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Summary Report

Verification Of the Determination of Rebaudioside M (Bestevia) by High Performance Liquid Chromatography (HPLC) and Purity Analysis of Five Production Samples

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Approved by: _____

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I. Study Identification

1. Study Title:

Method Verification of the Determination of Rebaudioside M by High Performance Liquid Chromatography (HPLC), and Purity Analysis of Six Production Samples

2. Study Objective:

The objective of this study is to verify the assay for rebaudioside M in the Blue California supplied Bestevia powder with JECFA 2010 Rebaudioside A and related Steviol Glycosides method (modified).

3. Study Coordinator/Performing Laboratory:
Eurofins Scientific, Inc.

4. Study Monitors:

Blue California.

5. Method References:

Steviol glycosides, Prepared at the 73rd JEFCA (2010) published in FAO JECFA Monographs 10 (2010) superseding specification prepared in the 68th JEFCA (2008), published in FAO JECFA Monographs 5 (2008). An ADI of 0-4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008).

II. Study Description

1. Scope:
This method is applicable to the determination and quantitation of rebaudioside M, in raw materials and *Stevia rebaudiana* plant extracts. Rebaudioside M quantitation is determined using the USP stevioside standard with a molecular weight correction from stevioside to rebaudioside M. This convention is applied to related steviol glycoside materials. This study is referred to in the validation package for JECFA 2010 performed in 2013 for Blue California. Carbosynth rebaudioside M reference material was not found to be suitable for quantitative purposes.

2. Test Materials:
Stevia rebaudiana Leaf extracts

(1) Eurofins sample **740-2015-00020004**, Bestevia Reb M 95%, Powder, Lot #M195-151128, for method verification

(2) Eurofins sample **740-2015-00020005**, Bestevia Reb M 95%, Powder, Lot #M195-151127

(3) Eurofins sample **740-2015-00020006**, Bestevia Reb M 95%, Powder, Lot #M195-151165

(4) Eurofins sample **740-2015-00020007**, Bestevia Reb M 95%, Powder, Lot #20151123-D4
(5) Eurofins sample # **740-2015-00020008**, Bestevia Reb M 95%, Powder, Lot #20151115-C3

3. Test Reagents:

(1) Acetonitrile, HPLC Grade
Fisher P/N A998-4, VWR P/N JT9017-3

(2) Stevioside USP reference material, LOT F01080 C.A.S # 57817-89-1

(3) Rebaudioside A, ChromaDex, Lot # 00018226=5955 (96.2%). C.A.S number 58543-16-1

(5) Rebaudioside M, Carbosynth, Batch # OR448851401 (99%) C.A.S number 1220616-44-3
Carbosynth rebaudioside M reference material was found to not be suitable for quantitative purposes.

(6) Phosphoric Acid, Fischer Chemical Company P/N A260

4. Mobile Phase Preparation:

A. 80% HPLC grade acetonitrile: 20% Milli-Q water (pH adjusted to 3.0 with phosphoric acid) filtered through 0.5 µm filter (V/V).

5. Reference Standards:

Separate Standards (stevioside and rebaudioside M)

A. Stock standards.
1. Adjust standard concentration for purity and moisture levels (Carbosynth, USP, ChromaDex). Corrections are made based on suppliers C of A.

2. On a microbalance, accurately weigh 1 ± 0.4 mg of Carbosynth rebaudioside C reference material; qualitatively transfer to a 1-ml volumetric flask with mobile phase. Accurately weigh 5 ± 1 mg of

stevioside USP reference material standard and 5 ± 1 mg of rebaudioside A ChromaDex standard; quantitatively transfer to a 5-ml volumetric flask with mobile phase.
Dissolve using heat if necessary. Cool to room temperature and dilute to volume with mobile phase. Concentration is approximately 1 mg/ml rebaudioside M, stevioside and rebaudioside A. Adjust concentrations for vendor purity.

B. Calibration standards (Carbosynth rebaudioside C, ChromaDex rebaudioside A, USP stevioside (individual standards were used for this portion of the study). The range of quantitation will roughly be between 0.2 mg/ml and 1 mg/ml. A 3 point curve is utilized initially for determination of linearity for this study as well as routine quantitation that covers the range of sample concentrations defined by the method for future samples. Since this is a purity determination of the rebaudioside M material, a single point calibration of 5 replicate injections is used for purity determinations at a concentration of approximately 0.8 mg/ml. The 3 sample test concentrations will also be prepared at approximately 0.8 mg/ml, based on the expected test sample concentration of 95% purity dry weight basis or better.

C. Accuracy standard is determined by testing the pre-described control sample of known value used routinely for the JECFA 2010 method previously validated. The control sample contains small quantities of most steviol glycosides with rebaudioside A being the prominent steviol glycoside present. Since this study has determined that the JECFA 2010 method is capable of separating and quantitating rebaudioside M from other related steviol glycosides, the use the current accuracy check is accepted. Accuracy check results are reported as a percentage with 2 standard deviations (STD) Being valid/valid. Results and limits for the control sample follow

Result %(w/w)	2 STD acceptance Criteria	PASS/fail
96.616	94.8-103.0	PASS

D. System suitability standards, retention time confirmation rebaudioside A ChromaDex, Carbosynth rebaudioside M and USP Stevioside for system suitability were utilized.

6. Single Lab Verification Study Results:

A. Primary method: See provided method.

C. Linearity:

1. A three point calibration curve for both rebaudioside C, stevioside and rebaudioside A were developed. The stock standard was then injected at 5ul, stock standard, 2.5 ul, midpoint standard and 1 ul, low standard. The 3 point calibration curve for validation with relative concentrations for **rebaudioside M** as follows (adjusted for standard purity and moisture):

Stock Injection (uls)	Relative Concentration (ug/ml)
5	0.80100
2.5	0.40090
1	0.16036

Linearity Results Rebaudioside M:

Correlation Coefficient	**Specification**	**Result**
0.99991	**>/= 0.999**	**PASS**

Concentrations for **rebaudioside A** are as follows (adjusted for standard purity and moisture):

Stock (uls)	Concentration (mg/ml)
5	0.197500
2.5	0.493790
1	0.987490

Linearity Results Rebaudioside A:

Correlation Coefficient	**Specification**	**Result**
0.99999	**>/= 0.999**	**PASS**

Concentrations for **stevioside** are as follows (adjusted for standard purity and moisture):

Stock (mls)	Concentration (mg/ml)
1	1.11760
2	0.503820
1	0.201530

Linearity Results Stevioside:

Correlation Coefficient	**Specification**	**Result**
1.0000	**>/= 0.999**	**PASS**

Concentrations for **stevioside** single point calibration for purity determination are as follows (adjusted for standard purity and moisture):

Stock (uls)	Area Counts	Concentration (mg/ml)
5	1386.47	1.0076
5	1407.67	1.0076
5	1400.04	1.0076
5	1395.14	1.0076
5	1392.99	1.0076

Results Stevioside:

RSD	**Specification**	**Result**
0.568349	**</= 2.0**	**PASS**

a. The relative standard deviation (RSD) for the response factor (amount/area) mg/mL/mAU) was determined for the 1 mg/ml calibration level. The RSD expressed as a percent is to achieve a specification of </=2%. The %RSDs achieved for this calibration level was acceptable at 0.5683 for stevioside.
b. Likewise, correlation coefficients for both compounds met the criteria.

D. Selectivity: For purposes of this study, selectivity is specificity

1. Perform selectivity procedures:
   a. Analyze an acetonitrile blank.
   b. Analyze positive control sample and rebaudioside M, rebaudioside A and stevioside reference materials.

2. Results:
   a. Three blanks were tested throughout the duration of the study. Each chromatogram was free of interfering peaks while no additional peaks were present in the blank chromatograms.

   b. The positive control sample detected compounds of interest within the positive control with the exception of rebaudioside M. The internal positive control (11-1056) also serves as a confirmation of identification most components and shows that none of these components interfere with rebaudioside M. The closest eluting component is rebaudioside D with a retention time of approximately 3.237 minutes. Rebaudioside M has an approximate retention time of 3.7 minutes showing complete separation between these compounds.

   c. Positive control standard exhibits complete separation between the major steviol glycosides; stevioside and rebaudioside A and from the target compound, rebaudioside M. Additionally there was complete separation from all other minor glycosides as defined in the previous validation for rebaudioside A. Reference materials were also used to indicate

the retention times of the, rebaudioside M, stevioside and rebaudioside A and serve as identification of these components by retention time.

E. System Suitability:

1. Minimum of three injections of an approximately 1.0 mg/ml standard solution were injected during the analysis sequence for rebaudioside M was well as stevioside and rebaudioside A.

2. Acceptance criteria: The system is considered suitable if the retention times of the standard peaks do not deviate more than 1 minute during an analytical run and the RSD of the peak retention times are less than 2%. Results follow:

	Day 1
Retention time (Rt) Range (minutes)	3.687-3.740
Rt % RSD	0.72
Rebaudioside M Peak Area RSD	0.76
Number of Data Points	3

	Day 1
Retention time (Rt) Range (minutes)	7.3810-7.4017
Rt % RSD	0.12
Stevioside Peak Area RSD	0.57
Number of Data Points	5

	Day 1
Retention time (Rt) Range (minutes)	6.891-6.965
Rt % RSD	0.044
Rebaudioside A Peak Area RSD	0.14
Number of Data Points	5

Rebaudioside M, rebaudioside A and stevioside, retention time ranges meet the criteria for deviation of less than 1 minute, passing the criteria.
Rebaudioside M, rebaudioside A and stevioside A, retention time % RSD pass the criteria of less than 2%.
Rebaudioside M, rebaudioside A and stevioside Peak Area RSDs, are less than 2 percent passing the criteria.

3. An Extended Performance report was generated using Agilent Chem Station software to include resolution, tailing and theoretical plate counts, for rebaudioside M (Reb M). Results are as follows;

USP Resolution Reb M = 1.0625
USP Tailing Reb M = 1.08904
USP Plate Count Tangent Method, 8294

4. The retention time and identity for Rebaudioside M was confirmed using the Carbosynth rebaudioside A standard.

F. Accuracy:

Accuracy was determined by applying the analytical procedure to an analyte of known purity. For this purpose the internal control sample, that has had accuracy confirmed for validated JECFA 2010 methodology.

G. Repeatability (precision):

1. For the sample, perform 3 sample preparations. Rebaudioside M was prepared at 0.8 mg/ml. This concentration is based on a limited amount of available rebaudioside M reference standard. As a consideration of that issue samples for purity analysis were also prepared at approximately 0.8 mg/ml. % RSD for precision measurements shall be less than 2.

Sample Description/ Eurofins Sample Number	Approximate Amount (mg)	Final Volume	Approximate Concentration (mg/mL)	Reb M Result (% w/w) Average	Reb M % RSD (N=3)
Bestevia Reb M 95%, Powder, Lot #M195-151128 740-2015-00020004	32	40	0.8	98.5	0.585
Bestevia Reb M 95%, Powder, Lot #M195-151127 740-2015-00020005	32	40	0.8	97.9	0.392
Bestevia Reb M 95%, Powder, Lot #M195-151165 740-2015-00020006	32	40	0.8	97.8	0.317
Bestevia Reb M 95%, Powder, Lot #20151123-D4 740-2015-00020007	32	40	0.8	98.8	1.17
Bestevia Reb M 95%, Powder, Lot #20151115-C3 740-2015-00020008	32	40	0.8	98.7	0.766

Repeatability results:

All results meet acceptance criteria.

7. Purity Analysis of Five Bestevia Production Samples:

A. Five samples were analyzed for purity. Each sample was tested for rebaudioside M. Initially quantitation was scheduled to be determined against a Carbosynth rebaudioside M reference material standard. When purchasing the material only 5 mgs were available at the time of the study. Upon purchase of this standard and its use, the lab found that the material was delivered in an oversize vial with rebaudioside M material sticking to the sides of the vial. Enough material (0.844 mg) was available from the vial for analysis. As mentioned above this is the reason for setting the high standard concentration at approximately 0.8 mg/ml as well as the sample concentrations for the purity analysis.

Upon analysis of the standards using rebaudioside M reference material, percent purities well in excess of 100 percent were detected. Since this is not a possible outcome for a material that is either at 95 % pure or 98% pure, as these samples are quoted at and tested at, a different mode of quantitation was explored. The lab feels that the small amount of reference material likely picked up water when place in the oversize vial, skewing the purity results by picking up water. This effect on a reference standard has the result of overestimating purity values.

The convention in JECFA 2010 is to quantitate all steviol glycosides (with the exception of the rebaudioside A) as stevioside, using a correction for the molecular weight to stevioside. When this was investigated all samples tested at the 98 percent level. The molecular weight correction factor from stevioside to rebaudioside M is **1.6043**.

To confirm the weight percent quantitative rebaudioside M results, the more qualitative area percent results were quantitated for each sample run. Area percent results are calculated as the % area for the peak of interest on the chromatogram as compared to all other peaks on the chromatogram that are not in the blank and are not the peak of interest. On a pure sample matrix of this type it was hypothesized that the results of the area percent will closely (with-in 2 %) confirm the weight percent results. Results for the area percent calculation for rebaudioside M did confirm the weight percent as can be seen below.

The results for the five samples are reported in the table below. Each sample was tested 3 times. Average results and % relative standard deviation (% RSD) are also reported for each sample. An additional column for area percent results was also added with these results reported in red.

Sample 20004	Run 1	Run 2	Run 3		
Category	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside M	97.88	98.97	98.75	98.53	0.585
Amount Weighted	32.11	32.10	32.11	na	na
Concentration in Solution	0.803	0.802	0.803	na	na
Rebaudioside M Area Percent Purity	98.07	98.06	98.19	na	na
Sample 20005	Run 1	Run 2	Run 3		
Category	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside M	98.35	97.74	97.64	97.91	0.392
Amount Weighted	32.12	34.22	31.66	na	na
Concentration in Solution	0.803	0.856	0.792	na	na
Rebaudioside M Area Percent Purity	98.08	97.94	97.93	na	na
Sample 20006	Run 1	Run 2	Run 3		
Category	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside M	98.04	97.43	97.83	97.77	0.317
Amount Weighted	30.94	31.60	32.10	na	na
Concentration in Solution	0.774	0.790	0.802	na	na
Rebaudioside M Area Percent Purity	98.00	97.9	98.06	na	na
Sample 20007	Run 1	Run 2	Run 3		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside M	99.79	99.02	97.51	98.77	1.17
Amount Weighted	31.78	32.32	32.04	na	na
Concentration in Solution	0.794	0.808	0.801	na	na
Rebaudioside M Area Percent Purity	97.9	97.98	98.05	na	na

Sample 20008	Run 1	Run 2	Run 3		
Compound	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Result (%w/w) moisture corrected	Average	Relative Standard Deviation
Rebaudioside M	99.31	99.02	97.88	98.74	0.765
Amount Weighted	32.70	32.46	32.72	na	na
Concentration in Solution	0.818	0.812	0.818	na	na
Rebaudioside M Area Percent Purity	97.65	97.58	98.08	na	na

na, Not Applicable

9. Moisture Correction for Rebaudioside M:
All of the results in the above table have been adjusted for the moisture correction and reported on the dry weight basis.

The equation for moisture correction is as follows;
Rebaudioside A dry weight basis = rebaudioside A result as is / (100- % moisture / 100).

Results for the measured percent moisture using Karl Fischer titration are listed here;

Sample #	Measured Moisture (%)
20004	6.352
20005	5.366
20006	5.636
20007	5.000
20008	5.219

8. Conclusions:

The results generated meet and exceed the acceptance criteria as established for the study. All analyses were performed on Agilent 1200 series HPLC with Agilent Open Lab Chem Station software. The primary objective of the study has been to show that the method as designed can accurately determine the concentration of rebaudioside M in "Bestevia". The results show that the method is precise and accurate and can accurately determine the concentration of rebaudioside M.

Quantitation of rebaudioside M was accomplished using relative response factors to the USP stevioside reference material as described in the method and in JECFA 2010 for other related glycosides. It was found that at the time of the study an accurately presented

reference standard for rebaudioside M was not available most likely due to water levels. Regardless the rebaudioside M reference material is still useful for retention time determination and identification purposes.

Limit of detection and limit of quantitation were beyond the scope of this project due to the concentrated nature of the samples. However quantitation of the impurities can be performed at the low levels that are found in these samples. The ICH visual inspection method (ICH Validation of Analytical Procedures: Methodology, section 6.1) for determining limit of detection and limit of quantitation was utilized. Limit of detection and limit of quantitation for these compounds are roughly estimated at 0.1% and 0.5 percent respectively. In the future additional work can be performed to statistically determine these limits if requested.

Five lots of "Bestevia" were tested by this method. The results show that the method can accurately determine the concentration of rebaudioside M in this material while separating rebaudioside M from the other 2 major steviol glycosides and all minor glycosides. The results have shown accurate and precise determination and identification of rebaudioside M.