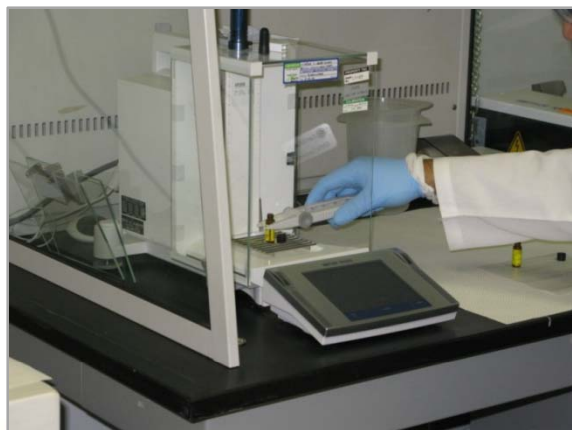
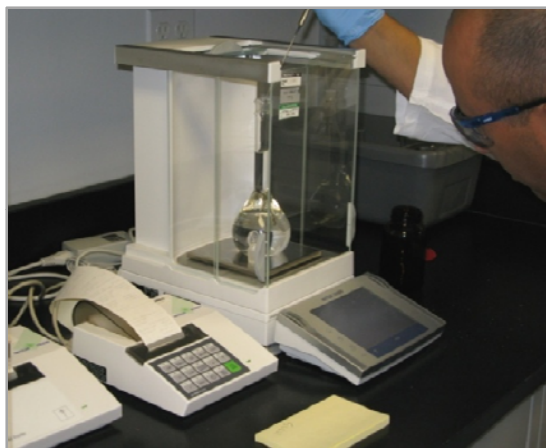
<b>Importance of Balance Selection and Mass Uncertainty</b>		
Sample Mass	Mass Uncertainty	
	5-place Balance	4-place Balance
1 mg	8.0%	45.0%
10 mg	0.80%	4.5%
100 mg	0.080%	0.45%
1000 mg	0.0080%	0.045%

<b>Cerilliant Minimum Weighing Requirements</b>				
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



# Gravimetric Approach Add Solvent by Weight

Gravimetric addition of diluent provides reproducibility



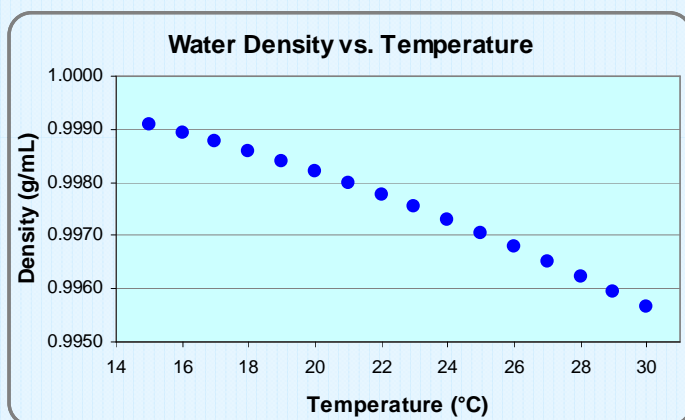
- Target solvent weight calculated from target volume by adjusting for density. Actual solvent weight can be calculated back into volume to report concentration in mg/mL
- Ensures lot-to-lot consistency – Measurement of volume by mass eliminates temperature dependence of flask accuracy and allows all 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



# Diluent Addition

## Gravimetric vs. Volumetric Methods

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

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

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 high quality, qualified, balance has lower error than Class-A volumetric flask





# Dispensing & Packaging

- Solution standards 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



# Analytical Verification & Certification

Concentration and  
Ampoule to Ampoule  
Consistency  
analytically verified  
post ampouling



- 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





# Solution Stability

Enhanced stability achieved with properly prepared ampouled solutions

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

Neat Material	98.4%
Solution Purity Time Zero	98.7%
Solution Purity 6 months	98.2%

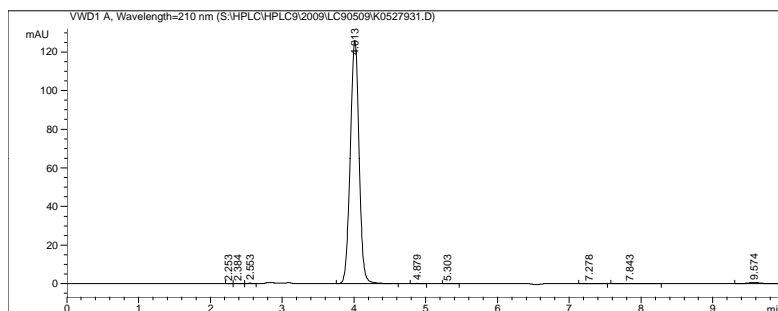


# Reb A & Reb A Impurities Solution Stability

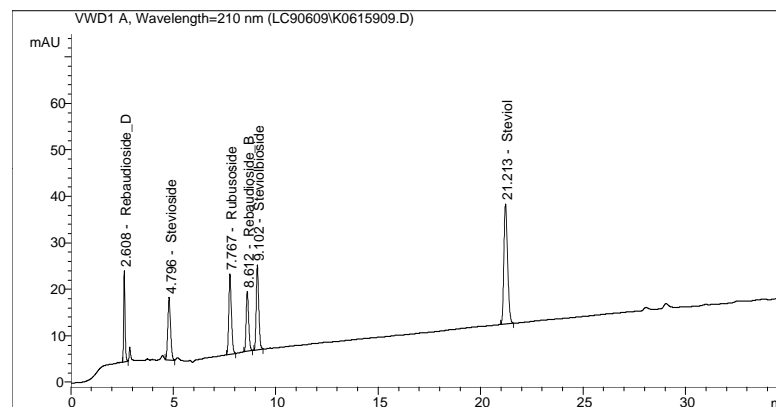
Reb A, 1 mg/mL  
50: 50 Acetonitrile: Water

Reb A Impurities Mix, 100 ug/mL  
50: 50 Acetonitrile: Water

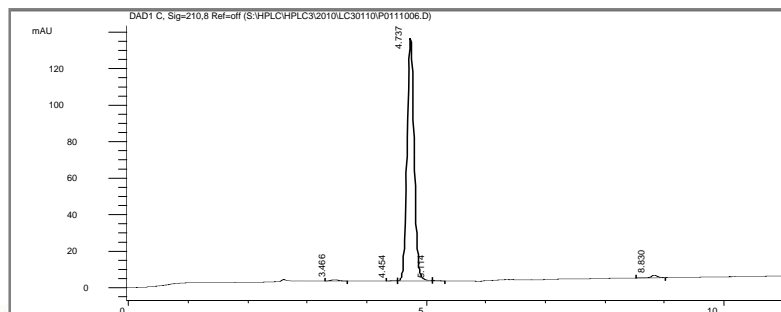
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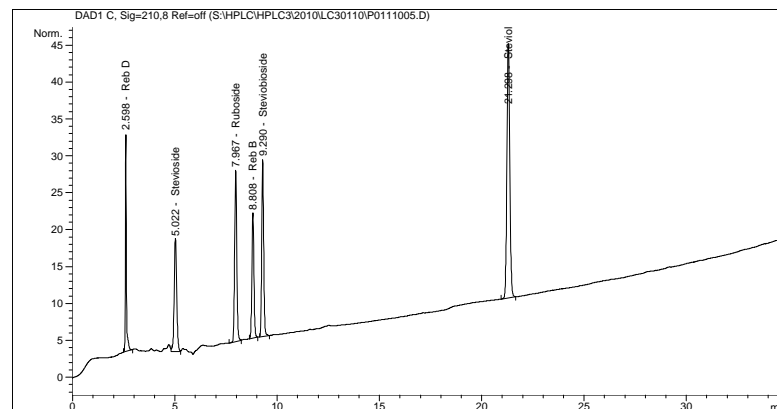
Time 0



6 Months



6 Months



# 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.*

- 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.
- 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.



**Traceability is Provided from Beginning to End**

# Certification and Uncertainty of Reb A & Reb A Impurities Solution Standards

Cerilliant's approach



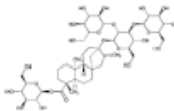
S-016  
FN051809-01  
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## Certificate of Analysis

### Rebaudioside A

*Kmr-16-ns-18-oic acid, 13-[O-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl ester, (4α)-(9C)*

Catalog Number: S-016  
Solution Lot: FN051809-01  
Re-test Date: July 2010  
Solvent: Acetonitrile:Water (1:1)  
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.



- Re-test Date - stability studies ongoing. Certificate of Analysis will be updated upon completion of retest.
- 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
Rebaudioside A	98.4%	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 balance and weighing technique.

• 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)		%RSD - Homogeneity	
		Actual Results	Acceptance Criteria	Actual Results	Acceptance Criteria
New Lot	FN051809-01	1.009	± 3%	0.2	± 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.

#### 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 when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.



*Lara Sparks*  
Lara Sparks, Quality Assurance Director

July 30, 2009  
Date

S-016  
FN051809-01  
Revision 2  
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#### Standard Solution Assay Parameters

Analysis Method: HPLC/UV  
Column: Prodigy Sp, 250 x 4.6 mm  
Mobile Phase: Acetonitrile:0.1% Phosphoric Acid in Water (35:65)  
Flow Rate: 1.0 mL/min  
Wavelength: 210 nm

#### Calibration Curve

Calibration Curve: Linear Regression  
Number of Points: 4  
Linearity (r): 0.999

#### Neat Material Data

Compound Name: Rebaudioside A  
Compound Lot: FN051809-01

Chemical Formula: C<sub>40</sub>H<sub>68</sub>O<sub>23</sub>  
CAS Number: 58543-16-1  
Molecular Weight: 976.01

#### Neat Material Characterization Summary

Analytical Test	Method	Results
Primary Chromatographic Purity by HPLC/PDA Analysis	SP10-0102	98.4%
Secondary Purity by Thin Layer Chromatography	SP10-0106	Single Spot, R <sub>f</sub> = 0.62
Identity by LC/MS Analysis	SP10-0107	Consistent with Structure
Identity by <sup>1</sup> H-NMR Analysis	USP <761>, SP10-0116	Consistent with Structure
Residual Solvent Analysis by GC/FID Headspace	AM1087 <sup>1</sup>	1.19%
Residual Water Analysis by Karl Fischer Coulometry	USP <921>, SP10-0103	5.58%
Inorganic Content by Microassay Analysis	SP10-0135	< 0.1%
Heavy Metals Analysis by ICP/MS	Outsourced	Not Detected for Lead and Arsenic
Specific Rotation	SP10-0133	[α] <sub>D</sub> <sup>25</sup> = -30.2° (c = 0.39, ethanol:water (50:50))
Purity Factor		91.7%

- Primary purity is calculated as the average of two independently performed analyses utilizing two different methods. Acceptance criteria requires the purity values to be within 0.5% of each other.
- The primary chromatographic purity value is used to calculate the Purity Factor.
- A secondary chromatographic purity method is utilized as a control.
- Purity Factor = [(100 - w% residual solvent - w% residual water - w% residual inorganics) x Chromatographic Purity]/100.
- Purity factor does not include adjustment for chiral and/or isotopic purity.

<sup>1</sup> Validated analytical method

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S-017  
FN060409-01  
Revision 0  
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## Certificate of Analysis

Certified Quality  
ISO 9001:2008  
ISO 17025  
ISO 9001:2000

### Rebaudioside A Impurities Mix-6

Catalog Number: S-017  
Solution Lot: FN060409-01  
Retest Date: August 2010  
Solvent: Acetonitrile:Water (1:1)  
Volume per Ampule: Not less than 1 mL  
Storage: Refrigerate or freeze.  
Intended Use: For laboratory use only. Not suitable for human or animal consumption.

- Retest Date - stability studies ongoing. Certificate of Analysis will be updated upon completion of retest.
- Ampoules are overfilled to ensure a minimum 1 mL volume. We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.

Component	Chromatographic Purity	Concentration
Rebaudioside B	96%	100.1 ± 0.6 µg/mL
Rebaudioside D	92%	100.1 ± 0.6 µg/mL
Steviol	99%	100.0 ± 0.6 µg/mL
Stevioside	92%	100.1 ± 0.6 µg/mL
Steviolbioside	92%	100.1 ± 0.6 µg/mL
Rubusoside	98%	100.1 ± 0.6 µg/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 balance and weighing technique.

• Concentration is corrected for chromatographic purity, residual water, residual solvents and residual inorganics.

#### 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 when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.



*Lara Sparks*  
Lara Sparks, Quality Assurance Director

July 1, 2009  
Date

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FN060409-01  
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#### Standard Solution Assay Parameters

Analysis Method: HPLC/UV  
Column: Prodigy ODS 3, 4.6 x 250 mm  
Mobile Phase: Acetonitrile:0.1% H<sub>3</sub>PO<sub>4</sub> in water (35:65)  
Flow Rate: 1.0 mL/min  
Wavelength: 210 nm

#### Calibration Curve

Calibration Curve: Linear Regression  
Number of Points: 4

#### Solution Standard Verification and Homogeneity

Compound	Verified Concentration		%RSD - Homogeneity	
	µg/mL	Acceptance Criteria	%	Acceptance Criteria
Rebaudioside B	99.9	± 5%	0.1	≤ 3%
Rebaudioside D	99.1	± 5%	0.2	≤ 3%
Steviol	101.9	± 5%	0.2	≤ 3%
Stevioside	99.5	± 5%	0.3	≤ 3%
Steviolbioside	100.9	± 5%	0.2	≤ 3%
Rubusoside	100.6	± 5%	0.2	≤ 3%

- Concentration is verified through multiple analyses and is calculated as the average of multiple analyses compared to an independently prepared calibration curve.
- Homogeneity 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.

#### Net Material Data

Compound	Lot Number	Chemical Formula	Molecular Weight	Chromatographic Purity	Residual Solvent	Residual Water	Residual Inorganics	Purity Factor
Rebaudioside B	PN021209-02	C <sub>44</sub> H <sub>80</sub> O <sub>18</sub>	804.87	86.11%	0.06%	4.17%	<0.1%	82.47%
Rebaudioside D	PN021209-03	C <sub>40</sub> H <sub>68</sub> O <sub>18</sub>	1129.15	91.51%	0.22%	4.57%	<0.1%	87.12%
Steviol	PN021209-07	C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>	318.45	99.22%	1.74%	1.55%	<0.1%	95.52%
Stevioside	PN021209-06	C <sub>38</sub> H <sub>64</sub> O <sub>14</sub>	804.87	92.33%	0.86%	4.44%	<0.1%	87.44%
Steviolbioside	PN021209-05	C <sub>42</sub> H <sub>72</sub> O <sub>14</sub>	642.73	92.02%	0.45%	9.86%	<0.1%	82.53%
Rubusoside	PN021209-04	C <sub>42</sub> H <sub>72</sub> O <sub>13</sub>	642.73	98.32%	0.42%	3.90%	<0.1%	94.07%

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# A Comparison of Approaches

	Cerilliant Snap-N-Shoot® Certified Solution Standards	Certified Neat Reference Material (solutions from neat materials)
Accuracy & Stability		
Lot to lot consistency / Reproducibility	One lot available for <b>an extended time</b> providing consistency throughout supply chain analysis	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
Stability over time	Years	Weeks-months
Cost Efficiencies		
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 (dilute) and Shoot"	Weigh, dilute, verify

## Problem

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

## Solution

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







**Cerilliant®**  
Analytical Reference Standards



science, smarter.®

Cerilliant Quality

ISO GUIDE 34

ISO/IEC 17025

ISO 9001:2008

GMP/GLP