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November 15, 2018

#823

Food and Drug Administration  
Center for Food Safety & Applied Nutrition  
Office of Food Additive Safety (HFS-255)  
5001 Campus Drive  
College Park, MD 20740-3835

Attention: Dr. Paulette Gaynor  
Re: GRAS Notification—Rebaudioside E

Dear Dr. Gaynor:

GRAS Associates, LLC, acting as the Agent for Blue California is submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS notification for *Rebaudioside E*. Along with Blue California's determination of safety, an Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into food in general, other than infant formulas and meat and poultry products. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)



William J. Rowe  
President  
Agent for Blue California  
GRAS Associates, LLC  
27499 Riverview Center Blvd., Suite 212  
Bonita Springs, FL 34134  
[wrowe@nutrasource.ca](mailto:wrowe@nutrasource.ca)  
Enclosure: GRAS Notification for Blue California – *Rebaudioside E*



**FDA USE ONLY**

DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Food and Drug Administration

**GENERALLY RECOGNIZED AS SAFE (GRAS) NOTICE** (Subpart E of Part 170)

GRN NUMBER  
000 823

DATE OF RECEIPT  
**RECEIVED**  
INTENDED USE FOR INTERNET  
NOV 27 2018  
OFFICE JF  
FOOD ADDITIVE SAFETY

ESTIMATED DAILY INTAKE

NAME FOR INTERNET

KEYWORDS

Transmit completed form and attachments electronically via the Electronic Submission Gateway (see Instructions); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

**SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION**

1. Type of Submission (Check one)

New       Amendment to GRN No. \_\_\_\_\_       Supplement to GRN No. \_\_\_\_\_

2.  All electronic files included in this submission have been checked and found to be virus free. (Check box to verify)

3. Most recent presubmission meeting (if any) with FDA on the subject substance (yyyy/mm/dd): N/A

**SECTION B – INFORMATION ABOUT THE NOTIFIER**

<b>1a. Notifier</b>	Name of Contact Person Hadi Omrani	Position or Title Technical Director - Regulatory Affairs
	Organization (if applicable) Blue California	
	Mailing Address (number and street) 30111 Tomas	

City Rancho Santa Margarita	State or Province California	Zip Code/Postal Code 92688	Country United States of America
Telephone Number 949-635-1991 X29	Fax Number 949-635-1984	E-Mail Address hadi@bluecal-ingredients.com	

<b>1b. Agent or Attorney (if applicable)</b>	Name of Contact Person William J. Rowe	Position or Title President
	Organization (if applicable) GRAS Associates	
	Mailing Address (number and street) 27499 Riverview Center Blvd., Suite 212	

City Bonita Springs	State or Province Florida	Zip Code/Postal Code 34134	Country United States of America
Telephone Number 239-444-1724	Fax Number 239-444-1723	E-Mail Address wrowe@nutrasource.ca	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

BESTEVIATM Rebaudioside E (Rebaudioside E; Reb E)

2. Submission Format: (Check appropriate box(es))

Electronic Submission Gateway  Electronic files on physical media

Paper  
If applicable give number and type of physical media

4. Does this submission incorporate any information in CFSAN's files? (Check one)

Yes (Proceed to Item 5)  No (Proceed to Item 6)

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- 

6. Statutory basis for conclusions of GRAS status (Check one)

Scientific procedures (21 CFR 170.30(a) and (b))  Experience based on common use in food (21 CFR 170.30(a) and (c))

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

Yes (Proceed to Item 8)  
 No (Proceed to Section D)

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SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Intended to be used as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into foods in general, other than infant formulas and meat and poultry products, at per serving levels reflecting good manufacturing practices and principles, in that the quantity added to foods should not exceed the amount reasonably required to accomplish its intended technical effect.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

Yes  No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

Yes  No. you ask us to exclude trade secrets from the information FDA will send to FSIS.

**SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE**

*(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)*

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes     No

Did you include this other information in the list of attachments?

Yes     No

**SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS**

1. The undersigned is informing FDA that Blue California  
*(name of notifier)*

has concluded that the intended use(s) of BESTEVIATM Rebaudioside E (Rebaudioside E; Reb E)  
*(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Blue California *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

30111 Tomas, Rancho Santa Margarita, CA 92688  
*(address of notifier or other location)*

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official,	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6)	Katrina Emmel on behalf of William J. Rowe, President	11/15/2018

**SECTION G – LIST OF ATTACHMENTS**

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Multiple Appendices -- Appendices 1 through 15	

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, . (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.



**GRAS Notification**

of

**Rebaudioside E**

**Food Usage Conditions for General Recognition of Safety**

on behalf of

**Blue California**

**30111 Tomas  
Rancho Santa Margarita, CA 92688**

11/15/18

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## FOREWORD

Blue California based our Generally Recognized as Safe (GRAS) assessment primarily on the composite safety information on steviol glycosides, i.e., scientific procedures with corroboration from history of use. The safety/toxicity of steviol glycosides, history of use of steviol glycosides, and compositional details, specifications, and method of preparation of the subject ingredient were reviewed. In addition, a search of the scientific and regulatory literature was conducted through September 24, 2018, with particular attention paid to adverse reports, as well as those that supported conclusions of safety. Those references that were deemed pertinent to this review are listed in Part 7. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

At Blue California's request, GRAS Associates, LLC ("GA") convened an Expert Panel to complete an independent safety evaluation of Blue California's high purity rebaudioside E ( $\geq 85\%$  Reb E) product. Blue California's high purity rebaudioside E preparation is synthesized from *Stevia rebaudiana* extract by a genetically-modified yeast that is purified to yield a  $\geq 95\%$  total steviol glycosides product with the principle component of Reb E ( $\geq 85\%$ ). The purpose of the evaluation is to ascertain whether Blue California's conclusion that the intended food uses of rebaudioside E as described in Part 3 are generally recognized as safe, i.e., GRAS, under the intended conditions of use. In addition, Blue California has asked GRAS Associates to act as Agent for the submission of this GRAS notification.

## PART 1. SIGNED STATEMENTS AND CERTIFICATION

### A. Basis of Exclusion from the Requirement for Premarket Approval Pursuant to Subpart E of 170<sup>1</sup>

Blue California has concluded that our high purity rebaudioside E, referred to as "Reb E" and "BESTEVIA® Rebaudioside E," and which meet the specifications described below, is GRAS in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). This determination was made in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the intended conditions of food use for the designated high purity rebaudioside E ( $\geq 95\%$  total steviol glycosides primarily composed of Reb E) preparation.

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<sup>1</sup> See 81 FR 54960, 17 August 2016. Accessible at: <https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf> (Accessed 9/4/18).

Signed:

(b) (6)



Agent for Blue California

William J. Rowe  
President  
GRAS Associates, LLC  
27499 Riverview Center Blvd.  
Suite 212  
Bonita Springs, FL 34134

Date: 11/15/18

## **B. Name and Address of Responsible Parties**

Blue California  
30111 Tomas  
Rancho Santa Margarita, CA 92688

As the Responsible Party, Blue California accepts responsibility for the GRAS conclusion that has been made for our high purity rebaudioside E ( $\geq 95\%$  total steviol glycosides primarily composed of Reb E) preparation, BESTEVIA® Rebaudioside E, as described in the subject safety evaluation; consequently, the purified steviol glycosides preparations having acceptable steviol glycosides compositions which meet the conditions described herein, are not subject to premarket approval requirements for food ingredients.

## **C. Common Name and Identity of Notified Substance**

The common name of the ingredient to be used on food labels is high purity rebaudioside E, which can be also be abbreviated as Reb E or reb E. Blue California also plans to market our high purity rebaudioside E preparations under the trade name BESTEVIA® Rebaudioside E.

## **D. Conditions of Intended Use in Food**

Blue California's BESTEVIA® Rebaudioside E ( $\geq 95\%$  total steviol glycosides primarily composed of Reb E) preparation is intended for use as a general-purpose sweetener in foods, excluding meat and poultry products and infant formulas, at levels determined by current good manufacturing practices (CGMPs).

## **E. Basis for GRAS Conclusion**

Pursuant to 21 CFR 170.30(a) and (b), Blue California's BESTEVIA® Rebaudioside E ( $\geq 95\%$  total steviol glycosides primarily composed of Reb E) has been concluded to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

Purified steviol glycosides are not subject to premarket approval requirements of the FD&C Act based on Blue California's conclusion that the substance is GRAS under the conditions of its intended food use.

Blue California certifies, to the best of our knowledge, that this GRAS notice is a complete, representative, and balanced assessment that includes all relevant information, both favorable and unfavorable, available and pertinent to the evaluation of safety and GRAS status of purified steviol glycosides.

## **F. Availability of Information**

The data and information that serve as the basis for this GRAS Notice will be maintained at the offices of Blue California, located at 30111 Tomas, Rancho Santa Margarita, CA 92688, and will be made available during customary business hours.

Blue California certifies that no data or information contained herein are exempt from disclosure under the Freedom of Information Act (FOIA). No non-public, safety-related data were used by the Expert Panel to reach a GRAS conclusion.

## **PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT**

### **A. Chemical Identity of Ingredient**

"Rebaudioside E" is the common or usual name of the non-nutritive sweetener derived from an extract of *Stevia rebaudiana* Bertoni by genetically-modified yeast. The compositional features of the BESTEVIA® Rebaudioside E are described in more detail in this section. Rebaudioside E and Reb E are the terms used by Blue California in referring to the notified substance. The preparation is also marketed as BESTEVIA® Rebaudioside E.

The general chemistry of steviol glycosides and enzyme modified steviol glycosides has previously been reviewed in a number of GRAS Notifications, including those previously submitted by Blue California, specifically GRN 667 (Blue California, 2016) and GRN 715 (Blue California, 2017).

No known toxins have been identified in stevia or stevia-derived products.

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## 1. Chemistry of Rebaudioside E

Rebaudioside E is a minor, naturally occurring steviol glycoside obtained from the leaves of *Stevia rebaudiana* Bertoni; it is reported to be 150-200 times sweeter than sugar (Prakash, 2013). Similar to the other steviol glycosides, Reb E is an *ent*-kaurane diterpene glycoside with a steviol backbone.

**Chemical name:** 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester

**Synonyms:** Rebaudioside E, Reb E

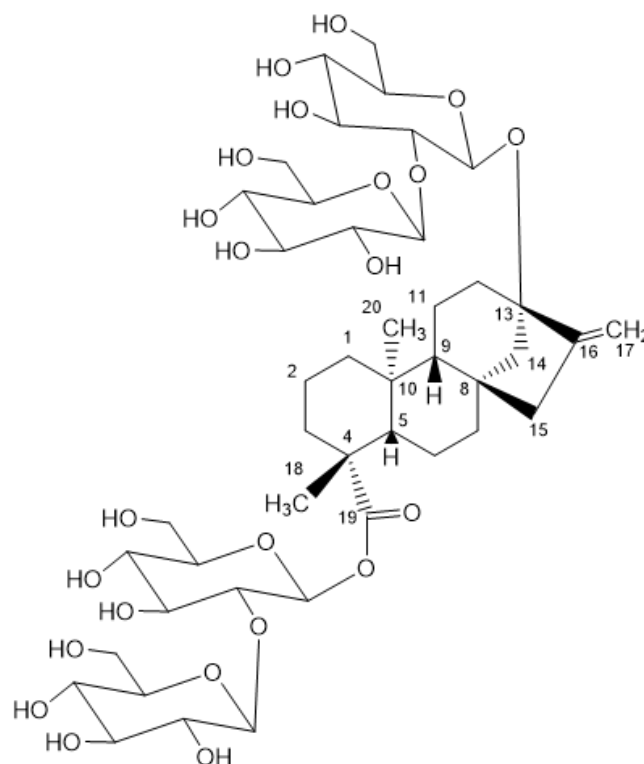
**Chemical formula:** C<sub>44</sub>H<sub>70</sub>O<sub>23</sub>

**Molecular weight (MW):** 967.02 daltons

**CAS Number:** 63279-14-1

The chemical structure of rebaudioside E is presented in Figure 1.

**Figure 1. Chemical Structure of Rebaudioside E**



**Reb E**

## 2. Chemistry of the Yeast Vector

Blue California's manufacturing process for its high purity Reb E preparation uses a set of enzymes to carry out catalytic bio-conversion in a process similar to that previously described in GRN 715 for the production of Rebaudioside D (Blue California, 2017). The enzymes are produced by a nonpathogenic and nontoxic strain of wild-type *Pichia pastoris* from the Saccharomycetaceae family. This strain was originally isolated from harvested plant material, cultured, and studied extensively by other groups, and it has a history of use in food production. It is commonly found in a variety of food products, including cheese and wine.

The parental strain used by Blue California is closely related to *P. pastoris* ATCC 20864. It was converted to production strains by site-specific DNA integration.

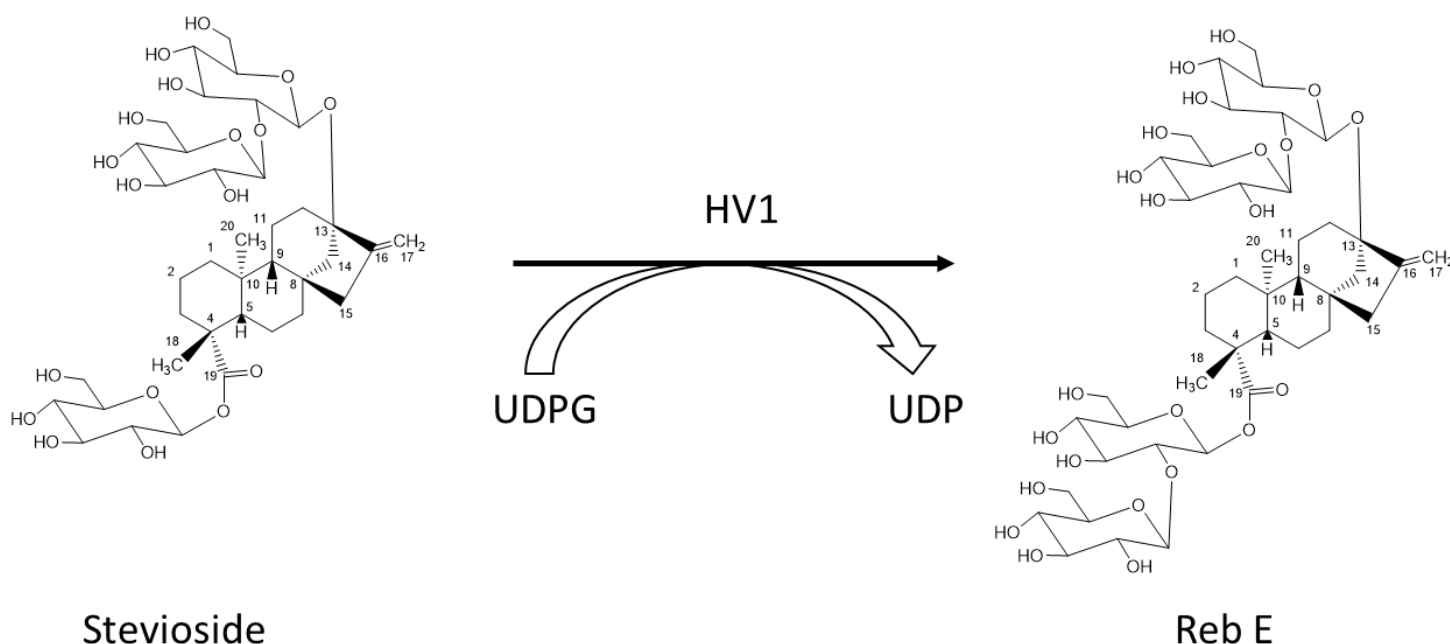
The enzymes are produced by a microorganism that is a unicellular yeast that is widely used in the biotechnology industry. It can be commonly found in nature, and it can grow in a simple, inexpensive medium. Its morphology, physiology, and growth conditions have been widely studied and reported. The detailed transformation protocol and plasmid information have been reported in Blue California's published patents, which are listed in Appendix 1.

UGT-A is a fusion enzyme including FLAG tag, HV1-SUS fusion enzyme, and GCw61 anchor. HV1 is a member of the 5'-diphosphouridine-glucosyltransferase (UGT) family, which was identified from *Hordeum vulgare subsp. Vulgare*. HV1 produces Reb E from stevioside via 1,2-19-O-glucose glycosylation, where a sugar moiety is transferred from uridine diphosphate-glucose (UDPG) to the C-2' of the 19-O-glucose moiety of stevioside (Figure 2).

Sucrose synthases (SUS) catalyze the conversion of uridine diphosphate (UDP) to UDPG in the presence of sucrose. Thus, for a glycosylation reaction catalyzed by UGT enzymes, SUS can be used to regenerate UDPG, thereby enhancing the efficiency of such a reaction.

For yeast-strain A, Blue California transformed the UGT-A into yeast cells.

**Figure 2. Biosynthesis Pathway from Stevioside to Reb E**



## B. Manufacturing Process

Blue California manufactures high purity rebaudioside E in a process similar to that which was described for rebaudioside M in GRN 667 (Blue California, 2016) and rebaudioside D in GRN 715 (Blue California, 2017). The multi-step biosynthesis pathway process to manufacture BESTEVIA Rebaudioside E uses a strain of *Pichia pastoris* yeast that contains uridine 5'-diphosphogucuronosyltransferase (UGT) enzymes that facilitate the transfer of glucose to small molecules via glycosidic bonds.

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## 1. Catalytic Bioconversion Process

To produce the enzymes used in the bio-conversion, the glycerol stock of Yeast Cell A (carrying the UGT-A gene) is removed from the -70°C freezer, thawed to room temperature, and grown in 50 mL yeast culture seed media. After 12 hours, the growing Seed Culture 1 is transferred to 2-L yeast culture seed media as Seed Culture 2. When the cells<sup>2</sup> read OD<sub>600</sub> = 10, they are transferred to 500-L fermenters. This level 3 Seed Culture is then transferred to a 60-ton production fermenter.

The yeast cells are cultured for 48 hours as described in Blue California's published patent (MAO et al., 2016). After confirming their catalytic activity in a small shaking flask, Yeast Cells A are harvested by centrifugation. The cells are then passed through a homogenizer to loosen the cell surface enzymes. The enzymes are separated by another centrifugation step and are re-suspended in a reaction buffer. For the catalytic reaction needed to convert stevia extract to Reb E, the enzymes are mixed in the reaction buffer in a large 60-ton reaction tank with slow agitation.

Blue California uses a ≥ 95% steviol glycosides starting material, which is derived from *Stevia rebaudiana* leaves. The steviol glycosides are extracted with a 70% ethanol/30% water solution, isolated, and purified through microfiltration. A manufacturing flow chart and product specifications for the ≥ 95% steviol glycosides starting material are provided in Appendix 2.

The stevia extract is fed into the tank containing the enzymes to allow the reaction to proceed. The reaction mixture is then heated to 85°C for 20 minutes to denature the enzymes in the supernatant, which is then removed for down-stream processing.

## 2. Extraction & Purification

The supernatant from the Catalytic Bioconversion Process, described above, is filtered to remove any remaining debris. The supernatant is then loaded onto large columns containing a macro-porous resin. The supernatant flows through the column by gravity and is bound to the resin. The column is then rinsed with a series of buffers. Reb E is eluted with food-grade ethanol a number of times. The eluent is collected and condensed in a wipe-film evaporator. Blue California evaporates out the ethanol, and Reb E remains in aqueous solution.

The condensate is chilled to allow Reb E to crystallize and precipitate from the solution. The wet crystals are collected, washed, and dissolved in ethanol. Blue California uses a recrystallization step to increase the concentration of Reb E, while removing impurities, including other steviol glycosides that exhibit higher solubilities than Reb E. Consequently, the other steviol glycosides (i.e., impurities) will remain in solution while Reb E precipitates out first, allowing Blue California to produce a higher purity Reb E product. Activated charcoal is used to purify the final product by adsorbing the non-steviol glycosides impurities. The resulting Reb E product is re-crystallized, dried, and processed to the final BESTEVIA® Rebaudioside E product.

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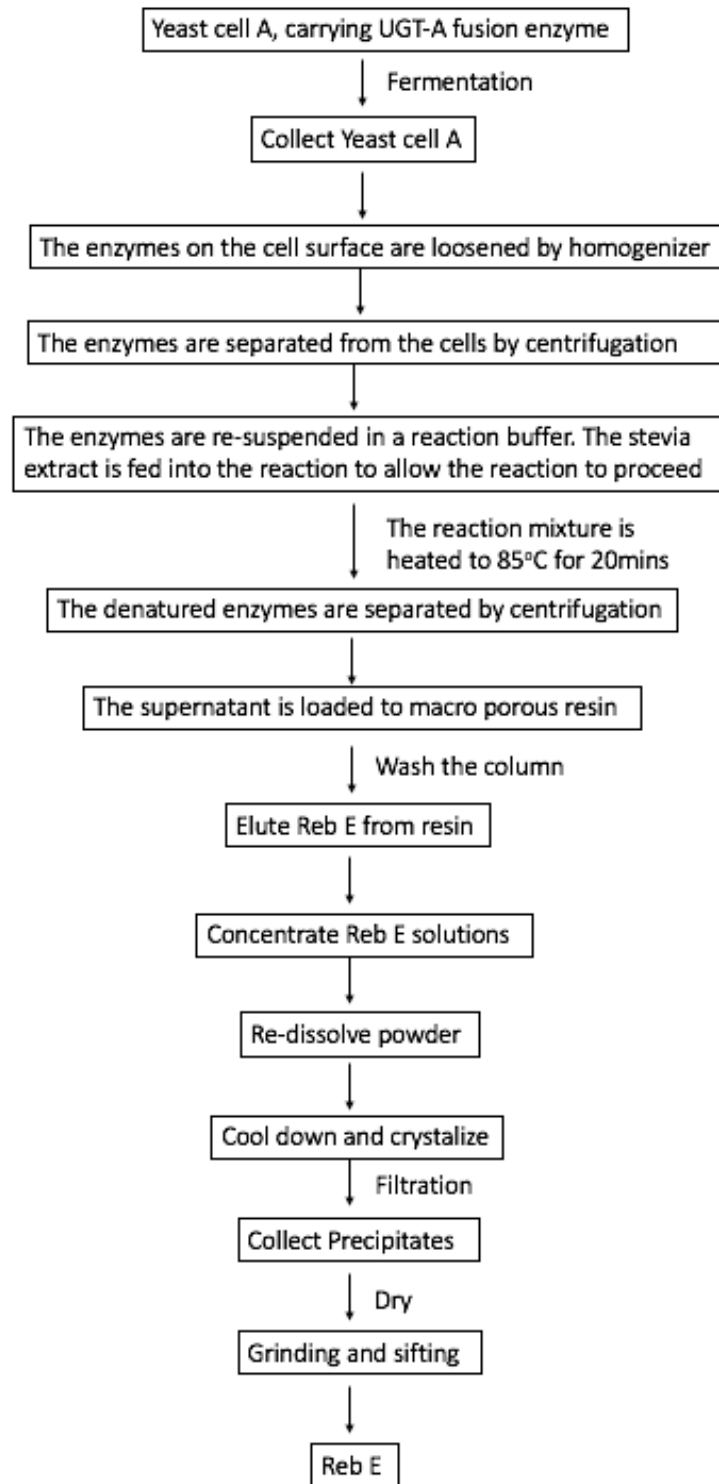
<sup>2</sup> Blue California uses older, larger cells to perform the measurement.

The manufacturing process is summarized in a flow chart provided in Figure 3.

All raw materials, processing aids, and additives used to manufacture Reb E are food-grade ingredients permitted by U.S. regulations or have previously been determined to be GRAS for their respective uses, as detailed in Appendix 3.



**Figure 3. Flow Chart of Manufacturing Process for Blue California’s BESTEVIA® Rebaudioside E**



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## **C. Product Specifications**

### **1. JECFA Specifications for Steviol Glycosides**

The compositions of extracts of *Stevia rebaudiana* Bertoni depend upon the compositions of the harvested leaves, which are, in turn, influenced by soil, climate, and the manufacturing process itself (FAO, 2007b).

The most recent JECFA monograph was published in 2017 (FAO, 2017), in which the steviol glycosides specifications were modified to include a minimum requirement of not less than 95% total steviol glycosides, on a dry basis, “determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni.”

JECFA’s 2017 monograph describes steviol glycosides as white-to-yellow powders that are odorless or have a slight characteristic odor that exhibit a sweetness that is 200-300 times greater than that of sucrose. The ingredient must consist of a minimum of 95% total steviol glycosides, as defined above. The steviol glycosides are freely soluble in a 50:50 mixture of ethanol and water, and the 1 in 100 solutions exhibit pH values between 4.5 and 7.0. The product should not have more than 1% ash with no more than a 6% loss on drying at 105°C for 2 hours. Any residual methanol levels should not exceed 200 mg per kg, and ethanol residues should not exceed 5,000 mg per kg. Arsenic and lead levels should not exceed 1 mg per kg. Microbiological criteria have also been established, with specifications of no more than 1,000 colony forming units (CFU) per g total plate count, not more than 200 CFU per g yeasts and molds, and *E. coli* and *Salmonella* negative in 1 g and 25 g, respectively.

### **2. Specifications for Blue California’s Rebaudioside E Preparation and Supporting Methods**

Blue California has adopted product specifications for its BESTEVIA® Rebaudioside E preparation that meet or exceed JECFA recommendations, while also complying with Food Chemicals Codex (FCC, 2010) specifications for rebaudioside A as a consumable human food substance. The compositions of five non-consecutive lots of Blue California’s BESTEVIA® Rebaudioside E are compared to the JECFA and FCC specifications in Table 1.

**Table 1. Specifications for Blue California’s BESTEVIA® Rebaudioside E**

PHYSICAL & CHEMICAL PARAMETERS	JECFA <sup>a</sup> SPECIFICATIONS STEVIOL GLYCOSIDES	FCC <sup>b</sup> SPECIFICATIONS REBAUDIOSIDE A	Blue California's Specifications for BESTEVIA® Rebaudioside E	BESTEVIA® REBAUDIOSIDE E REPRESENTATIVE LOTS				
				Lot 20131005	Lot 160202-1601	Lot 160921-1602	Lot 160921-1603	Lot 20170665-04
Appearance Form	Powder	Crystal, granule or powder	Powder	Pass	Pass	Pass	Pass	Pass
Appearance Color	White to light Yellow	White to off-white	White	Pass	Pass	Pass	Pass	Pass
Solubility <sup>d</sup>	Freely soluble in water: ethanol (50:50)	Freely soluble in water: ethanol (50:50)	Soluble in water (3.5% clear solution in 10 minutes)	Pass	Pass	Pass	Pass	Pass
Purity (HPLC Area)	≥95% Steviol Glycosides	≥ 95% Reb A	≥ 95% Steviol glycosides ≥ 85% Reb E	98.5% 90%	97% 90.5%	98.3% 91.2%	99.5% 86.7%	99.3% 85.4%
Residual Ethanol	NMT 5,000 mg/kg	NMT 0.5%	< 1,000 ppm	< 200 ppm	< 200 ppm	< 200 ppm	< 200 ppm	< 200 ppm
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	< 200 ppm	< 100 ppm	< 100 ppm	< 100 ppm	< 100 ppm	< 100 ppm
Loss on Drying (%)	NMT 6.0%	NMT 6.0%	≤ 5%	1.2%	1.48%	1.12%	1.29%	1.33%
pH, 1% Solution	4.5-7.0	4.5-7.0	4.5-7.0	5.2	5.5	5.2	4.95	5.6
Total Ash (%)	NMT 1%	NMT 1%	≤ 1%	0.12%	0.15%	0.15%	0.25%	0.1%
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Lead	NMT 1 mg/kg	NMT 1 mg/kg	< 0.5 ppm	< 0.05 ppm	< 0.05 ppm	< 0.05 ppm	< 0.05 ppm	< 0.05 ppm
Mercury	NS	NS	< 0.5 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm	< 0.1 ppm
Cadmium	NS	NS	< 0.5 ppm	< 0.25 ppm	< 0.25 ppm	< 0.25 ppm	< 0.25 ppm	< 0.25 ppm
Total Plate Count (cfu/g, max)	NMT 1,000	NS	< 1,000	< 1,000	< 1,000	< 1,000	< 1,000	< 1,000
Yeast & Mold (cfu/g, max)	NMT 200	NS	< 100	< 50	< 50	< 50	< 50	< 50
<i>E. coli</i> (mpn/g)	Negative in 1 g	NS	Negative	ND	ND	ND	ND	ND
<i>Salmonella spp.</i>	Negative in 25 g	NS	Negative	ND	ND	ND	ND	ND
Total coliform (cfu/g)	NS	NS	< 10	< 3	< 3	< 3	< 3	< 3

<sup>a</sup> Prepared at 84<sup>th</sup> JECFA (2017)

<sup>b</sup> Rebaudioside A monograph. Food Chemicals Codex (7th Ed.). (FCC, 2010)

NS = not specified; NA = not applicable; NMT = not more than; ND = not detected

Blue California analyzes its BESTEVIA® Rebaudioside E preparation by high performance liquid chromatography (HPLC), following the method presented in Appendix 4. In addition to the presentation of key specifications found in Table 1 for comparison with generally accepted purity standards, certificates of analysis for five representative lots of BESTEVIA® Rebaudioside E are provided in Appendix 5. The chromatograms for representative BESTEVIA® Rebaudioside E are provided in Appendix 6. Test reports for analysis of pesticide residues in representative lots of BESTEVIA® Rebaudioside E are located in Appendix 7. The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

#### **D. Physical or Technical Effect**

Blue California determined the relative sweetness of BESTEVIA® Rebaudioside E preparation to be 137 X sweeter than sucrose by organoleptic comparison to 1.0%, 3.0%, and 6.0% sucrose solutions, following the method outlined in Appendix 8.

#### **E. Stability**

##### **1. Stability Data on Steviol Glycosides**

The stabilities of steviol glycosides and enzyme modified steviol glycosides have previously been reviewed in a number of GRAS Notifications, including those previously submitted by Blue California, specifically GRN 667 (Blue California, 2016) and GRN 715 (Blue California, 2017).

Steviol glycosides have been reported to be stable over the pH range 3-9 and can be heated at 100°C for 1 hour and maintain stability, but, at pH levels greater than 9, they rapidly decompose (Kinghorn, 2002). In previously submitted GRAS Notifications, GRN 252 (Merisant, 2008), GRN 253 (Cargill, 2008), and GRN 304 (Sunwin/WILD, 2010), reported stability data indicated that Rebaudioside A is stable under the intended conditions of use.

Furthermore, in the over 50 GRAS Notifications that have been submitted to FDA to date on steviol glycosides, the presented stability data have supported the position that steviol glycosides are stable and well-suited for the intended uses in foods.

##### **2. Stability Data for Blue California's BESTEVIA® Rebaudioside E**

Blue California conducted a 6-month stability study of five lots of BESTEVIA® Rebaudioside E. The samples were stored at 40°C ± 2°C at a relative humidity of 75% ± 5%. BESTEVIA® Rebaudioside E was observed to be stable over the course of the accelerated stability study, as demonstrated in Table 2.

**Table 2. BESTEVIA® Rebaudioside E Storage Stability Data**

BESTEVIA® Lot# 160202-1601				
Duration	Appearance	Moisture (%)	Rebaudioside E (HPLC %)	Rebaudioside D (HPLC %)
t=0	Off White to White Powder	3.46	85.75	8.52
1 month	Off White to White Powder	3.41	85.73	8.43
2 months	Off White to White Powder	3.46	85.73	8.50
3 months	Off White to White Powder	3.53	85.72	8.51
4 months	Off White to White Powder	3.48	85.71	8.68
5 months	Off White to White Powder	3.60	85.78	8.50
6 months	Off White to White Powder	3.55	85.75	9.13
Average	Off White to White Powder	3.49	85.73	8.61
BESTEVIA® Lot# 160921-1602				
Duration	Appearance	Moisture (%)	Rebaudioside E (HPLC %)	Rebaudioside D (HPLC %)
t=0	Off White to White Powder	2.78	87.44	7.62
1 month	Off White to White Powder	3.01	87.46	6.63
2 months	Off White to White Powder	2.88	87.45	7.77
3 months	Off White to White Powder	2.93	87.44	7.55
4 months	Off White to White Powder	3.01	87.46	8.84
5 months	Off White to White Powder	3.11	87.44	9.21
6 months	Off White to White Powder	3.02	87.44	8.08
Average	Off White to White Powder	2.96	87.44	7.96
BESTEVIA® Lot# 160921-1603				
Duration	Appearance	Moisture (%)	Rebaudioside E (HPLC %)	Rebaudioside D (HPLC %)
t=0	Off White to White Powder	2.33	86.73	8.46
1 month	Off White to White Powder	2.58	86.72	8.21
2 months	Off White to White Powder	2.62	86.73	6.84
3 months	Off White to White Powder	2.66	86.75	8.30
4 months	Off White to White Powder	2.63	86.75	8.54
5 months	Off White to White Powder	2.71	86.78	7.80
6 months	Off White to White Powder	2.72	86.76	8.03
Average	Off White to White Powder	2.61	86.75	8.03

BESTEVIA® Lot# 20170665-04				
Duration	Appearance	Moisture (%)	Rebaudioside E (HPLC %)	Rebaudioside D (HPLC %)
t=0	White Powder	3.27	87.16	8.28
1 month	White Powder	3.32	87.13	8.79
2 months	White Powder	3.34	87.18	8.13
3 months	White Powder	3.43	87.16	8.62
4 months	White Powder	3.52	87.14	7.70
5 months	White Powder	3.42	87.13	8.79
6 months	White Powder	3.44	87.15	8.16
Average	Off White to White Powder	3.39	87.15	8.35
BESTEVIA® Lot# 20131005				
Duration	Appearance	Moisture (%)	Rebaudioside E (HPLC %)	Rebaudioside D (HPLC %)
t=0	White Powder	3.06	86.65	8.43
1 month	White Powder	3.17	86.63	8.88
2 months	White Powder	2.98	86.61	9.51
3 months	White Powder	3.11	86.63	8.40
4 months	White Powder	3.09	86.57	8.89
5 months	White Powder	3.12	86.61	8.82
6 months	White Powder	3.16	86.63	9.51
Average	Off White to White Powder	3.10	86.62	8.92

The stability data in the scientific literature for stevioside, the JECFA report, and the extensive stability testing for the structurally similar rebaudioside A as presented by Merisant, Cargill, and Sunwin & WILD Flavors, along with Blue California’s stability testing results, support the position that Blue California’s BESTEVIA® Rebaudioside E preparation is well-suited for the intended food uses.

**PART 3. DIETARY EXPOSURE**

The subject Blue California BESTEVIA® Rebaudioside E preparation, containing rebaudioside E as the principal component (≥85%), is intended to be used as a table top sweetener and general-purpose non-nutritive sweetener in various foods other than infant formulas and meat and poultry products. The intended use will be as a non-nutritive sweetener as defined in 21 CFR

170.3(o)(19).<sup>3</sup> The intended use levels will vary by actual food category, but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations. However, the amounts of Blue California’s high purity rebaudioside E preparation to be added to foods will not exceed the amounts reasonably required to accomplish its intended technical effect in foods as required by FDA regulation.<sup>4</sup>

**A. Estimate of Dietary Exposure to the Substance**

There have been many scholarly estimates of potential dietary intake replacement of sweeteners, including steviol glycosides, that have been published (FSANZ, 2008; WHO, 2003; Renwick, 2008) or submitted to FDA (Merisant, 2008). These are summarized in Appendix 11. In GRAS notification 301, a simplified estimate was proposed to, and accepted by, FDA based on the estimates of exposure in “sucrose equivalents” (Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90<sup>th</sup> percentile consumer of a sweetener which is 100 times as sweet as sucrose when used as a total sugar replacement would be a maximum of 9.9 mg per kg body weight (bw) per day for any population subgroup.

The estimated sweetness intensity for Blue California’s BESTEVIA® Rebaudioside E preparation is approximately 137-fold that of sucrose (Part 2.D). Therefore, the highest 90<sup>th</sup> percentile consumption by any population subgroup of Blue California’s BESTEVIA® Rebaudioside E preparation would consume approximately 7.23 mg per kg steviol glycosides bw per day. Based on an estimate that Reb E preparations consist of approximately 33% steviol equivalents,<sup>5</sup> the consumption would be less than 2.38 mg per kg bw per day on a steviol equivalents basis for any population group. These calculations are summarized in Table 3.

**Table 3. Daily Intake of Sweeteners (in Sucrose Equivalents) & Estimated Daily Intakes of BESTEVIA® Rebaudioside E**

Population Group	Intakes of Sweeteners (mg sucrose/kg bw/day) <sup>a</sup>		Calculated Intake of BESTEVIA® Rebaudioside E (mg/kg bw/day) <sup>b</sup>		Calculated Intake of BESTEVIA® Rebaudioside E as Steviol Equivalents (mg/kg bw/day)	
	Low	High	Low	High	Low	High
Healthy Population	255	675	1.86	4.93	0.61	1.62
Diabetic Adults	280	897	2.04	6.55	0.67	2.15
Healthy Children	425	990	3.10	7.23	1.02	2.38

<sup>3</sup> Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity

<sup>4</sup> See 21 CFR 182.1(b)(1)

<sup>5</sup> Calculated as percent of molecular weight of steviol to molecular weight of rebaudioside E

Population Group	Intakes of Sweeteners (mg sucrose/kg bw/day) <sup>a</sup>		Calculated Intake of BESTEVIA <sup>®</sup> Rebaudioside E (mg/kg bw/day) <sup>b</sup>		Calculated Intake of BESTEVIA <sup>®</sup> Rebaudioside E as Steviol Equivalents (mg/kg bw/day)	
	Low	High	Low	High	Low	High
Diabetic Children	672	908	4.91	6.63	1.61	2.18

<sup>a</sup> From Renwick (2008)

<sup>b</sup> Calculated by dividing the sucrose intake by the minimum average relative sweetness value of 137 for BESTEVIA<sup>®</sup> Rebaudioside E

The values in Table 3 assume Blue California’s BESTEVIA<sup>®</sup> Rebaudioside E preparation constitutes the entire sweetener market, which makes these estimates extremely conservative since the likelihood of that occurrence is minimal. For the general healthy adult population, the estimated maximum intake of purified steviol glycosides is 4.93 mg per kg bw per day, or 1.62 mg per kg steviol equivalents. For healthy children, the estimated maximal intake is 7.23 mg per kg bw per day, or 2.38 mg per kg as steviol equivalents. In all population groups, the estimated daily intake of purified steviol glycosides, expressed as steviol equivalents, is well below the JECFA-established acceptable daily intake (ADI) of 4.0 mg per kg bw per day steviol equivalents.

**B. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed In or On Food**

This section is not applicable to Blue California’s BESTEVIA<sup>®</sup> Rebaudioside E product, which would be chemically stable under conditions of use.

**C. Dietary Exposure to Contaminants or Byproducts**

While a recent publication by Kumari et al. (2016) investigated the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Antioxidant Capacity (TAC) in *S. rebaudiana* leaf --- and the observed activity has been attributed to naturally-occurring phytochemicals such as phenolics, flavonoids, and pigments in the plant --- the study has minimal relevance with regard to the safety considerations of highly purified stevia extract, of which ≥ 95% consists of the most familiar steviol glycosides and their glucosylated steviosides. These phytochemical contaminants, if present, are in low amounts and were likely similarly present in purified test materials that were used in the toxicology studies summarized in Appendix 10.

Furthermore, no concerns regarding dietary exposure to contaminants or byproducts have been raised by expert regulatory bodies, including the World Health Organization/Joint FAO/WHO Expert Committee on Food Additives (WHO/JECFA), European Food Safety Authority (EFSA), Food Standards Australia New Zealand (FSANZ), and FDA, since JECFA’s first steviol glycosides review was performed in 2000 (WHO, 2000).



#### **PART 4. SELF-LIMITING LEVELS OF USE**

It has been well-documented in the published literature that the use of steviol glycosides is self-limiting due to organoleptic factors and consumer taste considerations (Kochikyan et al., 2006; Carakostas et al., 2008; Brandle et al., 1998; Prakash et al., 2008; Gupta et al., 2016; Gerwig et al., 2016). These organoleptic factors include bitterness and astringency, as well as a lingering metallic aftertaste (Gerwig et al., 2016).

#### **PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958**

##### **A. Other Information on Dietary Exposure**

###### **1. History of Traditional Medicinal and Human Food Use**

Stevia has been used as a traditional medicine and sweetener by native Guarani tribes for centuries (Esen, 2016; Gerwig et al., 2016; Brusick, 2008; Brandle et al., 1998). Hawke (2003) reported that stevia is commonly used as a treatment for type 2 diabetes in South America. However, for its therapeutic effects, elevated doses in the range of 1 gram per person per day or more were reported to be necessary (Gregersen et al., 2004).

For about 30 years, consumers in Japan and Brazil, where stevia has long been approved as a food additive, have been using stevia extracts as non-caloric sweeteners (Raintree, 2012). It was previously reported that 40% of the artificial sweetener market in Japan had been stevia based and that stevia is commonly used in processed foods in Japan (Lester, 1999). Use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and as a natural health product in Canada. It has wide use in China and Japan in food and in dietary supplements. In 2005, it was estimated that sales of stevia in the US reached \$45 million (Newsday, 2006).

NewHope360 reported that the global market for stevia in 2014 was \$347 million, and that is expected to increase to \$565.2 million by 2020. In addition, consumption is expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).

Most recently, Nutritional Outlook reported that Mintel data indicated a 48% increase in stevia-containing products over the last five years (Decker and Prince, 2018). According to Zion Market Research (2018), the global stevia market was valued at approximately \$416.6 million in 2017 and is expected to generate revenue of approximately \$721 million by the end of 2024.

##### **B. Summary of Regulatory History of Steviol Glycosides**

Stevia-derived sweeteners are permitted as food additives in South America and in several countries in Asia, including China, Japan, and Korea. In recent years, these sweeteners have received food usage approvals in Mexico, Australia, New Zealand, Switzerland, France, Peru, Uruguay, Colombia, Senegal, Russia, Malaysia, Turkey, Taiwan, Thailand, Israel, Canada, and

Hong Kong (EFSA, 2010; Watson, 2010; Health Canada, 2012). In the United States, steviol glycosides have been used as a dietary supplement since 1995 (Geuns, 2003).

A brief overview of the most recent regulatory activity regarding steviol glycosides is presented below in Part 5.B. Sections 1-5; a more detailed historical overview is provided in Appendix 11.

### **1. U.S. Regulatory History**

Based on available information from FDA’s GRAS Notice Inventory website (FDA, 2018) as of October 8, 2018, FDA has issued 54 “no questions” letters on GRAS notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme treatment. A comprehensive list is provided in Table 11-1 in Appendix 11.

In addition, the Flavor and Extract Manufacturers Association (FEMA) includes nine steviol glycosides preparations, one of which is for an enzymatically modified stevia extract, on their GRAS lists.

### **2. Canadian Regulatory History**

On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012). In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (Health Canada, 2014). On January 15, 2016, Health Canada approved the use of Reb M for use as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (Health Canada, 2016).

Most recently, Health Canada’s Food Directorate has updated its List of Permitted Sweeteners to allow for the use of steviol glycosides as a sweetener in ‘unstandardized snack bars,’ including granola bars, cereal bars, fiber bars, and protein isolate-based bars (Health Canada, 2017b). Health Canada (2017a) also modified the List of Permitted Sweeteners to include “all the steviol glycosides in the *Stevia rebaudiana* Bertoni plant (stevia plant).”

### **3. European Regulatory History**

An amendment to the EU food additives regulation 231/2012, which became active on November 3, 2016, removed the previous requirement for stevia blends to contain at least 75% Reb A or stevioside. In addition, the updated regulation ---(EU) 2016/1814---now permits the following steviol glycosides in stevia blends: stevioside, rebaudiosides A, B, C, D, E, F and M (Reb A; Reb B; Reb C; Reb D; Reb E; Reb F; Reb M), steviolbioside, rubusoside, and dulcoside (Searby, 2016).

The EFSA Panel of Food Additives and Nutrient Sources reviewed an application for glucosylated steviol glycoside preparations for use as a new food additive. The Panel concluded that the data

supplied by the applicant were “insufficient to assess the safety” of the glucosylated steviol glycosides preparation. It should be noted that no safety concerns were raised by the EFSA Panel, and that their decision was based on the “limited” data provided in the dossier submitted by the applicant (EFSA, 2018).

Recently, JECFA updated the steviol glycosides specifications to include a minimum requirement of not less than 95% total steviol glycosides, on a dry basis, “determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni.” Microbiological criteria were also established, with specifications of no more than 1,000 CFU per g total plate count, not more than 200 CFU per g yeasts and molds, and *E. coli* and *Salmonella* negative in 1 g and 25 g, respectively (FAO, 2017).

#### **4. Asian Regulatory History**

No regulatory updates have been identified in recent years. The Asian regulatory history for steviol glycosides through 2014 is presented in Appendix 11.

#### **5. Other Regulatory History**

FSANZ called for submissions on permitting all minor steviol glycosides extracted from stevia leaf to be included in the definition of steviol glycosides in the Food Standards Code, noting that “[no] evidence was found to suggest that the proposed changes pose any public health and safety concerns.” The submission period ended on December 19, 2016 (FSANZ, 2016b). Subsequently, on February 8, 2017, FSANZ approved a draft variation of the definition of steviol glycosides to include all steviol glycosides present in the *Stevia rebaudiana* leaf (FSANZ, 2017).

On July 20, 2018, FSANZ called for comments on the production of Reb M using enzymes derived from genetically modified yeast. A risk assessment by FSANZ found no health and safety concerns with the manufacturing method. The comment period closed on August 31, 2018 (FSANZ, 2018a).

On October 9, 2018, FSANZ called for comments on the addition of steviol glycosides to fruit drinks at a maximum permitted level of 200 mg per kg steviol equivalents. A risk assessment by FSANZ found no health and safety concerns with regard to the proposed levels of use in fruit drinks, and noted that “the impact...on total dietary exposure was determined to be small.” The deadline for submissions is November 6, 2018 (FSANZ, 2018b).

### **PART 6. NARRATIVE**

The biological, toxicological, and clinical effects of stevia and steviol glycosides have been extensively reviewed (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002). Additionally---and as noted earlier---the national and international regulatory agencies have thoroughly reviewed the

safety of stevia and its glycosides. Most notably, over the years, JECFA has evaluated purified steviol glycosides multiple times (WHO, 2000; WHO, 2006; WHO, 2007; WHO, 2008), and their findings have been summarized in Part 5.B.3. FSANZ (2008) also evaluated steviol glycosides for use in food. The JECFA reviews, as well as the other reviews completed before 2008, primarily focused on mixtures of steviol glycosides. These studies are summarized in Appendix 12.

Since the JECFA evaluation (WHO, 2008), FDA has received and not objected to over fifty GRAS notifications for steviol glycosides or enzyme modified steviol glycosides that have been submitted to FDA (Perrier et al., 2018). In each case, FDA has agreed with the conclusions that steviol glycosides are GRAS based largely on the 0-4 mg per kg bw per day ADI on a steviol equivalence basis that was established by JECFA. A recent publication by Roberts et al. (2016) indicates that the ADI could be higher, as discussed further in Appendix 9. Among the GRAS notifications submitted to FDA, several assessed purified preparations of rebaudioside A, and they were supported by additional toxicology and clinical studies that are summarized in Appendix 13. To date, 54 of the submitted notifications have had "no questions" letters of response from FDA (see Table 11-1 in Appendix 11).

Because of their sweetness characteristics, steviol glycosides have viable uses as a non-nutritive sweetener in foods.<sup>6</sup> Periodic reviews by JECFA over the years indicate the progression of knowledge on the toxicology of steviol glycosides. Several early safety-related studies on these compounds were performed on crude extracts of stevia. These studies also included multiple investigations with *in vivo* and *in vitro* models, which explored the biological activity of stevia extracts at high doses or high concentrations. These early investigations raised several concerns, including impairment of fertility, renal effects, interference with glucose metabolism, and inhibition of mitochondrial enzymes. In recent years, as more and more studies were performed on purified glycosides, the toxicology profile of steviol glycosides eventually proved to be rather unremarkable. A number of subchronic, chronic, and reproductive studies have been conducted in laboratory animals. These studies were well designed with appropriate dosing regimens and adequate numbers of animals to maximize the probability of detection of important effects. Notably, the initially reported concerns related to the effects of stevia leaves or crude extracts on fertility were refuted by the well-designed reproductive studies with purified steviol glycosides. All other concerns failed to manifest themselves at the doses employed in the long-term rat studies.

As discussed in Appendix 14 and elsewhere, at its 51<sup>st</sup> meeting, JECFA determined that there were adequate chronic studies in rats, particularly the study by Toyoda et al. (1997), to establish a

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<sup>6</sup> It has also been reported that steviol glycosides may have pharmacological properties, which can be used to treat certain disease conditions such as hypertension and type 2 diabetes. Chatsudhipong and Muanprasat (2009), as well as others, have published reviews where they note that such therapeutic applications have not been firmly established as being due to steviol glycosides. The reviewers point out that the effects occur at higher doses than would be used for sweetening purposes. Furthermore, many effects noted in older studies may have been due to impurities in preparations that do not meet the contemporary purity specifications established by JECFA for use as a sweetener. If oral doses of steviol glycosides impart pharmacological effects, such effects would undoubtedly occur due to actions of the principal metabolite, steviol, but the pharmacological effects of steviol have not been comprehensively investigated. For a more comprehensive discussion of this subject, see Section 7 of Appendix 14.

temporary ADI of 0 - 2 mg per kg bw per day with an adequate margin of safety (Toyoda et al., 1997). The committee also critically reviewed the lack of carcinogenic response in well-conducted studies. These studies validated the Committee conclusion that the *in vitro* mutagenic activity of steviol did not present a risk of carcinogenic effects *in vivo* and, therefore, all common steviol glycosides that likely share the same basic metabolic and excretory pathway and that use high purity preparations of various steviol glycosides, are safe as a sugar substitute. Subsequently, the additional clinical data reviewed by JECFA allowed the Committee to establish a permanent ADI of 0 - 4 mg per kg bw per day (based on steviol equivalents).

Recently, JECFA published a safety evaluation of a number of food additives, including steviol glycosides. The JECFA committee reviewed information supporting the safety of a *Yarrowia lipolytica* fermentation-produced rebaudioside A, which included a 90-day rat toxicity study and two *in vitro* genotoxicity studies, as well as *in vitro* colonic microflorae hydrolysis studies in several steviol glycosides, toxicokinetic studies of stevioside in humans and rats, and literature published since the 69<sup>th</sup> meeting (WHO, 2017).

The Committee noted that the most recent short-term toxicity studies were consistent with those reviewed at or prior to the 69<sup>th</sup> meeting, and that the new toxicokinetic study in humans did not have a large enough subject pool to provide reliable toxicokinetic estimates to derive an update ADI for steviol glycosides. The Committee confirmed the current ADI of 0-4 mg per kg bw steviol. In addition, the Committee prepared new “tentative” specifications for steviol glycosides, which was expanded to include “any mixture of steviol glycosides compounds derived from *S. rebaudiana* Bertoni” while retaining the requirement that the total percentage of steviol glycosides is  $\geq 95\%$  (WHO, 2017).

Blue California critically reviewed the JECFA assessments and agree with the calculation of the ADI for steviol glycosides.

Several published and unpublished studies (summarized in Appendix 13) on purified preparations of rebaudioside A showed an absence of toxicological effects in rats (Curry and Roberts, 2008; Nikiforov and Eapen, 2008) and dogs (Eapen, 2008) in subchronic studies, and an absence of reproductive (Curry et al., 2008; Slotter, 2008a) and developmental effects (Slotter, 2008b) in rats. Most notably, pharmacokinetic studies in rats (Roberts and Renwick, 2008) and humans (Wheeler et al., 2008) on purified rebaudioside A follow the same pathway of being degraded to steviol by intestinal bacteria with subsequent rapid glucosylation and elimination in urine and feces.

Purkayastha et al. (2015) compared the anaerobic *in vitro* metabolism of rebaudioside E with rebaudioside A using human fecal homogenates. Both rebaudioside E and rebaudioside A were hydrolyzed to steviol within 24 hours, with the majority of metabolism occurring within the first 16 hours. Metabolism of the rebaudiosides took longer at higher concentrations (2.0 mg per mL vs. 0.2 mg per mL), which is consistent with concentration-dependent hydrolysis observations on rebaudiosides B, D, and M reported by Purkayastha et al. (2014) in a previous study. There were

no marked differences in rate or extent of hydrolysis observed between genders, ethnicities (Asian vs. Caucasian), or the individual rebaudiosides (Purkayastha et al., 2015).

In a follow up study, Purkayastha et al. (2016) investigated the metabolic fate of two concentrations of steviolbioside, dulcoside A, and rebaudiosides A, B, C, D, E, F, and M in an *in vitro* study using pooled human fecal homogenates over the course of 24-48 hours. It was reported that the glycosidic side chains ---containing glucose, rhamnose, xylose, fructose, and those with deoxy-glucose including combinations of  $\alpha(1-2)$ ,  $\beta-1$ ,  $\beta(1-2)$ ,  $\beta(1-3)$ , and  $\beta(1-6)$  linkages---were mostly degraded to steviol within 24 hours. This observation supports the extrapolation of safety data for specific steviol glycosides and steviol to other steviol glycosides found in *Stevia rebaudiana* leaf extract. As previously observed, the rate of metabolism was slower at higher concentrations (2.0 mg per mL vs. 0.2 mg per mL). In addition, Purkayastha et al. (2016) reported that no appreciable differences in metabolism were observed between male and female, or homogenates of feces from individuals with different ethnicities.

Blue California concludes that the results of the studies in these two publications corroborate the presumption of safety of rebaudioside E, given the similarity of metabolism to rebaudioside A, in concert with the large number of toxicology studies with other steviol glycosides.

Blue California has also reviewed the findings from human clinical studies, noting that ---with regard to the clinical effects reported in humans--- in order to corroborate the observations in these studies that these effects of steviol glycosides only occur in patients with either elevated blood glucose or blood pressure (or both), JECFA called for studies in individuals who are neither hypertensive nor diabetic (WHO, 2006). The supplemental data presented to JECFA and also published by Barriocanal et al. (2008) demonstrate the lack of pharmacological effects of steviol glycosides at 11 mg per kg bw per day in normal individuals, or approximately slightly more than 4 mg per kg bw on the basis of steviol equivalents (Barriocanal et al., 2008). Clinical studies on purified rebaudioside A showed an absence of effects on blood pressure (Maki et al., 2008a) and blood glucose levels (Maki et al., 2008b) at doses slightly higher than the exposures expected in food. Blue California concludes that there will be no effects on blood pressure and glucose metabolism in humans at the doses of steviol glycosides expected from its use in food as a non-nutritive sweetener.

Two previously published studies summarized in Appendix 10 raised a potential concern regarding the toxicological effects of steviol glycosides. In one study, DNA damage was seen in a variety of organs as assessed by Comet assay in rats given drinking water containing 4 mg per mL steviol glycosides for up to 45 days (Nunes et al., 2007a). Several experts in the field have since questioned the methodology used in this study (Geuns, 2007a; Williams, 2007; Brusick, 2008). Blue California has reviewed the cited publications, along with the responses made by the authors (Nunes et al., 2007b; Nunes et al., 2007c), and concurs with the challenges to the methodology utilized by Nunes et al. (2007a), thereby discounting the validity and importance of this study.

In another study with stevioside in rats, (TRAP) levels were measured and found to be significantly decreased at doses as low as 15 mg per kg bw (Awney et al., 2011). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages, and dendritic cells. This enzyme was not measured in any previous toxicology studies on steviol glycosides, nor has it been adequately vetted for application in toxicological studies. Critical reviews of this study by Carakostas (2012) and Waddell (2011) revealed a poor study design that included: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection (which affects many chemistry and hematological values); no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. Additionally, the report did not adequately describe mean or individual organ weight data, and it lacked comparison of study findings against laboratory historical control data.

Urban et al. (2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in two relatively recent publications (Brahmachari et al., 2011; Tandel, 2011) in which the authors concluded that additional testing is necessary to adequately address the genotoxicity profile (Urban et al., 2013). The review aimed to address this matter by evaluating the specific genotoxicity studies of concern, while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in these publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside A are genotoxic. This comprehensive review with overwhelming negative genotoxicity findings in the literature combined with a paucity of evidence for neoplasm development in rat bioassays, provide a weight-of-evidence position that clearly favors and establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

In addition, a paper by Shannon et al. (2016) raises a possible concern of endocrine disruption by steviol. Blue California reviewed the publication and notes that the effects on progesterone production and on the action of progesterone (both antagonistic and agonistic) were observed *in vitro* in sperm cells. Blue California concludes that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at receptors. Further, no adverse effects were observed in well-conducted reproductive toxicology studies in several species. Therefore, this study does not alter Blue California's opinion that steviol glycosides preparations are generally recognized as safe. A summary of this study is provided in Appendix 14.

In two *in vitro* metabolism studies using human fecal homogenates, Purkayastha et al. (2015; 2016) found that a number of steviol glycosides, including rebaudiosides A and E, were all hydrolyzed to steviol within 24 hours, with no marked differences in rate or extent of hydrolysis between individual rebaudiosides or between male and female fecal homogenates at that time point. In addition, the majority of metabolism occurred within the first 16 hours, and was concentration-dependent, where high concentrations (2.0 mg per mL) took longer to hydrolyze than lower concentrations (0.2 mg per mL).

Philippaert et al. (2017) demonstrated that stevioside, rebaudioside A, and steviol potentiate the activity of transient receptor potential cation channel subfamily melastatin member 5 (TRPM5), a  $\text{Ca}^{2+}$ -activated cation channel that is expressed in type II taste receptor cells and pancreatic  $\beta$ -cells. The authors found that the steviol glycosides increased the perception of bitter, sweet, and umami tastes and also enhanced glucose-induced insulin secretion in a TRPM5-dependent manner. Furthermore, *in vivo* studies indicated that daily consumption of stevioside prevents high-fat-induced diabetic hyperglycemia development in wild-type mice. No adverse events or animal deaths were discussed.

A commercially available steviol glycoside extract (>99%, composition and brand unknown) was used to investigate genotoxicity in human peripheral blood lymphocytes. Uçar et al. (2017) observed no significant differences in chromosomal aberration induction or micronuclei between the control and treatment groups at 24 and 48 h. These data support previous findings that steviol glycosides are not genotoxic.

Panagiotou et al. (2018) observed that steviol and steviol glycosides exert glucocorticoid receptor-mediated effects in human leukemic T-cells (Jurkat cells) but not in normal human peripheral blood mononuclear cells, which they concluded was due to a cell-type specific manner of glucocorticoid receptor-modulation.

Thøgersen et al. (2018) investigated the effect of rebaudioside A, stevioside, and steviol on porcine cytochrome p450 (CYP) expression and activity to assess their potential food-drug interactions in the IPEC-J2 cell line, which is a non-transformed cell line derived from intestinal porcine epithelial cells and in primary hepatocytes. The authors reported that there were no changes in CYP mRNA expression following treatment of IPEC-J2 cells with rebaudioside A, stevioside, and steviol compared with control. Treatment of primary hepatocytes resulted in increases in CYP329 mRNA at low concentrations of rebaudioside A and steviol, and at all concentrations of stevioside. The authors reported that while treatment with the steviol glycosides tested over 24 hours resulted in minor increases in CYP3A29 mRNA expression (<2.0-fold), “no direct effect on CYP activity” was observed. The authors concluded that rebaudioside A, stevioside, and steviol are unlikely to cause a food-drug interaction but noted that the study could not predict long term effects and effects *in vivo*.

A recently published study by Silva et al. (2016) addressed the genotoxic activity of stevia (Svetia™, purity not reported<sup>7</sup>). Human lymphocytes were treated with 5% and 0.5% Svetia™ for 2 hours. No statistically significant difference in genetic damage was observed following treatment with 0.5% Svetia™ compared to the negative control, while the 5% Svetia™ showed a statistically significant difference ( $P < 0.0001$ ) compared to the control, with a decrease in migration average. The authors described the effect as being beneficial. Human lymphocytes treated with

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<sup>7</sup> While the purity of the material used for the study was not reported by Silva et al. (2016), a search of the manufacturer's website ([www.svetia.us](http://www.svetia.us)) indicates that the trademarked material is a blend of cane sugar and 97% pure Reb A.



10% Svetia™ demonstrated significant ( $P < 0.0001$ ) genotoxic activity compared to the control; however, at treatment concentrations of 0.05%, 0.5%, and 5% Svetia™, a significant ( $P < 0.0001$ ) decrease in average migration of DNA was observed compared to the control. The authors conclude that these results demonstrate the absence of genotoxicity at concentrations up to 5% Svetia™ (Silva et al., 2018). It should be noted that these observations are inconsistent with data reported by Nunes et al. (2007a); however, as discussed above, the validity and importance of the Nunes et al. study has been discounted given the questions surrounding the methodology.

Blue California agrees with the safety conclusions of the 54 GRAS Expert Panels in the notifications for steviol glycosides previously submitted to FDA that resulted in "no questions" responses from FDA (as summarized in Appendix 11), JECFA (WHO, 2006; WHO, 2008), and Renwick (2008) that a sufficient number of good quality health and safety studies support the determination that purified preparations of steviol glycosides, when added to food at levels up to full replacement of sucrose on a sweetness equivalency basis, meet FDA's definition of safe.

Blue California concludes that it is reasonable to apply the JECFA ADI of 4 mg per kg bw per day for steviol glycosides (expressed on a steviol basis) to Blue California's BESTEVIA® Rebaudioside E. Therefore, with the steviol equivalence values shown in Table 3, Blue California concludes that, for the general population, the estimated maximum daily intake of Blue California's BESTEVIA® Rebaudioside E is 7.23 mg per kg bw or 2.38 mg per kg expressed as steviol equivalents. Based upon these calculations, the intake of Blue California's BESTEVIA® Rebaudioside E safely falls within the 4 mg per kg bw per day ADI expressed as steviol equivalents as determined by JECFA.

Blue California's BESTEVIA® Rebaudioside E preparations contain not less than 95% total steviol glycosides with rebaudioside E as the principal component ( $\geq 85\%$ ). Given the structural similarities with rebaudioside A, stevioside, and other steviol glycosides, and considering analogous metabolic pathways for all these substances, the safety data on stevia and its other components have a direct bearing on the present safety assessment for BESTEVIA® Rebaudioside E. This is further supported by over a decade and a half of scientific studies on the safety of these substances, along with the fact that the major regulatory bodies view the results of toxicology studies on either stevioside or rebaudioside A as applicable to the safety assessment of all known steviol glycosides, since all are metabolized and excreted by similar pathways, with steviol being the common metabolite for each. The foundational safety of Reb A, other steviol glycosides and steviol has been summarized, with key studies detailed in Appendices, 12, 13, and 14.

In addition, Blue California affirms that its BESTEVIA® Rebaudioside E preparation is manufactured under cGMP conditions with raw materials and processing aids that meet the appropriate food grade regulations. Blue California has established sufficient rigorous product specifications based upon FCC and JECFA monographs---which are consistent with other steviol glycosides on the market---and has demonstrated batch-to-batch consistency against these specifications.

Blue California notes that the relative sweetness intensity of BESTEVIA® Rebaudioside E was determined to be somewhat lower than values reported elsewhere (137 vs. 150-200); however, the use levels needed to provide adequate sweetness in food are still within the ADI.

Furthermore, Blue California has reviewed this safety information and has concluded that BESTEVIA® Rebaudioside E is generally recognized as safe for the proposed uses.

## A. GRAS Criteria

FDA defines “safe” or “safety” as it applies to food ingredients as:

“...reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use.”<sup>8</sup>

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA’s operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

“...General recognition of safety requires common knowledge, throughout the expert scientific community knowledgeable about the safety of substances directly or indirectly added to food, that there is reasonable certainty that the substance is not harmful under the conditions of its intended use.”

“‘Common knowledge’ can be based on either “scientific procedures” or on experience based on common use of a substance in food prior to January 1, 1958.”<sup>9</sup>

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called “common knowledge element,” in terms of the two following component elements:<sup>10</sup>

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is

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<sup>8</sup> See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: <https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe> (Accessed on 9/8/18)

<sup>9</sup> See 81 FR 54959 Available at: <https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe> (Accessed on 9/8/18)

<sup>10</sup> See Footnote 1

established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

The apparent imprecision of the terms “appreciable,” “at the time,” and “reasonable certainty” demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

As noted below, this safety assessment to ascertain GRAS status for high purity steviol glycosides for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

## **B. Expert Panel Findings on Safety of Blue California’s BESTEVIA® Rebaudioside E**

An evaluation of the safety and GRAS status of the intended use of Blue California’s BESTEVIA® Rebaudioside E preparations has been conducted by an Expert Panel convened by GRAS Associates; the Panel consisted of Robert Kapp, Ph.D., Fellow Academy of Toxicological Sciences (ATS), Fellow Royal Society of Biology (FRSB) & European Registered Toxicologist (ERT, UK); Kara Lewis, Ph.D.; and Katrina Emmel, Ph.D., as Panel Chair. The Expert Panel reviewed Blue California’s dossier as well as other publicly available information available to them. The individuals who served as Expert Panelists are qualified to evaluate the safety of foods and food ingredients by merit of scientific training and experience.

The GRAS Expert Panel report is provided in Appendix 15.

## **C. Common Knowledge Elements for GRAS Conclusions**

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS conclusion requires that consensus exists within the broader scientific community.

### **1. Public Availability of Scientific Information**

The majority of the studies reviewed on steviol glycosides and steviol have been published in the scientific literature as summarized in Appendices 10, 13, and 14. Most of the literature relied upon

by JECFA has also been published---most importantly, the chronic rat studies on steviol glycosides. JECFA did make limited use of unpublished studies, and they were summarized in the two JECFA monographs. Moreover, JECFA publicly releases the results of their safety reviews, and their meeting summaries and monographs are readily available on their website.

With regard to the safety documentation, the key pharmacokinetic data establish that steviol glycosides are not absorbed through the gastrointestinal (GI) tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but rapidly metabolized and excreted (Gardana et al., 2003; Koyama et al., 2003b). The action of bacteria in the large intestine is directly supported by the published study that showed that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003b; Renwick and Tarka, 2008).

The ADI for steviol glycosides has been set largely based on a published chronic study in rats (Toyoda et al., 1997) and several published clinical studies that there are no pharmacological effects in humans at doses several fold higher than the ADI (Barriocanal et al., 2006; Barriocanal et al., 2008; Wheeler et al., 2008). As mentioned above, Roberts et al. (2016) noted that the ADI could be higher using a chemical-specific adjustment factor (CSAF), as defined by the WHO in 2005, determined by comparative studies in rats and humans, which they conclude can justify an ADI Value of 6-16 mg per kg bw per day for steviol glycosides.

The toxicity of the metabolite steviol has been well-reviewed in the published literature and by authoritative agencies (Geuns, 2003; WHO, 2006; Urban et al., 2013).

In addition, there is a large, publicly available, collection of GRNs regarding steviol glycosides on FDA's website.

## **2. Scientific Consensus**

The second common knowledge element for a GRAS conclusion requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use.

A number of well-respected regulatory agencies, including JECFA, EFSA, FSANZ, WHO, the Switzerland Office of Public Health, and Health Canada, as well as numerous well-respected individual scientists, have indicated that steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (FAO, 2010; EFSA, 2010; FSANZ, 2008; Health, 2008; Health Canada, 2012; Xili et al., 1992; Toyoda et al., 1997; Geuns, 2003; Williams, 2007). Since December 2008, over fifty GRAS notifications have been submitted to FDA for highly purified stevia-derived sweetener products, and FDA's detailed reviews have consistently yielded "no questions" letters.

In summary, a compelling case can be made that scientific consensus exists regarding the safety of steviol glycosides when of sufficiently high purity. The central role of conversion to steviol and

subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which the various steviol glycosides molecules are metabolized and eliminated from the body. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, Blue California believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as evidenced by several publications (Carakostas, 2012; Geuns, 2007a; Urban et al., 2013; Waddell, 2011; Williams, 2007; Brusick, 2008) that refute safety concerns expressed by a minority of scientists. Roberts et al. (2016) suggests that the ADI could be higher than has been previously accepted by the scientific community.

#### **D. Conclusion**

In consideration of the aggregate safety information available on naturally occurring steviol glycosides, Blue California concludes that BESTEVIA® Rebaudioside E ( $\geq 95\%$  total steviol glycosides with rebaudioside E as the principle component) defined in the subject notification are safe for use as a general-purpose non-nutritive sweetener in foods other than infant formulas and meat and poultry products. The JECFA ADI for steviol glycosides of 4 mg per kg bw per day (as steviol equivalents) can be applied to Blue California's BESTEVIA® Rebaudioside E preparation. Based on published dietary exposure data for other approved sweeteners and adjusting for relative sweetness intensity, intake was estimated for healthy non-diabetic children and adults, and diabetic children and adults with the following findings.

The worst-case estimated intakes of Blue California's BESTEVIA® Rebaudioside E for several population groups summarized in Part 3.A. are no greater than 2.38 mg per kg steviol equivalents per bw per day, which is below the ADI of 4 mg per kg bw expressed as steviol equivalents as established by JECFA. The dietary levels from anticipated food consumption is not likely to exceed the ADI when BESTEVIA® Rebaudioside E is used as a general non-nutritive sweetener.

Accordingly, BESTEVIA® Rebaudioside E as produced by Blue California and declared within the subject notification meet FDA's definition of safety in that there is "reasonable certainty of no harm under the intended conditions of use" as described herein and, therefore, is generally recognized as safe (GRAS).

## **PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE.**

### **A. Acronyms and References**

#### **1. List of Acronyms**

µg	Microgram
ADI	Acceptable daily intake
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATS	Academy of Toxicological Sciences
AUC	Area under the plasma-concentration time curve
AVA	Agri-food and Veterinary Authority of Singapore
BP	Blood pressure
bw	Body weight
C	Celsius
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
CGMPs	Current Good Manufacturing Practice
C <sub>max</sub>	Maximum (peak) serum concentration of substance is observed
Co.	Company
CSAF	Chemical-specific adjustment factor
CYP	Cytochrome P450
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
EDI	Estimated daily intake
EFSA	European Food Safety Authority
ERT	European Registered Toxicologist
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCC	Food Chemicals Codex
FD&C Act	Federal Food Drug and Cosmetics Act
FDA	Food and Drug Administration
FEMA	Flavor Extract Manufacturers Association
FOIA	Freedom of Information Act
FR	Federal Register
FRSB	Fellow Royal Society of Biology
FSANZ	Food Standards Australia New Zealand
FSSAI	Food Safety and Standards Authority of India
g	Gram
GA	GRAS Associates
GEMS	Global Environment Monitoring System
GGT	Gamma-glutamyltransferase
GI	Gastrointestinal
GPT	Glutamic-pyruvate transaminase
gpt	Guanine phosphoribosyltransferase
GRAS	Generally Recognized as Safe
GRN	GRAS Notification
h or hr	Hour

HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
Hg	Mercury
HPLC	High-Performance Liquid Chromatography
HR	Heart rate
IADSA	International Alliance of Dietary/Food Supplement Associations
IPEC-J2	Non-transformed intestinal porcine epithelial cells
JEFCA	Joint FAO/WHO Expert Committee on Food Additives
kg	Killogram
L	Liter
LD <sub>50</sub>	Median (50%) lethal dose
LDL	Low-density lipoprotein
LLC	Limited Liability Corporation
Ltd.	Limited
MAP	Mean arterial pressure
mg	Milligram
Mins	Minutes
mL	Milliliter
mm	Millimeter
MPL	Maximum permitted level
MW	Molecular weight
n	number
NA	Not applicable
ND	Not detected
ng	Nanogram
NHANES	National Health and Nutrition Examination Surveys
NHP	Natural Health Products
NMT	Not more than
No.	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NS	Not specified
OD <sub>600</sub>	Optical density at 600 nm
PCV%	Packed cell volume
Ph.D.	Doctor of Philosophy
PND	Post natal day
ppm	Parts per million
RBC	Red blood cell
Reb A	Rebaudioside A
Reb B	Rebaudioside B
Reb C	Rebaudioside C
Reb D	Rebaudioside D
Reb E	Rebaudioside E
Reb F	Rebaudioside F
Reb M	Rebaudioside M
SBP	Systolic blood pressure
SCF	European Commission's Scientific Committee on Food
SUS	Sucrose synthases
t <sub>1/2</sub>	Half-life

TAC	Total Antioxidant Capacity
tds	Total dissolved solids
TFC	Total Flavonoid Content
TK	Toxicokinetic
T <sub>max</sub>	Time at which maximum (peak) plasma concentration (C <sub>max</sub> ) of substance is observed
TPC	Total Phenolic Content
TRAP	Tartrate-resistant alkaline phosphatase
TRPM5	Transient receptor potential cation channel subfamily melastatin member 5
U.S.	United States
UDP	Uridine diphosphate
UDPG	Uridine diphosphate-glucose
UGT	Uridine 5'-diphosphouridine-glucosyltransferase
UK	United Kingdom
VLDL	Very low density lipoprotein
WBC	White blood cell
WHO	World Health Organization
WHO/JECFA	World Health Organization/Joint FAO/WHO Expert Committee on Food Additives

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## B. Appendices

## Appendix 1 List of Scientific Publications Regarding the Synthesis of Rebaudioside E

1. Bioconversion of Rebaudioside I from Rebaudioside A. *Molecules* (2014) 19:17345-17355; (enzymatic bioconversion).
2. Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of *Stevia rebaudiana*. *The Plant Journal* (2005) 41:56–67 (identification of UGTs (UGT76G1, UGT74G1 and UGT85C2) involve in steviol glycoside biosynthesis from stevia).
3. Isolation and characterization of a novel rebaudioside M isomer from a bioconversion reaction of rebaudioside A and NMR comparison studies of rebaudioside M isolated from *Stevia rebaudiana* Bertoni and *Stevia rebaudiana* Morita. *Biomolecules* (2014) 4:374-389; (Produce Reb M2 from Reb A by UGT enzyme).
4. Synthesis of rebaudioside-A by enzymatic transglycosylation of stevioside present in the leaves of *Stevia rebaudiana* Bertoni. *Food Chemistry* (2016) 200:154–158 (Conversion of stevioside to Reb A by stevia leaf crude protein).
5. WO 2014/122227 A2 Methods for improved production of rebaudioside D and rebaudioside M. Evolva. (improve D and M bioconversion in yeast). US Patent application: EP2954058A2. Available online: <https://patents.google.com/patent/EP2954058A2>
6. WO2013/176738A1. High-purity steviol glycosides. Purecircle and coca-cola company. (Identify UGTs involve in biosynthesis of D and X (M)). US Patent application: US20130071339A1. Available online: <https://patents.google.com/patent/US20130071339A1/en>
7. WO 2015/007748A1. Diterpene production. DSM. (De novo biosynthesis of Reb M). Available online: <https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2013110673&tab=PCTBIBLIO&maxRec=1000>

## Appendix 2 Steviol Glycosides Raw Material Flow Chart and Specifications

### **Appendix 2.1 Steviol Glycosides Raw Material Flow Chart**

### **Appendix 2.2 Steviol Glycosides Raw Material Specifications**

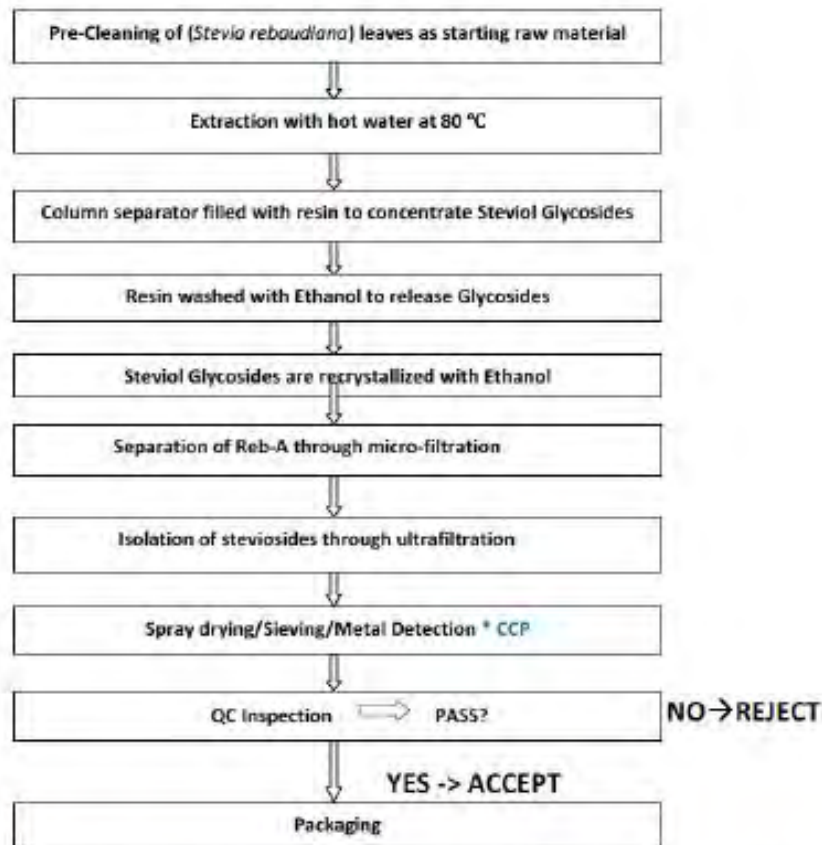
### Appendix 2.1 Steviol Glycosides Raw Material Flow Chart



**A Perfect Blend of Science and Nature**

Product Name: **Steviol Glycosides 95%**

#### PROCESS DIAGRAM



\* CCP: Metal detection. Physical Hazard: Metal impurities. Criterion: Metal equipment used in production line, such as mesh screens. In order to prevent chronic intoxication by metal impurities, product has to go through magnet and metal detector.

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Website: [www.bluecal-ingredients.com](http://www.bluecal-ingredients.com)

**Appendix 2.2 Steviol Glycosides Raw Material Specifications**



*Blue California*<sup>®</sup>

30111 Tomas  
Rancho Santa Margarita, CA 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**PRODUCT SPECIFICATION**

**Product: Stevia Extract 95% ( *Stevia rebaudiana*, leaves )**  
**Item# ST0301238**

<b>Country of Origin:</b>	<b>China</b>	<b>Extraction Solvent:</b>	<b>Not Applicable</b>
<b>Grade:</b>	<b>68th JECFA 2007</b>	<b>Shelf life:</b>	<b>2 Years</b>
<b>ATTRIBUTES</b>	<b>SPECIFICATION</b>	<b>METHODS</b>	
APPEARANCE	WHITE POWDER	VISUAL	
FOREIGN MATTER	ABSENT	VISUAL	
ODOR	CHARACTERISTIC	OLFACTORY	
TASTE	CHARACTERISTIC	GUSTATORY	
<b>STEVIOL GLYCOSIDES</b>	<b>≥ 95%</b>	<b>HPLC</b>	
<b>SOLUBILITY IN WATER</b>	<b>Excellent</b>	<b>USP</b>	
LOSS ON DRYING	< 5%	USP	
HEAVY METALS	< 10 ppm	USP	
LEAD	< 1 ppm	ICP-MS	
ARSENIC	< 1 ppm	ICP-MS	
CADMIUM	< 1 ppm	ICP-MS	
MERCURY	< 1 ppm	ICP-MS	
Solvent Residue: Methanol	< 200 mg/kg	GC	
pH	4.5-7.0	USP	
ASH	< 1%	USP	
BULK DENSITY	> 0.2 g/ml	USP	
TAP DENSITY	≥ 0.3 g/ml	USP	
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP	
TOTAL PLATE COUNT	< 5,000 cfu/gm	AOAC	
TOTAL COLIFORM	< 100 cfu/gm	AOAC	
YEAST AND MOLDS	< 100 cfu/gm	AOAC	
E. COLI	NEGATIVE	AOAC	
SALMONELLA	NEGATIVE	AOAC	

Approved by: ( QA/QC Manager ) Revised date: 06-25-2015

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

### Appendix 3 Regulatory Status of Raw Materials and Processing Aids Used in the Manufacture of BESTEVIA® Rebaudioside E

Raw Material	Use	Regulatory Status	
		21 CFR	Approved Uses
Yeast extract	Fermentation and Culture Media	184.1983	Used as a flavoring agent and adjuvant at levels not to exceed 5% in food.
Yeast Peptone	Fermentation and Culture Media	184.1553	Peptones are GRAS affirmed for use as processing aids
Glycerol	Fermentation and Culture Media		GRAS; standard material used within food industry
Potassium phosphate	Fermentation Medium		GRAS; standard materials used within enzyme industry
Ferric chloride	Fermentation Medium	184.1297	Used as a nutrient supplement and processing aid with no limitation other than cGMP
Ammonia	Fermentation Medium	184.1139	Used as a leavening agent, pH control agent, surface- finished agent, and boiler water additive with no limitation other than cGMP
Sucrose/ sugar	Reaction Medium		GRAS; standard material used within food industry
UDP-glucose	Reaction Medium		FDA's approval of UDP-Glucose is noted in GRAS notice # 00045, GRN 000626 and GRN 000106
Ethanol	Elution solvent Crystallization	182.1	GRAS when used in accordance with cGMP JECFA specifications for steviol glycosides specify a level of not more than 5,000 ppm for ethanol residues
Methanol	Fermentation media and Crystallization	182.1	GRAS when used in accordance with cGMP JECFA specifications for steviol glycosides specify a level of not more than 200 ppm for methanol residues
Activated charcoal	Decolorizing agent		GRAS; standard material used within the food industry
Microporous resin	Purification		Used in accordance with §173.25

## Appendix 4 Analytical Method



Eurofins Scientific, Inc.  
1365 Redwood Way  
Petaluma, CA 94954

### Summary Report

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

Prepared by: (b) (6)  
Hong You, Ph.D., Principal Scientist  
Eurofins Scientific, Inc.

(b) (6)  
Darlene Enriquez, QA Manager  
Eurofins Scientific, Inc.

(b) (6)  
Jason Mulligan, Business Unit Manager  
Eurofins Scientific, Inc.

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Approved by: \_\_\_\_\_  
Hadi Omrani  
Technical Manager-Regulatory Affairs  
Blue California

Date Issued: December 11, 2017





## **I. Study Identification**

### **1. Study Title:**

Method Verification of the Determination of Rebaudioside E by High Performance Liquid Chromatography (HPLC) and Purity Analysis of Five Production Samples

### **2. Study Objective:**

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

### **3. Study Coordinator/Performing Laboratory:**

Hong You, Ph.D., Principal Scientist  
Eurofins Scientific, Inc.

Bailey Ireland, Associate Scientist III  
Eurofins Scientific, Inc.

Darlene Enriquez, QA Manager  
Eurofins Scientific, Inc.

Jason Mulligan, Business Unit Manager  
Eurofins Scientific, Inc.

### **4. Study Monitors:**

Hadi Omrani, Technical Manager-Regulatory Affairs  
Blue California

### **5. Method References:**

Steviol Glycosides Monograph, Prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010), superseding specifications prepared at the 69th JECFA (2008) and published in FAO JECFA Monographs 5 (2008).

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## **II. Study Description**

### **1. Scope:**

This method is applicable to the determination and quantification of rebaudioside E, in *Stevia rebaudiana* raw materials. Rebaudioside E quantitation is determined using the USP stevioside standard with a molecular weight adjustment from stevioside to rebaudioside E. This paradigm is applied to other related steviol glycosides except stevioside and rebaudioside A that are determined by corresponding stevioside and rebaudioside A standard. This study is referred to the method verification packages for rebaudioside M and rebaudioside D that Eurofins performed for Blue California in 2013 and 2016 using JECFA 2010 method.



**2. Test Materials:**

*Stevia rebaudiana* leaf extracts

(1) Eurofins sample 740-2017-10300057, BESTEVIA-E, Powder,  
Lot #20170665-04

(2) Eurofins sample 740-2017-10300058, BESTEVIA-E, Powder,  
Lot #160921-1603

(3) Eurofins sample 740-2017-10300059, BESTEVIA-E, Powder,  
Lot #20131005

(4) Eurofins sample 740-2017-10300060, BESTEVIA-E, Powder,  
Lot #160202-1601

(5) Eurofins sample 740-2017-10300061, BESTEVIA-E, Powder,  
Lot #160921-1602

**3. Test Reagents:**

(1) Acetonitrile (HPLC Grade), Fisher Catalog #: A998-4  
C.A.S #: 75-05-8

(2) Stevioside, USP reference material, Lot # F0I080 (97% purity)  
C.A.S #: 57817-89-1

(3) Rebaudioside A, ChromaDex, Lot # 18226-1008-005 (98.17% purity)  
C.A.S #: 58543-16-1

(4) Rebaudioside E, ChromaDex, Lot # 18235-410 (85.0 % purity)  
C.A.S #: 63279-14-1

Note: This rebaudioside E reference material was only used for identification,  
spiking recovery, and system suitability purposes.

**4. Mobile Phase Preparation:**

A. 32% acetonitrile : 68% sodium phosphate buffer (10 mmol/L, pH = 2.6) (v/v).

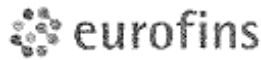
**5. Reference Standards:**

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A. Stock standards.

1. Adjust standard concentration for purity and moisture levels  
(ChromaDex, USP). Corrections were made based on suppliers'  
Certificate of Analysis.

2. On a microbalance, accurately weighed  $5.0 \pm 0.5$  mg of stevioside USP  
standard and  $5.0 \pm 0.5$  mg of rebaudioside A ChromaDex standard;  
quantitatively transferred to a 5-ml volumetric flask with prep solvent  
(70%/30%, water/acetonitrile, v/v).



Sonicated for 15 ± 5 min to dissolve. When warming occurred during sonication, the solution was allowed to cool to room temperature.

Concentration was approximately 1 mg/ml stevioside, rebaudioside A.

B. Calibration standards were USP stevioside and ChromaDex rebaudioside A. The range of quantitation was approximately between 0.2 mg/mL and 1 mg/mL in solution. A 3 point curve was utilized for determination of linearity for this study as well as routine quantitation that covers the range of sample concentrations defined by the method for the current and future samples. The sample test concentration was approximately 1 mg/mL rebaudioside E, based on the expected test sample concentration. The adjusted-stevioside standard curve covered the targeting rebaudioside E sample concentration.

C. System suitability standards (ChromaDex Rebaudioside E, ChromaDex rebaudioside A, USP Stevioside), retention time confirmation standard (ChromaDex Rebaudioside E) and calibration standards (ChromaDex rebaudioside A, USP Stevioside) were utilized. See results section for concentrations.

**6. Single Lab Verification Study Results:**

**A. Primary method: See provided method.**

**B. System Suitability:**

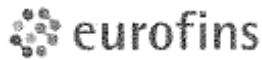
1. Minimum of five injections of an approximately 1.0 mg/ml standard solution were injected during the analysis sequence for rebaudioside E as well as stevioside and rebaudioside A.

2. Acceptance criteria: The system is considered suitable if  
 USP tailing factor of the standard peak must be  $T \leq 2.0$   
 Critical resolution must be  $> 1.5$   
 Standard peak area %RSD  $\leq 2.0$   
 Standard retention time %RSD  $\leq 2.0$

Standard peak area and retention time results are as follows:

	Reb E	Reb A	Stevioside
Retention time (RT) Range (minutes)	2.98 -3.02	6.53-6.77	6.92-7.11
RT % RSD	0.464	1.45	1.03
Peak area range	1272-1300	1406-1418	1631-1641
Peak area RSD	0.619	0.293	0.247
Number of Data Points	12	6	6

**Rebaudioside E, rebaudioside A, and stevioside standard retention time %RSD passed the criteria of less than 2%.**



**Rebaudioside E, rebaudioside A, and stevioside standard peak area %RSD passed the criteria of less than 2%.**

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

Beginning of run;  
**USP Tailing Reb E = 1.08**

End of run;  
**USP Tailing Reb E = 1.06**

There is a slight coelution between rebaudioside E and rebaudioside D in all testing samples; **critical resolution for rebaudioside E was 0.862** which is smaller than the 1.5 criteria. However, the error that is generated by the peak coelution has been minimized by using “New Exponential” Tangent Skim Mode in the peak integration ChemStation setup (Bicking M., Integration Errors in Chromatographic Analysis, Part I: Peaks of Approximately Equal Size. LCGC North America, 2006, Volume 24, Issue 4, p. 402–414).

4. The retention time and identity for Rebaudioside E and Rebaudioside D (major impurity peak in the testing samples) were confirmed using the ChromaDex Rebaudioside E and historical Rebaudioside D’s retention time record.

**C. Linearity:**

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

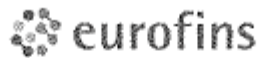
Stock (uL)	Relative Concentration (mg/mL)
5	1.16586
2.5	0.58293
1	0.23317

**Linearity Results Rebaudioside E:**

Correlation Coefficient	Criteria	PASS/FAIL
<b>0.99999</b>	<b>&gt; 0.999</b>	<b>PASS</b>

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

Stock (uL)	Concentration (mg/mL)
5	0.97155
2.5	0.48578
1	0.19431



**Linearity Results Stevioside:**

Correlation Coefficient	Criteria	PASS/FAIL
0.99999	>0.999	PASS

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

Stock (uL)	Concentration (mg/mL)
5	0.97856
2.5	0.48928
1	0.19571

**Linearity Results Rebaudioside A:**

Correlation Coefficient	Criteria	PASS/FAIL
1.0000	>0.999	PASS

2. The relative standard deviation (RSD) for the response factor ((amount/area) mg/mL/MAU) was determined between calibration levels. The RSD expressed as a percent is to achieve a specification of <5%. The %RSDs achieved between calibration levels was acceptable at **1.3639%** for rebaudioside E, **1.3633%** for stevioside, and **0.58272%** for rebaudioside A.

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

1. Perform selectivity procedures:
  - a. Analyze at least one prep solvent blank.
2. Results:
  - a. **Three preparation solvent blanks were tested throughout the duration of the study. The chromatograms were free of interfering peaks.**

**E. Accuracy (Recovery):**

C. Accuracy test was determined by spiking a sample of known value (740-2017-10300057) with standard stock solution in multiple levels at the beginning of the study. The low level (0.5 mL) spike was tested in duplicate and the high level (1 mL) spike was tested in triplicate. The analyzed final results were used to compare to their theoretical results for the percentage recovery result. This test was used to determine if the method can accurately determine the analyte results without significant matrix interference. According to the AOAC Single Laboratory Validation Guideline, 95-102% was set as passing criteria. Results and Acceptance Criteria are as follows:

<u>Spiked stock(mL)</u>	<u>Avg. Recovery%</u>	<u>Acceptance criteria</u>	<u>PASS/FAIL</u>
0.5 (in duplicate)	100.2	95-102%	PASS
1.0 (in triplicate)	102.3	95-102%	PASS



**F. Repeatability (Precision):**

Five Bestevia testing samples were analyzed for purity. Stevioside stock standard was prepared at about 1 mg/mL (5 uL, 2.5 uL and 1 uL were injected into HPLC for establishing standard curve), which leads to a range of 0.23-1.17 mg/mL for the converted standard curve for rebaudioside E determination (conversion factor = 1.2 according the conversion paradigm of JECFA 2010). The testing samples were prepared at approximately 1 mg/mL. % RSD for precision measurements shall be less than 2.

A rebaudioside D signal and two additional trivial signals (stevioside, steviolbioside) can be detected in all the Bestevia samples.

Sample results are as follows:

740-2017-10300057					
Compound	Run 1 Result (%w/w)	Run 2 Result (%w/w)	Run 3 Result (%w/w)	Average	Relative Standard Deviation
Rebaudioside E	77.446	77.431	77.280	77.386	0.119
Rebaudioside D	10.280	10.217	10.238	10.245	0.312
Stevioside	0.370	0.332	0.308	0.337	9.283
Steviolbioside	1.287	1.166	1.150	1.201	6.271
Total	89.384	89.145	88.975	89.168	0.230
740-2017-10300058					
Compound	Run 1 Result (%w/w)	Run 2 Result (%w/w)	Run 3 Result (%w/w)	Average	Relative Standard Deviation
Rebaudioside E	77.196	77.079	76.765	77.013	0.289
Rebaudioside D	10.447	10.393	10.356	10.398	0.440
Stevioside	0.339	0.310	0.350	0.333	6.146
Steviolbioside	1.225	1.326	1.035	1.195	12.359
Total	89.206	89.108	88.506	88.940	0.426
740-2017-10300059					
Compound	Run 1 Result (%w/w)	Run 2 Result (%w/w)	Run 3 Result (%w/w)	Average	Relative Standard Deviation
Rebaudioside E	77.025	77.061	77.025	77.037	0.027
Rebaudioside D	10.343	10.396	10.399	10.379	0.300
Stevioside	0.337	0.340	0.371	0.349	5.455
Steviolbioside	1.365	1.315	0.965	1.215	17.955
Total	89.070	89.111	88.760	88.981	0.216



740-2017-10300060					
	Run 1	Run 2	Run 3		
Compound	Result (%w/w)	Result (%w/w)	Result (%w/w)	Average	Relative Standard Deviation
Rebaudioside E	76.498	76.590	76.038	76.375	0.387
Rebaudioside D	11.225	11.234	10.688	11.049	2.829
Stevioside	0.346	0.332	0.338	0.339	1.940
Steviolbioside	0.989	0.880	1.096	0.988	10.899
Total	89.058	89.036	88.160	88.751	0.577
740-2017-10300061					
	Run 1	Run 2	Run 3		
Compound	Result (%w/w)	Result (%w/w)	Result (%w/w)	Average	Relative Standard Deviation
Rebaudioside E	77.605	77.756	78.027	77.796	0.275
Rebaudioside D	10.599	10.647	10.712	10.653	0.532
Stevioside	0.267	0.268	0.274	0.270	1.279
Steviolbioside	1.003	0.984	0.943	0.977	3.186
Total	89.474	89.656	89.955	89.695	0.270

**G. Moisture Correction:**

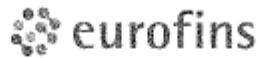
All of the results in above tables were adjusted for the moisture content and reported on the dry weight basis. Stevioside standard was weighed out and analyzed after 2 hours of oven-drying to eliminate moisture influence. The moisture content of Rebaudioside A standard was adjusted according to supplier’s Certificate of Analysis. Moisture contents of positive control sample and testing samples were measured using Karl Fischer titration on the same day that the experiment was performed.

The equation for moisture correction is as follows:

$$\text{Dry weight basis} = \text{testing result as is} / (100 - \% \text{ moisture} / 100).$$

Dry-basis results are listed below:

740-2017-10300057						
	Moisture	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 E	11.157	87.172	87.155	86.984	87.104	0.119
Rebaudioside D		11.571	11.500	11.523	11.531	0.312
Stevioside		0.417	0.373	0.347	0.379	9.283
Steviolbioside		1.449	1.312	1.294	1.352	6.271
Total		100.608	100.340	100.149	100.366	0.230



Method Verification JEFCA 2010, Modified for Rebaudioside E  
Page 9 of 10

740-2017-10300058						
Moisture	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 E	11.142	86.876	86.743	86.391	86.670	0.289
Rebaudioside D		11.756	11.696	11.654	11.702	0.440
Stevioside		0.381	0.349	0.393	0.375	6.146
Steviolbioside		1.378	1.493	1.165	1.345	12.359
Total		100.392	100.281	99.604	100.092	0.426
740-2017-10300059						
Moisture	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 E	11.083	86.626	86.666	86.626	86.639	0.027
Rebaudioside D		11.632	11.691	11.695	11.673	0.300
Stevioside		0.379	0.382	0.417	0.393	5.455
Steviolbioside		1.536	1.479	1.085	1.367	17.955
Total		100.17	100.22	99.82	100.07	0.216
740-2017-10300060						
Moisture	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 E	10.849	85.807	85.910	85.291	85.669	0.387
Rebaudioside D		12.591	12.601	11.989	12.394	2.829
Stevioside		0.388	0.373	0.380	0.380	1.940
Steviolbioside		1.109	0.987	1.229	1.109	10.899
Total		99.90	99.87	98.89	99.55	0.577
740-2017-10300061						
Moisture	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 E	11.016	87.212	87.382	87.686	87.427	0.275
Rebaudioside D		11.911	11.966	12.038	11.971	0.532
Stevioside		0.300	0.302	0.308	0.303	1.279
Steviolbioside		1.128	1.106	1.059	1.098	3.186
Total		100.55	100.75	101.09	100.80	0.270





## 7. Conclusions:

The results generally met or exceed the acceptance criteria as established in the method verification proposal. All analyses were performed on Agilent 1100 series HPLCs with Agilent ChemStation software. The primary objective of the study was to show that the method as designed can be used to quantitatively determine the concentration of rebaudioside E. The results showed that the JECFA 2010 method is suitable, linear, specific, precise and accurate for testing rebaudioside E in "BESTEVIA-E, Powder".

Quantification of rebaudioside E was accomplished using conversion factor-adjusted USP stevioside reference material calibration curve as described in the modified JECFA 2010 method. Limit of detection and limit of quantitation were beyond the scope of this project due to the concentrated nature of the samples. Additional work can be performed to determine these limits upon requested.

The control material of JECFA monograph method was not ran as planned for determining method Accuracy. This is because rebaudioside E is out of the method's original scope. Instead, Accuracy test was demonstrated by spiking a sample of known value (740-2017-10300057) with rebaudioside E reference material solution in multiple levels.

Five lots of "BESTEVIA-E, Powder" were tested using this method. The results have shown accurate and precise determination of rebaudioside E as well as identification and quantitation of three additional peaks (rebaudioside D, stevioside, and steviolbioside). After moisture correction, rebaudioside E dry-basis results are between 85.7-87.4% across the five "BESTEVIA-E, Powder" lots. There is a modest peak coelution between rebaudioside E and rebaudioside D in all sample chromatograms (ChemStation resolution between rebaudioside E and rebaudioside D was 0.862). The impact was considered as trivial and has been minimized by using "New Exponential" Tangent Skim Mode in the ChemStation peak integration setup (Bicking M., Integration Errors in Chromatographic Analysis, Part I: Peaks of Approximately Equal Size. LCGC North America, 2006, Volume 24, Issue 4, p. 402-414).

## Appendix 5      Certificates of Analysis for Multiple Lots of BESTEVIA® Rebaudioside E

**Appendix 3.1 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 3.2 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 3.3 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 3.4 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 3.5 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 3.1 BESTEVIA® Rebaudioside E (b) (6)**



*Blue California*™

30111 Tomas  
Rancho Santa Margarita, CA 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**CERTIFICATE OF ANALYSIS**

**Product: BESTEVIA® Rebaudioside E**  
**Item# BE17075E1**

<b>Lot No:</b> (b) (6)	<b>Original Manufacturer:</b> Blue California Co.
<b>Date of Manufacturing:</b> October 5-2013	<b>Expiration/Re-test date:</b> October 5-2015
<b>QC acceptance date:</b> October 24-2013	<b>Country of Origin:</b> China

This product has NOT been treated by irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	WHITE POWDER	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	CHARACTERISTIC	GUSTATORY	PASS
STEVIOL GLYCOSIDES	≥ 95%	HPLC	98.5%
REBAUDIOSIDE E	≥ 85%	HPLC	90%
LOSS ON DRYING	≤ 5%	USP 34	1.2%
HEAVY METALS	< 10 ppm	USP 34	PASS
ARSENIC	< 0.5 ppm	ICP-MS	< 0.5 ppm
CADMIUM	< 0.5 ppm	ICP-MS	< 0.25 ppm
LEAD	< 0.5 ppm	ICP-MS	< 0.05 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 100 ppm
METHANOL	< 200 ppm	USP 34	< 200 ppm
ASH	≤ 1%	USP 34	0.12%
PH	4.5-7	USP 34	5.2
SOLUBILITY IN WATER	3.5% clear solution in 10 minutes		PASS
BULK DENSITY	≥ 0.15 g/ml	USP 34	PASS
TAP DENSITY	≥ 0.30 g/ml	USP 34	PASS
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 34	PASS
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 10 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	Not detected
SALMONELLA	NEGATIVE	AOAC	Not detected
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Issue date: 10-08-2018

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

**Appendix 3.2 BESTEVIA® Rebaudioside E** (b) (6)



*Blue California*®

30111 Tomas  
Rancho Santa Margarita, CA. 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**CERTIFICATE OF ANALYSIS**

**Product: BESTEVIA® Rebaudioside E**  
**Item# BE17073E1**

Lot No:	(b) (6)	Original Manufacturer:	Blue California Co.
Date of Manufacturing:	February 02-2016	Expiration/Re-test date:	February 02-2018
QC acceptance date:	February 17-2016	Country of Origin:	China

This product has NOT been treated by irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	Off white to white powder	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	CHARACTERISTIC	GUSTATORY	PASS
STEVIOL GLYCOSIDES	≥ 95%	HPLC	97%
REBAUDIOSIDE E	≥ 85%	HPLC	90.5%
LOSS ON DRYING	≤ 5%	USP 34	1.48%
HEAVY METALS	< 10 ppm	USP 34	PASS
ARSENIC	< 0.5 ppm	ICP-MS	< 0.5 ppm
CADMIUM	< 0.5 ppm	ICP-MS	< 0.25 ppm
LEAD	< 0.5 ppm	ICP-MS	< 0.05 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 200 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
ASH	≤ 1%	USP 34	0.15%
PH	4.5-7	USP 34	5.5
SOLUBILITY IN WATER	3.5% clear solution in 10 minutes		PASS
BULK DENSITY	≥ 0.15 g/ml	USP 34	PASS
TAP DENSITY	≥ 0.30 g/ml	USP 34	PASS
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 34	PASS
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 10 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	Not detected
SALMONELLA	NEGATIVE	AOAC	Not detected
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revision date: 10-08-2018

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

**Appendix 3.3 BESTEVIA® Rebaudioside E (b) (6)**



*Blue California*™

30111 Tomas  
Rancho Santa Margarita, CA 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**CERTIFICATE OF ANALYSIS**

**Product: BESTEVIA® Rebaudioside E**  
**Item# BE17073E1**

<b>Lot No:</b>	(b) (6)	<b>Original Manufacturer:</b>	Blue California Co.
<b>Date of Manufacturing:</b>	September 21-2016	<b>Expiration/Re-test date:</b>	September 21-2018
<b>QC acceptance date:</b>	October 25-2016	<b>Country of Origin:</b>	China

This product has NOT been treated by irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	Off white to white powder	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	CHARACTERISTIC	GUSTATORY	PASS
STEVIOL GLYCOSIDES	≥ 95%	HPLC	98.3%
REBAUDIOSIDE E	≥ 85%	HPLC	91.2%
LOSS ON DRYING	≤ 5%	USP 34	1.12%
HEAVY METALS	< 10 ppm	USP 34	PASS
ARSENIC	< 0.5 ppm	ICP-MS	< 0.5 ppm
CADMIUM	< 0.5 ppm	ICP-MS	< 0.25 ppm
LEAD	< 0.5 ppm	ICP-MS	< 0.05 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 200 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
ASH	≤ 1%	USP 34	0.15%
PH	4.5-7	USP 34	5.2
SOLUBILITY IN WATER	3.5% clear solution in 10 minutes		PASS
BULK DENSITY	≥ 0.15 g/ml	USP 34	PASS
TAP DENSITY	≥ 0.30 g/ml	USP 34	PASS
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP 34	PASS
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 10 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	Not detected
SALMONELLA	NEGATIVE	AOAC	Not detected
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 10-08-2018

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

**Appendix 3.4 BESTEVIA® Rebaudioside E (b) (6)**



*Blue California*™

30111 Tomas  
Rancho Santa Margarita, CA. 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**CERTIFICATE OF ANALYSIS**

**Product: BESTEVIA® Rebaudioside E**  
**Item# BE17075E1**

Lot No: (b) (6) Original Manufacturer: Blue California  
Date of Manufacturing: November 01-2016 Expiration/Re-test date: November 01-2018  
QC acceptance date: November 18-2016 Country of Origin: China  
This product has NOT been treated by irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	Off white to white powder	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	CHARACTERISTIC	GUSTATORY	PASS
STEVIOL GLYCOSIDES	≥ 95%	HPLC	99.5%
REBAUDIOSIDE E	≥ 85%	HPLC	86.7%
LOSS ON DRYING	≤ 5%	USP 34	1.29%
HEAVY METALS	< 10 ppm	USP 34	PASS
ARSENIC	< 0.5 ppm	ICP-MS	< 0.5 ppm
CADMIUM	< 0.5 ppm	ICP-MS	< 0.25 ppm
LEAD	< 0.5 ppm	ICP-MS	< 0.05 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 200 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
ASH	≤ 1%	USP 34	0.25 %
PH	4.5-7	USP 34	4.95
SOLUBILITY IN WATER	3.5% clear solution in 10 minutes		PASS
BULK DENSITY	≥ 0.15 g/ml	USP 34	PASS
TAP DENSITY	≥ 0.30 g/ml	USP 34	PASS
PARTICLE SIZE:	> 95% through Mesh #60 Sieve	USP 34	PASS
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 10 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	Not detected
SALMONELLA	NEGATIVE	AOAC	Not detected
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised: 10-08-2018

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

**Appendix 3.5 BESTEVIA® Rebaudioside E** (b) (6)



*Blue California*™

30111 Tomas  
Ramirez Santa Margarita, CA. 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**CERTIFICATE OF ANALYSIS**

**Product: BESTEVIA® Rebaudioside E**  
**Item# BE17075E1**

<b>Lot No:</b>	(b) (6)	<b>Original Manufacturer:</b>	Blue California
<b>Date of Manufacturing:</b>	June 30-2017	<b>Expiration/Re-test date:</b>	June 30-2020
<b>QC acceptance date:</b>	July 19-2017	<b>Country of Origin:</b>	China

This product has NOT been treated by irradiation or ETO

ATTRIBUTES	SPECIFICATION	METHODS	RESULTS
APPEARANCE	Off white to white powder	VISUAL	PASS
FOREIGN MATTER	ABSENT	VISUAL	PASS
ODOR	CHARACTERISTIC	OLFACTORY	PASS
TASTE	CHARACTERISTIC	GUSTATORY	PASS
STEVIOL GLYCOSIDES	≥ 95%	HPLC	99.3%
REBAUDIOSIDE E	≥ 85%	HPLC	85.4%
LOSS ON DRYING	≤ 5%	USP 34	1.33%
HEAVY METALS	< 10 ppm	USP 34	PASS
ARSENIC	< 0.5 ppm	ICP-MS	< 0.5 ppm
CADMIUM	< 0.5 ppm	ICP-MS	< 0.25 ppm
LEAD	< 0.5 ppm	ICP-MS	< 0.05 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 200 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
ASH	≤ 1%	USP 34	0.1%
PH	4.5-7	USP 34	5.6
SOLUBILITY IN WATER	3.5% clear solution in 10 minutes		PASS
BULK DENSITY	≥ 0.15 g/ml	USP 34	PASS
TAP DENSITY	≥ 0.30 g/ml	USP 34	PASS
PARTICLE SIZE:	> 95% through Mesh #60 Sieve	USP 34	PASS
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 10 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	Not detected
SALMONELLA	NEGATIVE	AOAC	Not detected
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 10-08-2018

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- \* THIS PRODUCT SHOULD BE STORED SEALED IN A COOL AND DRY PLACE.

## Appendix 6 Analytical Chromatograms for Multiple Production Lots of BESTEVIA® Rebaudioside E

Appendix 6.1 BESTEVIA® Rebaudioside E (b) (6)

Appendix 6.2 BESTEVIA® Rebaudioside E (b) (6)

Appendix 6.3 BESTEVIA® Rebaudioside E (b) (6)

Appendix 6.4 BESTEVIA® Rebaudioside E (b) (6)

Appendix 6.5 BESTEVIA® Rebaudioside E (b) (6)

**Note: The following key identifies corresponding analytical sample and production batch numbers**

(1) Eurofins sample 740-2017-10300057, BESTEVIA-E, Powder,  
(b) (6) ) (6)

(2) Eurofins sample 740-2017-10300058, BESTEVIA-E, Powder,  
(b) (6)

(3) Eurofins sample 740-2017-10300059, BESTEVIA-E, Powder,  
(b) (6)

(4) Eurofins sample 740-2017-10300060, BESTEVIA-E, Powder,  
(b) (6)

(5) Eurofins sample 740-2017-10300061, BESTEVIA-E, Powder,  
(b) (6)



**Appendix 6.1 BESTEVIA® Rebaudioside E (b) (6)**

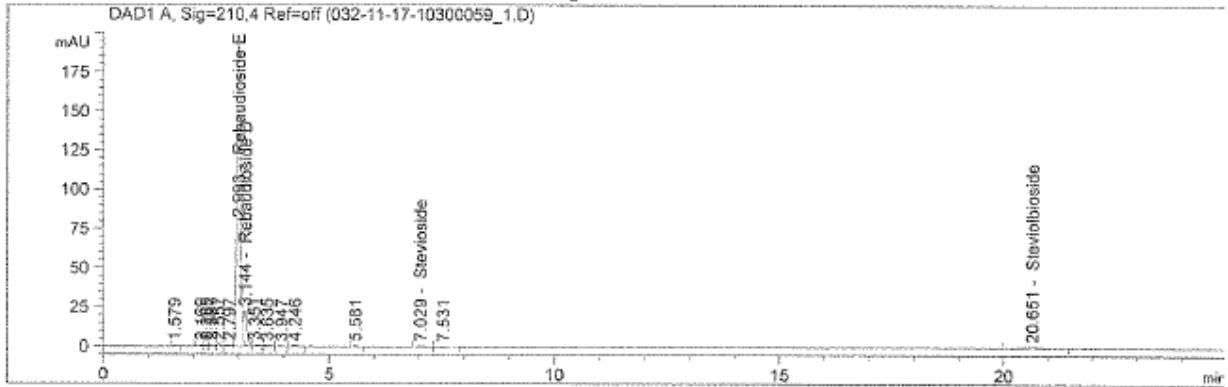
Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\032-11-17-10300059\_1.D  
Sample Name: 17-10300059\_1

```

=====
Acq. Operator   : Bailey Ireland           Seq. Line : 32
Acq. Instrument : HPLC-07                     Location  : 11
Injection Date  : 11/28/2017 5:50:16 AM      Inj       : 1
                                           Inj Volume: 5.000 µl

Sequence File   : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\KK262-17-2210.S
Acq. Method     : D:\Chem32\3\Data\KK262-17-2210 2017-11-27 13-29-31\LCKK262.M
Last changed    : 11/28/2017 1:36:07 AM by Bailey Ireland
Analysis Method : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\LCKK262.M (Sequence
Method)
Last changed    : 12/11/2017 4:22:36 PM by Hong You
Method Info     : JBCPA kk262

ECM Server      : http://us05apvp001/ecmwg
ECM Operator    : Hong You
ECM Path        : \Petaluma\LC\HPLC-07\Data\KK262-17-2210 2017-11-27 13-29-31.SC.SSIzip
ECM Version     : 13 (modified after loading)
    
```



=====  
ESTD Percent Report  
=====

```

Sorted By      : Signal
Calib. Data Modified : 12/11/2017 4:22:36 PM
Multiplier     : 1.0000
Dilution       : 40.0000
Sample Amount  : 40.25000 [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs
    
```

Signal 1: DAD1 A, Sig=210,4 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
2.993	VV R	1072.11487	7.22929e-4	37.024862		Rebaudioside E
3.144	VV E	123.40147	8.43417e-4	10.343244		Rebaudioside D
6.733		-	-	-		Rebaudioside A
7.029	BV	5.62568	6.02447e-4	0.335813		Stevioside
8.217		-	-	-		Rebaudioside F
8.917		-	-	-		Rebaudioside C
9.704		-	-	-		Dulcoside A
13.344		-	-	-		Rubusoside
18.279		-	-	-		Rebaudioside B
20.651	BB	28.50772	4.81953e-4	1.365403		Steviolbioside

Sample Name: (b) (6)

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
Totals :				89.070322		

2 Warnings or Errors :

- Warning : Calibration warnings (see calibration table listing)
- Warning : Calibrated compound(s) not found

\*\*\*\*\*  
\*\*\* End of Report \*\*\*

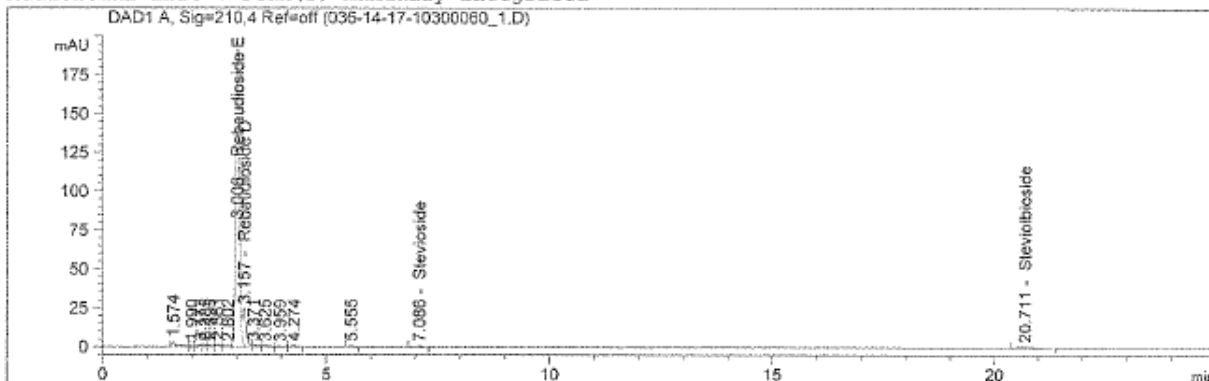
**Appendix 6.2 BESTEVIA® Rebaudioside E** (b) (6)

Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\036-14-17-10300060\_1.D  
Sample Name: (b) (6)

```

=====
Acq. Operator   : Bailey Ireland           Seq. Line : 36
Acq. Instrument : HPLC-07                   Location  : 14
Injection Date  : 11/28/2017 7:56:51 AM    Inj       : 1
                                           Inj Volume: 5.000 µl
Sequence File   : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\KK262-17-2210.S
Acq. Method     : D:\Chem32\3\Data\KK262-17-2210 2017-11-27 13-29-31\LCKK262.M
Last changed    : 11/28/2017 1:36:07 AM by Bailey Ireland
Analysis Method : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\LCKK262.M (Sequence
Method)
Last changed    : 12/11/2017 4:22:36 PM by Hong You
Method Info     : JECFA kk262

ECM Server      : http://us05apvp001/ecmwg
ECM Operator    : Hong You
ECM Path        : \Petaluma\LC\HPLC-07\Data\KK262-17-2210 2017-11-27 13-29-31.SC.SSIzip
ECM Version     : 13 (modified after loading)
Additional Info : Peak(s) manually integrated
    
```



=====  
ESTD Percent Report  
=====

```

Sorted By      : Signal
Calib. Data Modified : 12/11/2017 4:22:36 PM
Multiplier     : 1.0000
Dilution       : 40.0000
Sample Amount  : 40.31000 [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs
    
```

Signal 1: DAD1 A, Sig=210,4 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
3.006	VV R	1066.36182	7.22929e-4	75.497507		Rebaudioside E
3.157	VV E	134.12343	8.43417e-4	11.225262		Rebaudioside D
6.733		-	-	-		Rebaudioside A
7.086	BV	5.77949	6.02447e-4	0.345506		Stevioside
8.217		-	-	-		Rebaudioside F
8.917		-	-	-		Rebaudioside C
9.704		-	-	-		Dulcoside A
13.344		-	-	-		Rubusoside
18.279		-	-	-		Rebaudioside B

Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\036-14-17-10300060\_1.D  
Sample Name: (b) (6)

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
20.711	BB	20.68238	4.81953e-4	0.989127		Steviolbioside
Totals :				89.057342		

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)  
Warning : Calibrated compound(s) not found

\*\*\*\*\*  
\*\*\* End of Report \*\*\*

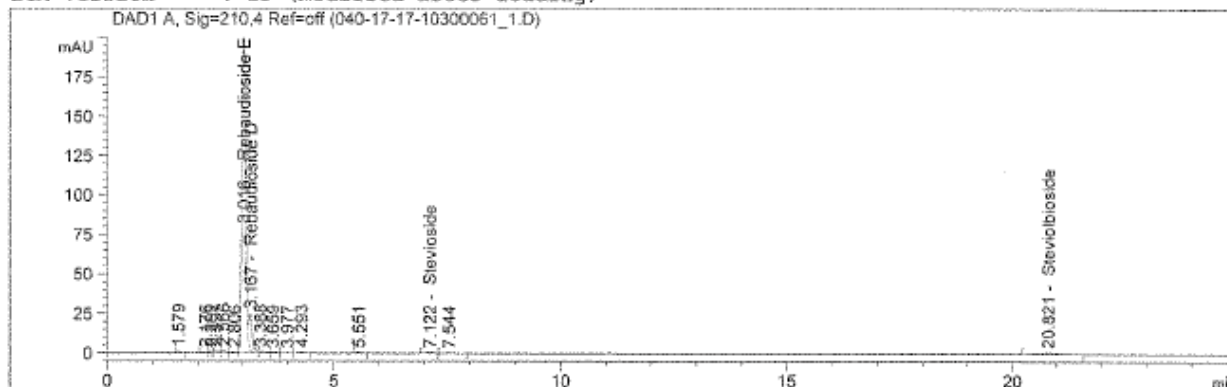
**Appendix 6.3 BESTEVIA® Rebaudioside E** (b) (6)

Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\040-17-17-10300061\_1.D  
Sample Name: (b) (6)

```

=====
Acq. Operator   : Bailey Ireland           Seq. Line : 40
Acq. Instrument : HPLC-07                     Location  : 17
Injection Date  : 11/28/2017 10:03:18 AM      Inj       : 1
                                           Inj Volume: 5.000 µl
Sequence File   : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\KK262-17-2210.S
Acq. Method     : D:\Chem32\3\Data\KK262-17-2210 2017-11-27 13-29-31\LCKK262.M
Last changed    : 11/28/2017 1:36:07 AM by Bailey Ireland
Analysis Method : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\LCKK262.M (Sequence
Method)
Last changed    : 12/11/2017 4:22:36 PM by Hong You
Method Info     : JECFA kk262

ECM Server      : http://us05apvp001/ecmwg
ECM Operator    : Hong You
ECM Path        : \Petaluma\LC\HPLC-07\Data\KK262-17-2210 2017-11-27 13-29-31.SC.SSizip
ECM Version     : 13 (modified after loading)
    
```



ESTD Percent Report

```

Sorted By      : Signal
Calib. Data Modified : 12/11/2017 4:22:36 PM
Multiplier     : 1.0000
Dilution       : 40.0000
Sample Amount  : 43.20000 [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs
    
```

Signal 1: DAD1 A, Sig=210,4 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
3.016	VV R	1159.36157	7.22929e-4	72.605169		Rebaudioside E
3.167	VV E	135.71658	8.43417e-4	10.598673		Rebaudioside D
6.733		-	-	-		Rebaudioside A
7.122	BV	4.78804	6.02447e-4	0.267087		Stevioside
8.217		-	-	-		Rebaudioside F
8.917		-	-	-		Rebaudioside C
9.704		-	-	-		Dulcoside A
13.344		-	-	-		Rubusoside
18.279		-	-	-		Rebaudioside B
20.821	BB	22.48650	4.81953e-4	1.003465		Steviolbioside

Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\040-17-17-10300061\_1.D  
Sample Name: (b) (6)

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
Totals :				89.474395		

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)  
Warning : Calibrated compound(s) not found

=====  
\*\*\* End of Report \*\*\*

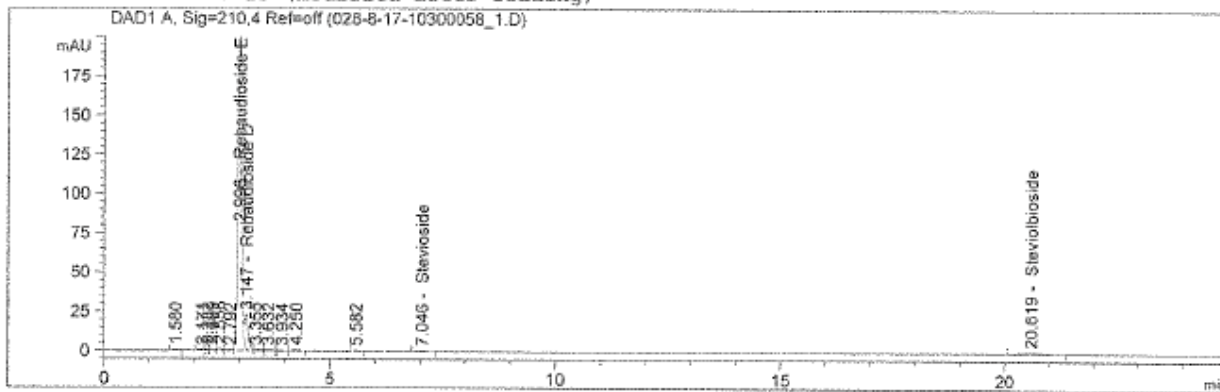
**Appendix 6.4 BESTEVIA® Rebaudioside E** (b) (6)

Sample Name: (b) (6)

```

=====
Acq. Operator   : Bailey Ireland           Seq. Line : 28
Acq. Instrument : HPLC-07                   Location  : 8
Injection Date  : 11/28/2017 3:43:56 AM    Inj       : 1
                                           Inj Volume: 5.000 µl
Sequence File   : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\KK262-17-2210.S
Acq. Method     : D:\Chem32\3\Data\KK262-17-2210 2017-11-27 13-29-31\LCKK262.M
Last changed    : 11/28/2017 1:36:07 AM by Bailey Ireland
Analysis Method : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\LCKK262.M (Sequence
Method)
Last changed    : 12/11/2017 4:22:36 PM by Hong You
Method Info     : JECFA kk262

ECM Server      : http://us05apvp001/ecmwg
ECM Operator    : Hong You
ECM Path        : \Petaluma\LC\HPLC-07\Data\KK262-17-2210 2017-11-27 13-29-31.SC.SSizip
ECM Version     : 13 (modified after loading)
    
```



ESTD Percent Report

```

Sorted By      : Signal
Calib. Data Modified : 12/11/2017 4:22:36 PM
Multiplier     : 1.0000
Dilution       : 40.0000
Sample Amount  : 40.94000 [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs
    
```

Signal 1: DAD1 A, Sig=210,4 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
2.996	VV R	1092.91797	7.22929e-4	77.196076		Rebaudioside E
3.147	VV E	126.76997	8.43417e-4	10.446501		Rebaudioside D
6.733		-	-	-		Rebaudioside A
7.046	BV	5.75810	6.02447e-4	0.338930		Stevioside
8.217		-	-	-		Rebaudioside F
8.917		-	-	-		Rebaudioside C
9.704		-	-	-		Dulcoside A
13.344		-	-	-		Rubusoside
18.279		-	-	-		Rebaudioside B
20.619	BB	26.01128	4.81953e-4	1.224837		Steviolbioside

Sample Name: (b) (6)

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
Totals :				89.206344		

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

Warning : Calibrated compound(s) not found

\*\*\* End of Report \*\*\*



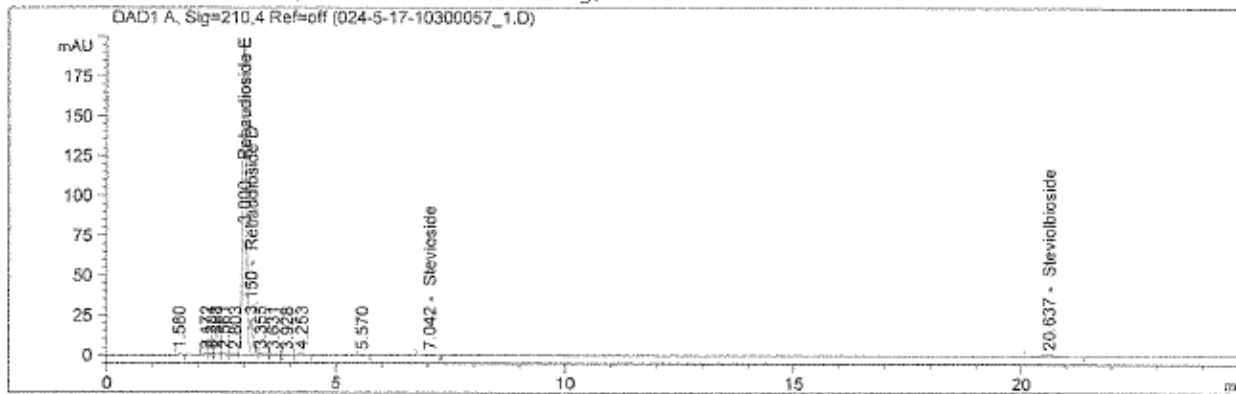
**Appendix 6.5 BESTEVIA® Rebaudioside E (b) (6)**

Sample Name: (b) (6)

```

=====
Acq. Operator   : Bailey Ireland           Seq. Line :   24
Acq. Instrument : HPLC-07                       Location  :    5
Injection Date  : 11/28/2017 1:37:34 AM    Inj       :    1
                                           Inj Volume: 5.000 µl
Sequence File   : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\KK262-17-2210.S
Acq. Method     : D:\Chem32\3\Data\KK262-17-2210 2017-11-27 13-29-31\LCKK262.M
Last changed    : 11/28/2017 1:36:07 AM by Bailey Ireland
Analysis Method : d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\LCKK262.M (Sequence
Method)
Last changed    : 12/11/2017 4:22:36 PM by Hong You
Method Info     : JECFA kk262

ECM Server      : http://us05apvp001/ecmwg
ECM Operator    : Hong You
ECM Path        : \Petaluma\LC\HPLC-07\Data\KK262-17-2210 2017-11-27 13-29-31.SC.SSIzip
ECM Version     : 13 (modified after loading)
    
```



ESTD Percent Report

```

Sorted By      : Signal
Calib. Data Modified : 12/11/2017 4:22:36 PM
Multiplier     : 1.0000
Dilution       : 40.0000
Sample Amount  : 39.51000 [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs
    
```

Signal 1: DAD1 A, Sig=210,4 Ref=off

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
3.000	VV-R	1058.16357	7.22929e-4	77.446407		Rebaudioside E
3.150	VV-E	120.38830	8.43417e-4	10.279679		Rebaudioside D
6.733		-	-	-		Rebaudioside A
7.042	BV	6.06872	6.02447e-4	0.370143		Stevioside
8.217		-	-	-		Rebaudioside F
8.917		-	-	-		Rebaudioside C
9.704		-	-	-		Dulcoside A
13.344		-	-	-		Rubusoside
18.279		-	-	-		Rebaudioside B
20.637	BB	26.38303	4.81953e-4	1.287306		Steviolbioside

Data File d:\Chem32\6\Data\KK262-17-2210 2017-11-27 13-29-31 (a)\024-5-17-10300057\_1.D

Sample Name: (b) (6)

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount %	Grp	Name
Totals :				89.383535		

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

Warning : Calibrated compound(s) not found

=====  
\*\*\* End of Report \*\*\*

## Appendix 7 Pesticide Testing Reports for BESTEVIA® Rebaudioside E

**Appendix 7.1 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 7.2 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 7.3 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 7.4 BESTEVIA® Rebaudioside E** (b) (6)

**Appendix 7.5 BESTEVIA® Rebaudioside E** (b) (6)

Appendix 7.1 BESTEVIA® Rebaudioside E (b) (6)



Supplement Analysis Center

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January 09, 2018

Hadi Omrani  
Blue California Co.  
30111 Tomas  
Rancho Santa Margarita, CA 92688

CERTIFICATE OF ANALYSIS

AR-17-KK-014578-02

Batch #: EUCAPE-00095538

This analytical report supersedes AR-17-KK-014578-01.

Sample Identification:

Sample #: (b) (6)  
Description: Bestevia Rebaudioside E, Powder, Lot #20131005  
Condition: White powder in a ziplock bag received at room temperature.  
Date Received: October 30, 2017

QA12C: Pesticides - USP 561 Screen (USP 39)

Method Reference: USP 561

Completed: 11/08/2017

	Result
Acephate	<0.10 mg/kg
[Method performed by an outsource lab.]	
Alachlor	<0.02 mg/kg
Aldrin and Dieldrin (sum of)	<0.02 mg/kg
Azinphos-ethyl	<0.02 mg/kg
Azinphos-methyl	<0.05 mg/kg
Bromophos-ethyl	<0.02 mg/kg
Bromophos-methyl	<0.02 mg/kg
Bromopropylate	<0.05 mg/kg
Chlordane (sum of cis-, trans- and Oxychlordane)	<0.05 mg/kg
Chlorfenvinphos	<0.02 mg/kg
Chlorpyrifos-ethyl	<0.02 mg/kg
Chlorpyrifos-methyl	<0.02 mg/kg
Chlorthal-dimethyl	<0.01 mg/kg
Cyfluthrin (sum of)	<0.10 mg/kg
Cyhalothrin, lambda-	<0.02 mg/kg
Cypermethrin and isomers (sum of)	<0.1 mg/kg
DDT (total)	<0.02 mg/kg
Deltamethrin	<0.10 mg/kg
Diazinon	<0.02 mg/kg
Dichlofluanid	<0.02 mg/kg
Dichlorvos	<0.02 mg/kg
Dicofol	<0.02 mg/kg
Dimethoate/Omethoate (sum)	<0.10 mg/kg
Endosulfan (sum of isomers and endo. sulfate)	<0.02 mg/kg
Endrin	<0.02 mg/kg
Ethion	<0.02 mg/kg
Etrimfos	<0.05 mg/kg
Fenchlorphos (sum)	<0.10 mg/kg
Fenitrothion	<0.02 mg/kg
Fenpropathrin	<0.03 mg/kg

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Sample #: (b) (6)

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**QA12C: Pesticides - USP 561 Screen (USP 39)**

**Method Reference:** USP 561

**Completed:** 11/08/2017

	<b>Result</b>
Fensulfothion (sum of parent, -oxons and sulfones)	<0.05 mg/kg
Fenthion (sum of fenthion, -oxons, -sulfones)	<0.05 mg/kg
Fenvalerate	<0.20 mg/kg
Flucythrinate	<0.05 mg/kg
Fluvalinate, tau-	<0.05 mg/kg
Fonofos	<0.02 mg/kg
Heptachlor (heptachlor+ cis-, trans- h. epoxide)	<0.03 mg/kg
Hexachlorobenzene	<0.01 mg/kg
Hexachlorocyclohexane isomers (other than gamma)	<0.02 mg/kg
Lindane (gamma-HCH)	<0.01 mg/kg
Malathion and malaoxon (sum of)	<0.02 mg/kg
Mecarbam	<0.05 mg/kg
Methacriphos	<0.05 mg/kg
Methamidophos	<0.05 mg/kg
Methidathion	<0.02 mg/kg
Methoxychlor	<0.05 mg/kg
Mirex	<0.01 mg/kg
Monocrotophos	<0.10 mg/kg
Parathion-ethyl and Paraoxon-ethyl (sum of)	<0.20 mg/kg
Parathion-methyl and Paraoxon-methyl (sum of)	<0.20 mg/kg
Pendimethalin	<0.10 mg/kg
Pentachloranisole	<0.01 mg/kg
Permethrin and isomers (sum of)	<0.2 mg/kg
Phosalone	<0.04 mg/kg
Phosmet	<0.05 mg/kg
Piperonyl butoxide (PBO)	<1.0 mg/kg
Pirimiphos-ethyl	<0.05 mg/kg
Pirimiphos-methyl (incl. N-desethyl-)	<0.10 mg/kg
Procymidone	<0.10 mg/kg
Profenofos	<0.10 mg/kg
Prothiofos	<0.05 mg/kg
Pyrethrum (sum of cinerins, jasmolins, pyrethrins)	<3.0 mg/kg
Quinalphos	<0.05 mg/kg
Quintozene (sum quintozene,pentachloraniline,MPPS)	<0.1 mg/kg
S 421	<0.02 mg/kg
Tecnazene	<0.05 mg/kg
Tetradifon	<0.05 mg/kg
Vinclozolin	<0.05 mg/kg

**QA23Q: Bromide, inorganic (GC)**

**Method Reference:** EURL-SRM, Bromine Containing Fumigants

**Completed:** 11/08/2017

	<b>Result</b>
Bromide <i>[Method performed by an outsource lab.]</i>	<10 mg/kg

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Sample #: (b) (6)

Blue California Co.  
30111 Tomas  
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92688

**QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)**

**Method Reference:** J. Agric. Food Chem. Vol. 49 pp 2152, 2001

**Completed:** 11/08/2017

**Result**

Total Dithiocarbamates, as CS2

<0.01 mg/kg

*[Method performed by an outsource lab.]*

Results pertain only to the items tested.

All results are reported on an as-is basis unless otherwise stated.

Estimation of uncertainty of measurement is available upon request.

Results shall not be reproduced except in full without written permission from Eurofins Scientific, Inc.

(b) (6)

Darlene Enriquez  
Manager Scientific Support

Appendix 7.2 BESTEVIA® Rebaudioside E (b) (6)



Supplement Analysis Center

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January 09, 2018

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**CERTIFICATE OF ANALYSIS**

AR-17-KK-014577-02

Batch #: (b) (6)

*This analytical report supersedes AR-17-KK-014577-01.*

**Sample Identification:**

Sample #: (b) (6)  
Description: Bestevia Rebaudioside E, Powder, Lot #160202-1601  
Condition: White powder in a ziplock bag received at room temperature.  
Date Received: October 30, 2017

**QA12C: Pesticides - USP 561 Screen (USP 39)**

Method Reference: USP 561

Completed: 11/08/2017

	Result
Acephate	<0.10 mg/kg
<i>[Method performed by an outsource lab.]</i>	
Alachlor	<0.02 mg/kg
Aldrin and Dieldrin (sum of)	<0.02 mg/kg
Azinphos-ethyl	<0.02 mg/kg
Azinphos-methyl	<0.05 mg/kg
Bromophos-ethyl	<0.02 mg/kg
Bromophos-methyl	<0.02 mg/kg
Bromopropylate	<0.05 mg/kg
Chlordane (sum of cis-, trans- and Oxychlordane)	<0.05 mg/kg
Chlorfenvinphos	<0.02 mg/kg
Chlorpyrifos-ethyl	<0.02 mg/kg
Chlorpyrifos-methyl	<0.02 mg/kg
Chlorthal-dimethyl	<0.01 mg/kg
Cyfluthrin (sum of)	<0.10 mg/kg
Cyhalothrin, lambda-	<0.02 mg/kg
Cypermethrin and isomers (sum of)	<0.1 mg/kg
DDT (total)	<0.02 mg/kg
Deltamethrin	<0.10 mg/kg
Diazinon	<0.02 mg/kg
Dichlofluanid	<0.02 mg/kg
Dichlorvos	<0.02 mg/kg
Dicofol	<0.02 mg/kg
Dimethoate/Omethoate (sum)	<0.10 mg/kg
Endosulfan (sum of isomers and endo. sulfate)	<0.02 mg/kg
Endrin	<0.02 mg/kg
Ethion	<0.02 mg/kg
Etrimfos	<0.05 mg/kg
Fenchlorphos (sum)	<0.10 mg/kg
Fenitrothion	<0.02 mg/kg
Fenpropathrin	<0.03 mg/kg

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Sample #: (b) (6)

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**QA12C: Pesticides - USP 561 Screen (USP 39)**

**Method Reference:** USP 561

**Completed:** 11/08/2017

	<b>Result</b>
Fensulfothion (sum of parent, -oxons and sulfones)	<0.05 mg/kg
Fenthion (sum of fenthion, -oxons, -sulfones)	<0.05 mg/kg
Fenvalerate	<0.20 mg/kg
Flucythrinate	<0.05 mg/kg
Fluvalinate, tau-	<0.05 mg/kg
Fonofos	<0.02 mg/kg
Heptachlor (heptachlor+ cis-, trans- h. epoxide)	<0.03 mg/kg
Hexachlorobenzene	<0.01 mg/kg
Hexachlorocyclohexane isomers (other than gamma)	<0.02 mg/kg
Lindane (gamma-HCH)	<0.01 mg/kg
Malathion and malaoxon (sum of)	<0.02 mg/kg
Mecarbam	<0.05 mg/kg
Methacriphos	<0.05 mg/kg
Methamidophos	<0.05 mg/kg
Methodathion	<0.02 mg/kg
Methoxychlor	<0.05 mg/kg
Mirex	<0.01 mg/kg
Monocrotophos	<0.10 mg/kg
Parathion-ethyl and Paraoxon-ethyl (sum of)	<0.20 mg/kg
Parathion-methyl and Paraoxon-methyl (sum of)	<0.20 mg/kg
Pendimethalin	<0.10 mg/kg
Pentachloranisole	<0.01 mg/kg
Permethrin and isomers (sum of)	<0.2 mg/kg
Phosalone	<0.04 mg/kg
Phosmet	<0.05 mg/kg
Piperonyl butoxide (PBO)	<1.0 mg/kg
Pirimiphos-ethyl	<0.05 mg/kg
Pirimiphos-methyl (incl. N-desethyl-)	<0.10 mg/kg
Procymidone	<0.10 mg/kg
Profenofos	<0.10 mg/kg
Prothiofos	<0.05 mg/kg
Pyrethrum (sum of cinerins, jasmolins, pyrethrins)	<3.0 mg/kg
Quinalphos	<0.05 mg/kg
Quintozene (sum quintozene, pentachloroaniline, MPPS)	<0.1 mg/kg
S 421	<0.02 mg/kg
Tecnazene	<0.05 mg/kg
Tetradifon	<0.05 mg/kg
Vinclozolin	<0.05 mg/kg

**QA23Q: Bromide, inorganic (GC)**

**Method Reference:** EURL-SRM, Bromine Containing Fumigants

**Completed:** 11/08/2017

	<b>Result</b>
Bromide <i>[Method performed by an outsource lab.]</i>	<10 mg/kg





Sample #: (b) (6)

Blue California Co.  
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**QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)**

**Method Reference:** J. Agric. Food Chem. Vol. 49 pp 2152, 2001

**Completed:** 11/08/2017

**Result**

Total Dithiocarbamates, as CS2

<0.01 mg/kg

*[Method performed by an outsource lab.]*

Results pertain only to the items tested.

All results are reported on an as-is basis unless otherwise stated.

Estimation of uncertainty of measurement is available upon request.

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(b) (6)

Darlene Enriquez  
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Appendix 7.3 BESTEVIA® Rebaudioside E (b) (6)



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January 09, 2018

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**CERTIFICATE OF ANALYSIS**

AR-17-KK-014576-03

Batch #: (b) (6)

*This analytical report supersedes AR-17-KK-014576-02.*

**Sample Identification:**

Sample #: (b) (6)  
Description: Bestevia Rebaudioside E, Powder, (b) (6)  
Condition: White powder in a ziplock bag received at room temperature.  
Date Received: October 30, 2017

**QA12C: Pesticides - USP 561 Screen (USP 39)**

Method Reference: USP 561

Completed: 11/08/2017

	Result
Acephate	<0.10 mg/kg
[Method performed by an outsource lab.]	
Alachlor	<0.02 mg/kg
Aldrin and Dieldrin (sum of)	<0.02 mg/kg
Azinphos-ethyl	<0.02 mg/kg
Azinphos-methyl	<0.05 mg/kg
Bromophos-ethyl	<0.02 mg/kg
Bromophos-methyl	<0.02 mg/kg
Bromopropylate	<0.05 mg/kg
Chlordane (sum of cis-, trans- and Oxychlordane)	<0.05 mg/kg
Chlorfenvinphos	<0.02 mg/kg
Chlorpyrifos-ethyl	<0.02 mg/kg
Chlorpyrifos-methyl	<0.02 mg/kg
Chlorthal-dimethyl	<0.01 mg/kg
Cyfluthrin (sum of)	<0.10 mg/kg
Cyhalothrin, lambda-	<0.02 mg/kg
Cypermethrin and isomers (sum of)	<0.1 mg/kg
DDT (total)	<0.02 mg/kg
Deltamethrin	<0.10 mg/kg
Diazinon	<0.02 mg/kg
Dichlofluanid	<0.02 mg/kg
Dichlorvos	<0.02 mg/kg
Dicofol	<0.02 mg/kg
Dimethoate/Omethoate (sum)	<0.10 mg/kg
Endosulfan (sum of isomers and endo. sulfate)	<0.02 mg/kg
Endrin	<0.02 mg/kg
Ethion	<0.02 mg/kg
Etrinfos	<0.05 mg/kg
Fenchlorphos (sum)	<0.10 mg/kg
Fenitrothion	<0.02 mg/kg
Fenpropathrin	<0.03 mg/kg

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Sample #: (b) (6)

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**QA12C: Pesticides - USP 561 Screen (USP 39)**

**Method Reference:** USP 561

**Completed:** 11/08/2017

	<b>Result</b>
Fensulfothion (sum of parent, -oxons and sulfones)	<0.05 mg/kg
Fenthion (sum of fenthion, -oxons, -sulfones)	<0.05 mg/kg
Fenvalerate	<0.20 mg/kg
Flucythrinate	<0.05 mg/kg
Fluvalinate, tau-	<0.05 mg/kg
Fonofos	<0.02 mg/kg
Heptachlor (heptachlor+ cis-, trans- h. epoxide)	<0.03 mg/kg
Hexachlorobenzene	<0.01 mg/kg
Hexachlorocyclohexane isomers (other than gamma)	<0.02 mg/kg
Lindane (gamma-HCH)	<0.01 mg/kg
Malathion and malaoxon (sum of)	<0.02 mg/kg
Mecarbam	<0.05 mg/kg
Methacriphos	<0.05 mg/kg
Methamidophos	<0.05 mg/kg
Methidathion	<0.02 mg/kg
Methoxychlor	<0.05 mg/kg
Mirex	<0.01 mg/kg
Monocrotophos	<0.10 mg/kg
Parathion-ethyl and Paraoxon-ethyl (sum of)	<0.20 mg/kg
Parathion-methyl and Paraoxon-methyl (sum of)	<0.20 mg/kg
Pendimethalin	<0.10 mg/kg
Pentachloranisole	<0.01 mg/kg
Permethrin and isomers (sum of)	<0.2 mg/kg
Phosalone	<0.04 mg/kg
Phosmet	<0.05 mg/kg
Piperonyl butoxide (PBO)	<1.0 mg/kg
Pirimiphos-ethyl	<0.05 mg/kg
Pirimiphos-methyl (incl. N-desethyl-)	<0.10 mg/kg
Procymidone	<0.10 mg/kg
Profenofos	<0.10 mg/kg
Prothiofos	<0.05 mg/kg
Pyrethrum (sum of cinerins, jasmolins, pyrethrins)	<3.0 mg/kg
Quinalphos	<0.05 mg/kg
Quintozene (sum quintozene, pentachloraniline, MPPS)	<0.1 mg/kg
S 421	<0.02 mg/kg
Tecnazene	<0.05 mg/kg
Tetradifon	<0.05 mg/kg
Vinclozolin	<0.05 mg/kg

**QA23Q: Bromide, inorganic (GC)**

**Method Reference:** EURL-SRM, Bromine Containing Fumigants

**Completed:** 11/08/2017

	<b>Result</b>
Bromide	<10 mg/kg
<i>[Method performed by an outsource lab.]</i>	

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Sample #: (b) (6)

Blue California Co.  
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92688

**QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)**

**Method Reference:** J. Agric. Food Chem. Vol. 49 pp 2152, 2001

**Completed:** 11/08/2017

**Result**

Total Dithiocarbamates, as CS2

<0.01 mg/kg

*[Method performed by an outsource lab.]*

Results pertain only to the items tested.

All results are reported on an as-is basis unless otherwise stated.

Estimation of uncertainty of measurement is available upon request.

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(b) (6)

Darlene Enriquez  
Manager Scientific Support

Appendix 7.4 BESTEVIA® Rebaudioside E (b) (6)



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January 09, 2018

Hadi Omrani  
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CERTIFICATE OF ANALYSIS

AR-17-KK-014579-02

Batch #: (b) (6)

This analytical report supersedes AR-17-KK-014579-01.

Sample Identification:

Sample #: (b) (6)  
Description: Bestevia Rebaudioside E, Powder, Lot #160921-1603  
Condition: White powder in a ziplock bag received at room temperature.  
Date Received: October 30, 2017

QA12C: Pesticides - USP 561 Screen (USP 39)

Method Reference: USP 561

Completed: 11/08/2017

	Result
Acephate	<0.10 mg/kg
[Method performed by an outsource lab.]	
Alachlor	<0.02 mg/kg
Aldrin and Dieldrin (sum of)	<0.02 mg/kg
Azinphos-ethyl	<0.02 mg/kg
Azinphos-methyl	<0.05 mg/kg
Bromophos-ethyl	<0.02 mg/kg
Bromophos-methyl	<0.02 mg/kg
Bromopropylate	<0.05 mg/kg
Chlordane (sum of cis-, trans- and Oxychlordane)	<0.05 mg/kg
Chlorfenvinphos	<0.02 mg/kg
Chlorpyrifos-ethyl	<0.02 mg/kg
Chlorpyrifos-methyl	<0.02 mg/kg
Chlorthal-dimethyl	<0.01 mg/kg
Cyfluthrin (sum of)	<0.10 mg/kg
Cyhalothrin, lambda-	<0.02 mg/kg
Cypermethrin and isomers (sum of)	<0.1 mg/kg
DDT (total)	<0.02 mg/kg
Deltamethrin	<0.10 mg/kg
Diazinon	<0.02 mg/kg
Dichlofluanid	<0.02 mg/kg
Dichlorvos	<0.02 mg/kg
Dicofol	<0.02 mg/kg
Dimethoate/Omethoate (sum)	<0.10 mg/kg
Endosulfan (sum of isomers and endo. sulfate)	<0.02 mg/kg
Endrin	<0.02 mg/kg
Ethion	<0.02 mg/kg
Etrimfos	<0.05 mg/kg
Fenchlorphos (sum)	<0.10 mg/kg
Fenitrothion	<0.02 mg/kg
Fenpropathrin	<0.03 mg/kg

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Sample #: (b) (6)

Blue California Co.  
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Rancho Santa Margarita, CA  
92688

**QA12C: Pesticides - USP 561 Screen (USP 39)**

**Method Reference:** USP 561

**Completed:** 11/08/2017

	<b>Result</b>
Fensulfothion (sum of parent, -oxons and sulfones)	<0.05 mg/kg
Fenthion (sum of fenthion, -oxons, -sulfones)	<0.05 mg/kg
Fenvalerate	<0.20 mg/kg
Flucythrinate	<0.05 mg/kg
Fluvalinate, tau-	<0.05 mg/kg
Fonofos	<0.02 mg/kg
Heptachlor (heptachlor+ cis-, trans- h. epoxide)	<0.03 mg/kg
Hexachlorobenzene	<0.01 mg/kg
Hexachlorocyclohexane isomers (other than gamma)	<0.02 mg/kg
Lindane (gamma-HCH)	<0.01 mg/kg
Malathion and malaoxon (sum of)	<0.02 mg/kg
Mecarbam	<0.05 mg/kg
Methacriphos	<0.05 mg/kg
Methamidophos	<0.05 mg/kg
Methidathion	<0.02 mg/kg
Methoxychlor	<0.05 mg/kg
Mirex	<0.01 mg/kg
Monocrotophos	<0.10 mg/kg
Parathion-ethyl and Paraoxon-ethyl (sum of)	<0.20 mg/kg
Parathion-methyl and Paraoxon-methyl (sum of)	<0.20 mg/kg
Pendimethalin	<0.10 mg/kg
Pentachloranisole	<0.01 mg/kg
Permethrin and isomers (sum of)	<0.2 mg/kg
Phosalone	<0.04 mg/kg
Phosmet	<0.05 mg/kg
Piperonyl butoxide (PBO)	<1.0 mg/kg
Pirimiphos-ethyl	<0.05 mg/kg
Pirimiphos-methyl (incl. N-desethyl-)	<0.10 mg/kg
Procymidone	<0.10 mg/kg
Profenofos	<0.10 mg/kg
Prothiofos	<0.05 mg/kg
Pyrethrum (sum of cinerins, jasmolins, pyrethrins)	<3.0 mg/kg
Quinalphos	<0.05 mg/kg
Quintozene (sum quintozene, pentachloroaniline, MPPS)	<0.1 mg/kg
S 421	<0.02 mg/kg
Tecnazene	<0.05 mg/kg
Tetradifon	<0.05 mg/kg
Vinclozolin	<0.05 mg/kg

**QA23Q: Bromide, inorganic (GC)**

**Method Reference:** EURL-SRM, Bromine Containing Fumigants

**Completed:** 11/08/2017

	<b>Result</b>
Bromide	<10 mg/kg
<i>[Method performed by an outsource lab.]</i>	

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Sample #: (b) (6)

Blue California Co.  
30111 Tomas  
Rancho Santa Margarita, CA  
92688

**QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)**

**Method Reference:** J. Agric. Food Chem. Vol. 49 pp 2152, 2001

**Completed:** 11/08/2017

**Result**

Total Dithiocarbamates, as CS2

<0.01 mg/kg

*[Method performed by an outsource lab.]*

Results pertain only to the items tested.

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(b) (6)

Darlene Enriquez  
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Appendix 7.5 BESTEVIA® Rebaudioside E (b) (6)



Supplement Analysis Center

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January 09, 2018

Hadi Omrani  
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CERTIFICATE OF ANALYSIS

AR-17-KK-014580-02

Batch #: (b) (6)

This analytical report supersedes AR-17-KK-014580-01.

Sample Identification:

Sample #: (b) (6)  
Description: Bestevia Rebaudioside E, Powder, (b) (6)  
Condition: White powder in a ziplock bag received at room temperature.  
Date Received: October 30, 2017

QA12C: Pesticides - USP 561 Screen (USP 39)

Method Reference: USP 561

Completed: 11/08/2017

	Result
Acephate	<0.10 mg/kg
[Method performed by an outsource lab.]	
Alachlor	<0.02 mg/kg
Aldrin and Dieldrin (sum of)	<0.02 mg/kg
Azinphos-ethyl	<0.02 mg/kg
Azinphos-methyl	<0.05 mg/kg
Bromophos-ethyl	<0.02 mg/kg
Bromophos-methyl	<0.02 mg/kg
Bromopropylate	<0.05 mg/kg
Chlordane (sum of cis-, trans- and Oxychlordane)	<0.05 mg/kg
Chlorfenvinphos	<0.02 mg/kg
Chlorpyrifos-ethyl	<0.02 mg/kg
Chlorpyrifos-methyl	<0.02 mg/kg
Chlorthal-dimethyl	<0.01 mg/kg
Cyfluthrin (sum of)	<0.10 mg/kg
Cyhalothrin, lambda-	<0.02 mg/kg
Cypermethrin and isomers (sum of)	<0.1 mg/kg
DDT (total)	<0.02 mg/kg
Deltamethrin	<0.10 mg/kg
Diazinon	<0.02 mg/kg
Dichlofluanid	<0.02 mg/kg
Dichlorvos	<0.02 mg/kg
Dicofol	<0.02 mg/kg
Dimethoate/Omethoate (sum)	<0.10 mg/kg
Endosulfan (sum of isomers and endo. sulfate)	<0.02 mg/kg
Endrin	<0.02 mg/kg
Ethion	<0.02 mg/kg
Etrimfos	<0.05 mg/kg
Fenchlorphos (sum)	<0.10 mg/kg
Fenitrothion	<0.02 mg/kg
Fenpropathrin	<0.03 mg/kg

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Sample #: (b) (6)

Blue California Co.  
30111 Tomas  
Rancho Santa Margarita, CA  
92688

**QA12C: Pesticides - USP 561 Screen (USP 39)**

**Method Reference:** USP 561

**Completed:** 11/08/2017

	<b>Result</b>
Fensulfthion (sum of parent, -oxons and sulfones)	<0.05 mg/kg
Fenthion (sum of fenthion, -oxons, -sulfones)	<0.05 mg/kg
Fenvalerate	<0.20 mg/kg
Flucythrinate	<0.05 mg/kg
Fluvalinate, tau-	<0.05 mg/kg
Fonofos	<0.02 mg/kg
Heptachlor (heptachlor+ cis-, trans- h. epoxide)	<0.03 mg/kg
Hexachlorobenzene	<0.01 mg/kg
Hexachlorocyclohexane isomers (other than gamma)	<0.02 mg/kg
Lindane (gamma-HCH)	<0.01 mg/kg
Malathion and malaoxon (sum of)	<0.02 mg/kg
Mecarbam	<0.05 mg/kg
Methacriphos	<0.05 mg/kg
Methamidophos	<0.05 mg/kg
Methidathion	<0.02 mg/kg
Methoxychlor	<0.05 mg/kg
Mirex	<0.01 mg/kg
Monocrotophos	<0.10 mg/kg
Parathion-ethyl and Paraoxon-ethyl (sum of)	<0.20 mg/kg
Parathion-methyl and Paraoxon-methyl (sum of)	<0.20 mg/kg
Pendimethalin	<0.10 mg/kg
Pentachloranisole	<0.01 mg/kg
Permethrin and isomers (sum of)	<0.2 mg/kg
Phosalone	<0.04 mg/kg
Phosmet	<0.05 mg/kg
Piperonyl butoxide (PBO)	<1.0 mg/kg
Pirimiphos-ethyl	<0.05 mg/kg
Pirimiphos-methyl (incl. N-desethyl-)	<0.10 mg/kg
Procymidone	<0.10 mg/kg
Profenofos	<0.10 mg/kg
Prothiofos	<0.05 mg/kg
Pyrethrum (sum of cinerins, jasmolins, pyrethrins)	<3.0 mg/kg
Quinalphos	<0.05 mg/kg
Quintozene (sum quintozene, pentachloraniline, MPPS)	<0.1 mg/kg
S 421	<0.02 mg/kg
Tecnazene	<0.05 mg/kg
Tetradifon	<0.05 mg/kg
Vinclozolin	<0.05 mg/kg

**QA23Q: Bromide, inorganic (GC)**

**Method Reference:** EURL-SRM, Bromine Containing Fumigants

**Completed:** 11/08/2017

	<b>Result</b>
Bromide	<10 mg/kg

*[Method performed by an outsource lab.]*



Sample #: (b) (6)

Blue California Co.  
30111 Tomas  
Rancho Santa Margarita, CA  
92688

**QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)**

**Method Reference:** J. Agric. Food Chem. Vol. 49 pp 2152, 2001

**Completed:** 11/08/2017

**Result**

Total Dithiocarbamates, as CS2

<0.01 mg/kg

*[Method performed by an outsource lab.]*

Results pertain only to the items tested.

All results are reported on an as-is basis unless otherwise stated.

Estimation of uncertainty of measurement is available upon request.

Results shall not be reproduced except in full without written permission from Eurofins Scientific, Inc.

(b) (6)

Darlene Enriquez  
Manager Scientific Support

## Appendix 8 Relative Sweetness Intensity Method

### SWEETNESS EQUIVALENCY OF BESTEVIA REB-E

#### INTRODUCTION:

Sucrose, more commonly known as table sugar, is the standard by which sugar substitutes are compared to in terms of taste, texture, and caloric values. Bestevia-E, a trademarked product produced by Blue California, is made from isolating the sweetest compound of fermentation, Rebaudioside E, in order to create a non-caloric sweetener that can be used in similar applications to sucrose.

#### PURPOSE:

To determine the sweetness equivalence of Bestevia-E (Rebaudioside E) produced by Blue California in comparison to sucrose.

#### TEST SAMPLES:

Samples of BESTEVIA-E and Sucrose were prepared in water at room temperature respectively for comparison.

#### EQUIPMENT & MATERIALS:

Bestevia-E  
Sucrose  
Purified water  
Analytical Scale  
1000ml beakers  
Glass stirrers  
Plastic cups

#### PROCEDURE:

1. 13 participants were pre-screened for taste acuity prior to completing the taste panel
2. Sensory evaluation of Reb E was performed using sucrose as a control. The sucrose sample purchased from Sigma-Aldrich and prepared control samples at three different concentrations of 1.0%, 3.0%, and 6.0% sucrose in purified water (w/v) at room temperature.
3. The Bestevia-E at 300ppm for sensory evaluation was prepared by adding corresponding mass into a 1000 mL of bottled water.
4. The mixture was stirred at room temperature until complete dissolved.
5. The Bestevia-E sample was evaluated against several control sucrose samples at 1.0%, 3.0%, and 6.0% by a panel of 13 volunteers.

#### RESULTS:

All the value from tasters were averaged and converted to the sweetness equivalency comparing to sucrose. The blind results showed consistent results among volunteers. The result indicates that the Bestevia-E is 137 times sweeter to sucrose.

## Appendix 9 Estimated Daily Intake Levels of Steviol Glycosides Preparations

There have been continuing studies to estimate the intake of steviol glycosides. Most recently, Dewinter et al. (2016) investigated the dietary intake of non-nutritive sweeteners, including steviol glycosides, in children with type 1 diabetes. Using a phased tier approach, the tier 2 (maximum concentration) and tier 3 (maximum used concentrations) exposures were assessed based on survey data obtained from patients at the Pediatrics Department of the University Hospitals Leuven (Belgium). In both tier 2 and tier 3 exposure assessments, high consumers (P95) aged 4-6 years old were estimated to have a steviol glycosides intake higher than the ADI, calculated at 119% of ADI. The authors noted that the exposure assessment is a worst-case scenario since “it is assumed that all processed foods in which the food additive is authorized contain the food additive at the [maximum permitted levels].” Furthermore, Dewinter et al. conclude that there is little chance that children with type 1 diabetes will exceed ADIs for steviol glycosides.

### 1. Food Uses as Addressed by JECFA, Merisant & Cargill

As part of its safety deliberations, JECFA reviewed various estimates of possible daily intake of steviol glycosides (WHO, 2006). These estimates are presented in Table 9-1. Merisant also listed intended use levels of rebaudioside A for various food applications in their GRAS Notification (Table 9-2). Merisant utilized food consumption survey data from 2003-2004 National Health and Nutrition Examination Survey (NHANES) to determine the estimated daily intake from the proposed uses of rebaudioside A. On a per user basis, the mean and 90<sup>th</sup> percentile daily consumption levels of rebaudioside A were estimated as 2.0 and 4.7 mg per kg bw per day, respectively. In its notification, Cargill (2008) utilized a different approach in estimating dietary intake figures for rebaudioside A when incorporated as a general sweetener in a broad cross-section of processed foods. Cargill considered that, with a few minor exceptions, rebaudioside A uses and use levels would be comparable to those of aspartame uses in the US. Using post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (Renwick, 2008), Cargill performed a side-by-side consumption analysis for rebaudioside A versus aspartame. Findings from the above-described different sources along with FSANZ estimates and the intake estimates are presented in Table 9-3.

### 2. Estimated Daily Intake

The very conservative consumer intake estimates provided by JECFA as shown in Table 9-1 were utilized to gauge the potential human exposures of rebaudioside A and steviol glycosides and in foods as reported in the US and in other countries. As rebaudioside A is about twice as sweet as the mixed glycosides, these levels can be adjusted accordingly.

**Table 9-1. Food Uses of Steviol Glycosides Reported to JECFA with Calculated Steviol Equivalents**

FOOD TYPE	MAXIMUM USE LEVEL REPORTED <sup>a</sup> (MG STEVIOL GLYCOSIDES /KG OF FOOD)	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A <sup>b</sup> MG REBAUDIOSIDE A /KG OF FOOD	MAXIMUM USE LEVEL CALCULATED FOR REBAUDIOSIDE A <sup>b</sup> MG STEVIOL EQUIVALENTS /KG OF FOOD
Desserts	500	250	83
Cold confectionery	500	250	83
Pickles	1000	500	167
Sweet corn	200	100	33
Biscuits	300	150	50
Beverages	500	250	83
Yogurt	500	250	83
Sauces	1000	500	167
Delicacies	1000	500	167
Bread	160	80	27

<sup>a</sup> Reproduced from WHO (2006).

<sup>b</sup> Calculated by Expert Panel assuming twice the sweetness intensity for rebaudioside A and three-fold difference in molecular weight between rebaudioside A and steviol.

**Table 9-2. Proposed Uses & Levels of Rebaudioside A by Merisant<sup>a</sup>**

FOOD USES	REB A (PPM)
Tabletop sweeteners	30,000 <sup>b</sup>
Sweetened ready-to-drink teas	90-450
Fruit juice drinks	150-500
Diet soft drinks	150-500
Energy drinks	150
Flavored water	150
Cereals (oatmeal, cold cereal, cereal bars)	150
<sup>a</sup> Merisant (2008)	
<sup>b</sup> Reb A content of sachet prior to dilution and not representative of “as consumed.”	

Further consideration was given to anticipated human exposures as projected independently and with different approaches by JECFA (WHO, 2006), Merisant (2008), and Cargill (2008). As described below, the multiple approaches tended to converge to yield estimated daily intakes (EDIs) in the range of 1.3 – 4.7 mg per kg bw per day that, when compared to the acceptable daily intake (ADI), constitutes supporting information in the subject GRAS evaluation.

JECFA evaluated information on exposure to steviol glycosides as submitted by Japan and China. Additional information was available from a report on *Stevia rebaudiana* Bertoni plants and leaves that were prepared for the European Commission by the Scientific Committee on Food. JECFA used the GEMS/Food database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, which is 200:1. The intakes ranged from 1.3 mg per kg bw per day with the African diet to 3.5 mg per kg bw per day with the European diet. Additionally, JECFA also estimated the per capita exposure derived from disappearance (poundage) data supplied by Japan and China. The Committee evaluated exposures to steviol glycosides by assuming full replacement of all dietary sugars in the diets for Japan and the US. The exposures to steviol glycosides (as steviol) as evaluated or derived by the Committee are summarized in Table 9-4.

JECFA concluded that the replacement estimates were highly conservative---that is, the calculated dietary exposure over-estimates likely consumption---and that true dietary intakes of steviol glycosides (as steviol) would probably be 20 – 30% of these values or 1.0 - 1.5 mg per kg bw per day on a steviol basis or 3.0 – 4.5 mg per kg bw per day for rebaudioside A based on the molecular weight adjustment. Similarly, FSANZ (2008) estimated steviol glycoside dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario, which resulted in estimated exposures of 0.3 - 1.0 mg per kg bw per day for the mean and 90<sup>th</sup> percentile consumer, or 0.5 – 1.5 mg per kg bw per day for rebaudioside A when making both the molecular weight and sweetness equivalency calculations. FSANZ examined consumption in other age groups and concluded that there were no safety concerns for children of any age. Merisant also calculated a dietary estimate for Reb A of 2.0 mg per kg bw per day for the average consumer and 4.7 mg per kg bw per day for a 90<sup>th</sup> percentile consumer. On a steviol equivalent basis, the Merisant estimates would be 0.7 and 1.6 mg per kg bw per day, respectively. In another review conducted on behalf of Cargill and included in their GRAS notification, the intake of rebaudioside A when used as a complete sugar replacement was estimated at 1.3 – 3.4 mg per kg bw per day when calculated as Reb A (Renwick, 2008).

**Table 9-3. Summary of Estimated Daily Intake Assessments for Rebaudioside A & Calculation of Rebaudioside A Values from JECFA & FSANZ Estimates of EDI**

SCENARIOS	EDI		
	AS STEVIOL <sup>a</sup> (MG/KG BW/DAY)	AS REBAUDIOSIDE A <sup>b</sup> (MG/KG BW/DAY)	TOTAL DAILY INTAKE <sup>c</sup> (MG/DAY)
JECFA			
100% Reb A replacement of sugars	5.0	7.5	450
20-30% Reb A replacement of sugars	1.0 - 1.5	1.5 - 2.3	90 - 140
FSANZ			
100% Reb A replacement of sugars	0.3 - 1.0	0.5 - 1.5	30 - 90
MERISANT			
		2.0 - 4.7 <sup>d</sup>	120 - 282
CARGILL			
		1.3 - 3.4 <sup>d</sup>	78 - 204

- <sup>a</sup> Published values for mixed steviol glycosides consumption listed in this column were used for the calculation of Reb A consumption values appearing in next two columns.
- <sup>b</sup> Estimates for Reb A consumption were calculated from JECFA and FSANZ estimates as steviol by multiplying by 3 to correct for the molecular weight of Reb A compared to steviol and by subsequently dividing by 2 because of the increased inherent sweetness of Reb A compared to the mixed steviol glycosides.
- <sup>c</sup> Total daily intake figures were calculated for a 60 kg adult.
- <sup>d</sup> Published values are shown for comparison purposes.

**Table 9-4. Summary of Estimates of Exposure to Steviol Glycosides (as Steviol)**

ESTIMATE	EXPOSURE (mg/kg BW/DAY)
GEMS/Food (International) <sup>a</sup>	1.3 -3.5 (for a 60 kg person)
Japan, Per Capita	0.04
Japan, Replacement Estimate <sup>b</sup>	3
US, Replacement Estimate <sup>b</sup>	5

- <sup>a</sup> WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.
- <sup>b</sup> These estimates were prepared in parallel to those for the international estimates; it was assumed that all dietary sugars in diets in Japan and the US would be replaced by steviol glycosides on a sweetness equivalent basis, at a ratio of 200:1.

In October 2009, Cargill applied to FSANZ to increase the maximum usage levels of high purity steviol glycosides in the high-volume food categories of ice cream and various beverages. Cargill supported its application with increased usage levels by presenting market share analyses that overestimate actual intake while remaining well below the generally accepted ADI. In December 2010, FSANZ recommended accepting the increased usage levels as requested since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved the Cargill application to increase the allowed maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

On January 13, 2011, EFSA revised its dietary exposure assessment of steviol glycosides. For high consumers, revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent). For European children aged 1-14, revised intake estimates ranged from 1.7 to 16.3 mg per kg bw per day, and for adults, the range was reported to be from 5.6 to 6.8 mg per kg bw per day (EFSA, 2011b).

Most recently, Roberts et al. (2016) suggested that a higher ADI is justified based on metabolic factors to reduce the 100X safety factor. A chemical-specific adjustment factor (CSAF), as defined by the WHO in 2005, was determined by comparative studies in rats and humans. A CSAF that is less than the standard 100X safety factor will result in an increase in the ADI, independent of the NOAEL. The authors determined that using a CSAF can justify an ADI value of 6-16 mg per kg bw per day for steviol glycosides, depending on whether area under the plasma-concentration time curve (AUC) or  $C_{max}$  data are used when considering the 1,000 mg per kg bw per day NOAEL (which is equivalent to 400 mg per kg bw per day of steviol) for stevioside reported by Toyoda et al. (1997).

There have been many scholarly estimates of potential dietary intake of replacement sweeteners---including steviol glycosides---that have been published (FSANZ, 2008; Renwick, 2008; WHO, 2003) or submitted to FDA (Merisant, 2008). In GRN 301, a simplified estimate was proposed to and accepted by FDA based on the estimates of exposure in “sucrose equivalents” (Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90<sup>th</sup> percentile consumer of a sweetener which is 100 times as sweet as sucrose when used as a total sugar replacement would be a maximum of 9.9 mg per kg bw per day for any population subgroup.



## Appendix 10 Studies on Steviol Glycosides Preparations That Are Primarily Mixtures of Stevioside & Rebaudioside A

This appendix summarizes studies on stevioside or stevia extracts that were identified compositionally as predominantly stevioside. In some of the published literature, the terms stevia, stevioside, and stevia glycoside are used interchangeably. However, wherever possible, an attempt has been made to identify the specific substance studied.

### 1. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

Several studies in rats (Wingard Jr et al., 1980; Nakayama et al., 1986; Koyama et al., 2003b) and other animal models, including chickens (Geuns et al., 2003b), hamsters (Hutapea et al., 1999), and pigs (Geuns et al., 2003a), indicate that stevioside is not readily absorbed from the GI tract. Available evidence from *in vitro* metabolism studies suggests that bacteria in the colon of rats and humans can transform various stevia glycosides into steviol (Gardana et al., 2003). Steviol was shown to be more readily transported with *in vitro* intestinal preparations than various steviosides (Geuns, 2003; Koyama et al., 2003b). Slow absorption of steviol was indicated by detection in the plasma of rats given oral stevioside (Wang et al., 2004). However, Sung (2002) did not detect plasma steviol following oral administration of steviosides to rats. In studies with human and rat liver extracts, Koyama et al. (2003b) demonstrated that steviol can be converted to various glucuronides. Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats (Sung, 2002) and hamsters (Hutapea et al., 1999). Oral doses in pigs led to the detection of metabolites in feces but not in urine (Geuns et al., 2003a).

Koyama et al. (2003b) published an *in vitro* study in which  $\alpha$ -glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These are subsequently hydrolyzed to the aglycone, steviol, demonstrating that the metabolic fate of  $\alpha$ -glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of  $\alpha$ -glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides. Furthermore, as individual steviol glycosides show similar pharmacokinetics in the rat and humans, the results of toxicology studies on individual steviol glycosides are applicable to the safety of steviol glycosides in general.

In a human study with 10 healthy subjects, Geuns et al. (2006) measured blood, urine, and fecal metabolites in subjects that received 3 doses of 250 mg of purified stevioside (>97%) three times a day for 3 days. Urine was collected for 24 hours on day 3, and blood and fecal samples were also taken on day 3. Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine, and feces. Approximately 76% of the total steviol equivalents dosed were recovered in urine and feces. Based on these measurements, the authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.

Renwick and Tarka (2008) reviewed studies on microbial hydrolysis of steviol glycosides. The reviewers concluded that stevioside and Reb A are not absorbed directly, and both are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for Reb A than for stevioside. Studies have shown that steviol-16,17-epoxide is not a microbial metabolite.

## 2. Acute Toxicity Studies

The oral LD<sub>50</sub> studies of stevioside (purity, 96%) following administration of a single dose to rodents are summarized in Table 10-1. No lethality was noted within 14 days after the administration, and no clinical signs of toxicity, or morphological or histopathological changes were found, indicating that stevioside is relatively harmless.

**Table 10-1. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents**

Species	Sex	LD <sub>50</sub> (g/kg bw)	Reference
Mouse	Male and Female	>15	Toskulkac et al. (1997)
Mouse	Male	> 2	Medon et al. (1982)
Rat	Male and Female	>15	Toskulkac et al. (1997)
Hamster	Male and Female	>15	Toskulkac et al. (1997)

## 3. Subchronic Toxicity Studies

In five published studies, subchronic toxicity of stevioside was investigated in rats following oral administration. In addition, a reproduction study in hamsters included subchronic phases on the F<sub>0</sub>, F<sub>1</sub>, and F<sub>2</sub> generations. These studies are summarized in Table 10-2. One of these studies was particularly important because it served as a range-finding study for two subsequent chronic studies. In this 13-week toxicity study, Fischer 344 rats (10 per sex per group) were given doses of 0, 0.31, 0.62, 1.25, 2.5, or 5% in the diet (equivalent to 160, 310, 630, 1,300, and 2,500 mg per kg bw per day) to determine the appropriate doses for a two-year carcinogenicity study. None of the animals died during the administration period, and there was no difference in body-weight gain between the control and treated groups during administration or in food consumption in the latter part of the study. The activity of lactic dehydrogenase and the incidence of single-cell necrosis in the liver were increased in all groups of treated males. The authors considered these effects to be nonspecific, because of the lack of a clear dose-response relationship, the relatively low severity, and their limitation to males. Other statistically significant differences in hematological and biochemical parameters were also considered to be of minor toxicological significance. The authors concluded that a concentration of 5% in the diet was a suitable maximum tolerable dose of stevioside for a two-year study in rats (Aze et al., 1990).

In earlier 3-month rat studies reviewed by Geuns (2003)---the sample purity, doses, strain of rat were not reported---a no effect level was determined to be in excess of 2,500 mg per kg bw per

day and 7% of the diet, apparently due to lack of effects at the highest dose tested in both studies (Akashi and Yokoyama, 1975).

In a recently published exploratory subchronic toxicity study, Awney et al. (2011) investigated the effects of 97% pure stevioside on body weight, organ relative weight, hematological and biochemical parameters, and enzyme activities in Sprague Dawley rats. In this 12-week toxicity study, groups of male rats (8 per group) were given drinking water containing stevioside. The groups were assigned to drink distilled water (control), low-dose stevioside solution (15 mg per kg per day), high-dose stevioside solution (1,500 mg per kg per day), or low-dose stevioside (15 mg per kg per day) plus inulin solution for 12 weeks as the sole source of liquid. Fluid intake was recorded daily, and levels of test articles were adjusted weekly to receive the appropriate target concentration. Low-dose stevioside (15 mg per kg bw per day) administration, with or without inulin, for 12 weeks did not reveal any adverse effects on body weight, organs relative weight, hematological and biochemical parameters, or enzyme activities. However, treatment with high-dose stevioside was reported to cause significant changes in several investigated toxicological parameters. Among the hematological parameters, significant changes were noted in all except white blood cells (WBCs), red blood cells (RBCs), and packed cell volume (PCV%), and in all clinical chemistry parameters except proteins, total lipids, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These data support the NOEL of 15 mg per kg per day. However, critical review of the publication reveals that the study was poorly designed and implemented. Design deficiencies include: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water, resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection, which affects many chemistry and hematological values; no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. In addition to these study design deficiencies, the report fails to adequately present mean or individual organ weight data and, in general, there appears to be inadequate comparison of study findings against laboratory historical control data. Any one of these oversights could have adversely affected the results and/or interpretation of the hematological and chemistry data.

In addition to the above-described parameters, tartrate-resistant alkaline phosphatase (TRAP) levels were measured and found to be significantly decreased (Awney et al., 2011). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages, and dendritic cells. This enzyme was not measured in any previous steviol glycosides studies nor has it been adequately vetted for application in toxicological studies. These investigators did not identify the specific TRAP isomer measured, the methodology employed, the handling of the samples, or any historical data on TRAP levels. The significance and relevance of this poorly documented toxicological endpoint, which lacks histopathological confirmation, does not appear to have a distinct role in determining the toxicological profile of a material in a test animal. The data presented by Awney et al. (2011) are probably not representative of changes due to the subchronic dietary administration of steviol glycosides because of overall inadequate study design and reliance on the findings of the untested enzyme TRAP. The preponderance of the data from

several well-designed studies on steviol glycosides suggest that differences noted in hematological and chemistry data are probably random, nonspecific, and not toxicologically significant.

Critical reviews of the publication by Carakostas (2012) and Waddell (2011) revealed a poor study design that included: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection, which affects many chemistry and hematological values; no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. Additionally, the report did not adequately describe mean or individual organ weight data and lacked comparison of study findings against laboratory historical control data.

**Table 10-2. Summary of Subchronic Studies on Stevioside**

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST MATERIAL/ SAMPLE PURITY	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS AND REMARKS
Aze et al. (1990) <sup>a</sup>	F344 rat/ 10 females & 10 males in each of 6 groups	Stevioside/ Not reported	0, 0.31, 0.62, 1.25, 2.5, 5% in diet/13 weeks	Not reported	No effects observed on mortality, body weight or food consumption. Clinical chemistry investigation revealed increased LDH levels & histopathological investigation indicated increased incidence of single-cell liver necrosis in all male treated groups, but not in clear dose-response relationship. Investigators did not consider these changes to be treatment related due to small magnitude & low severity of changes, the lack of clear dose relationship & limitation to males only. Organ weights, urine chemistry & gross necropsy not discussed. Authors concluded that 5% stevioside in diet is tolerable dose for 2 year study.
Mitsuhashi (1976) <sup>b</sup>	Rat (strain not reported)	Stevioside/ Not reported	Dietary concentrations up to 7%/ 3 months	Not reported	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy & histopathology not discussed.
Akashi and Yokoyama (1975) <sup>b</sup>	Rat (strain not reported)	Stevioside/ Not reported	Oral doses up to 2,500 mg/kg bw/3 months	2,500	No effects noted at all doses tested. Experimental details such as body weight, organ weight, blood analysis, urine chemistry, gross necropsy & histopathology not discussed.
Awney et al. (2011)	Sprague Dawley rats	Stevioside 97%	Drinking water (15, 1,500 mg/kg bw /day)	15	Treatment with high dose stevioside caused significant changes in several investigated toxicological parameters. Among hematological parameters, significant changes noted in all except WBCs, RBCs & PCV% & in all clinical chemistry parameters except proteins, total lipids, ALT and AST.

<sup>a</sup> Abstract only. <sup>b</sup> As reported by Geuns (2003).

#### 4. Chronic Toxicity Studies

Chronic effects of stevioside have been studied in three separate studies (Table 10-3). No treatment-related increase in tumor incidence was seen in any of these studies. In the most recent and well-documented study (additional study details were presented to JECFA in 2006 (WHO, 2006), the apparent no observed adverse effect level (NOAEL) in F344 rats was the dietary level of 2.5% [test sample purity 96%, Toyoda et al. (1997)]. At 5% of the diet, statistically significant decreases in body weight, percent survival, and kidney weight were noted. The authors attributed these effects to various factors. The decrease in body weight was attributed to an inhibition of glucose utilization. The decrease in survival seemed to have been caused by an unusual late onset of large granular lymphocyte leukemia in high dose males. The authors reported that this tumor is rather common in F344 rats and that the overall incidence in male rats was actually within the historical control range experienced in the laboratory where studies were conducted. The authors attributed the decrease in kidney weight as probably due to a decrease in chronic inflammation found in the histopathological examination relative to control animals.

**Table 10-3. Summary of Chronic Toxicity Studies on Stevioside**

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST MATERIAL/ SAMPLE PURITY	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS AND REMARKS
Toyoda et al. (1997)	F344 rat/ 50 per sex per group	95.6% Stevioside	<i>Ad libitum</i> 0, 2.5, 5% of diet/~24 months (104 weeks)	Author did not assign a NOAEL.  (Mid-dose calculates to 970 in males; JECFA, 2006)	Significant decrease in survival rates in males receiving 5%. General condition, body weight, food intake, mortality, hematological, histopathological & organ weights observed. Body weight gains dose-dependently decreased in both sexes. Kidney weights significantly lower in 5% males& ovary, kidney, & brain weights significantly increased in 5% females. Tumors & non-neoplastic lesions found in all groups& not correlated to treatment. Conclusion--stevioside is not carcinogenic under these experimental conditions.
Xili et al. (1992) <sup>a</sup>	Wistar rat/ 45 per sex per group	85% Stevioside	0, 0.2, 0.6, 1.2 % of diet/24 months	794 (high dose)	After 6, 12 & 24 months 5 rats from each group sacrificed for analysis. No effects observed on growth, food utilization, general appearance, mortality, or lifespan. No changes in hematological, urinary, or clinical biochemical values. Histopathological analysis showed that the neoplastic and non-neoplastic lesions unrelated to level of stevioside in diet.
Yamada et al. (1985)	F344 rat/ 70 per sex per group, 30 per sex per group in	95.2% Steviol glycosides (75% stevioside; 16% Reb A)	0.1, 0.3, 1% of diet/22 months for males, 24 months for females	550 (high dose)	At 6 & 12 months, 10 males & 10 females sacrificed for analysis. General behavior, growth & mortality were same among groups throughout experiment. At 6 months, protein urea significantly increased in females, & blood glucose increased in both sexes, although urinary glucose not detected. Weights of liver, kidney, heart, prostate & testes increased in males at 6 months, & weight of ovaries decreased in females in

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST MATERIAL/ SAMPLE PURITY	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS AND REMARKS
	low-dose				dose-dependent manner. Histopathological examination showed differences in various organs at 6 months that were unrelated to stevioside dose. These differences not found at 12 months. Authors concluded that there were no significant changes after 2 years.

<sup>a</sup> Only abstract available.

### 5. Reproductive & Developmental Toxicity Studies

The use of *S. rebaudiana* as an oral contraceptive has been reported by Indians in Paraguay (Planas and Kuć, 1968; Schvartaman et al., 1977). In experimental studies in rats, crude stevia leaf extract has been shown to inhibit fertility (Planas and Kuć, 1968). Reproductive toxicity studies have been conducted with orally administered purified stevioside. No effect on fertility or reproductive parameters was seen in a three-generation study in hamsters at doses up to 2,500 mg per kg per day (Yodyingyuad and Bunyawong, 1991). There was an absence of statistically significant effects at doses up to 3% [equivalent to 3,000 mg per kg bw per day; sample purity 96%; Mori et al. (1981)]. Similar results were observed in an additional rat study that was reviewed by Geuns (2003) where limited information is available in English (Usami et al., 1994).

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1,000 mg per kg bw per day (only 12 animals at the highest dose) by gavage in corn oil on days 6 - 10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). However, no dose-dependent teratogenic effects were seen. The NOEL was 250 mg per kg bw per day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

No effect on pregnancy or developmental parameters were observed in Swiss albino mice with stevioside or aqueous stevia extract at doses up to 800 mg per kg bw per day in female mice (Kumar and Oommen, 2008). Further details on these studies to the extent available are presented in Table 10-4.

**Table 10-4. Summary of Reproductive Toxicity Studies on Steviol Glycosides**

STUDY	ANIMAL MODEL/ GROUP SIZE	TEST SAMPLE PURITY STEVIOSIDE (UNLESS OTHERWISE NOTED)	DOSES / DURATION	AUTHOR ASSIGNED NOAEL (mg/kg bw/day)	RESULTS & REMARKS
Kumar and Oommen (2008)	Swiss albino mice/ 4 groups of 5 females	Not reported	500 & 800 mg/kg bw/15 days	800	Stevioside & stevia extract (purity & composition not reported) did not have any effect on reproductive parameters in mice when administered to female mice before or during pregnancy. No changes seen in number of implantations or uterine resorptions. No gross anatomical or histopathologic effects seen in 16-day embryos.
Usami et al. (1994) <sup>a</sup>	Wistar Rat/4 groups of 25 or 26 pregnant rats	95.6% <sup>b</sup>	0, 250, 500, 1,000 mg/kg bw/10 days	1,000	Pregnant rats given doses of stevioside by gavage once/day on days 6-15 of gestation & were sacrificed on day 20 of gestation. Fetuses examined for malformations in addition to maternal & fetal body weight, number of live fetuses, sex distribution& numbers of resorptions or dead fetuses. No treatment-related effects observed. Authors concluded that orally administered stevioside not teratogenic in rats.
Yodyingyuad and Bunyawong (1991)	Hamster/ 10 male, 10 female per group (40 total)	90%	0, 500, 1,000, 2,500 mg/kg bw/day/ duration unclear/ 3 months	2,500	Males from each group mated to females from respective dose group. Each female allowed to bear 3 litters during course of experiment. Stevioside had no effect on pregnancies of females at any dose. The F <sub>1</sub> & F <sub>2</sub> hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents); showed normal growth & fertility. Histological examination showed no effect on reproductive organs at any dose.
Oliveira-Filho et al. (1989) <sup>a</sup>	Rat/ number not reported	Not reported (Dried Stevia Leaves)	0 or 0.67 g dried leaves/mL , 2 mL twice per day/ 60 days	Not reported	Prepubertal rats (25-30 days old) tested for glycemia; serum concentrations of thyroxine; tri-iodothyroxine; available binding sites in thyroid hormone-binding proteins; binding of <sup>3</sup> H-methyltrienolone (a specific ligand of androgen receptors) to prostate cytosol; zinc content of prostate, testis, submandibular salivary gland, & pancreas; water content of testes & prostate; body-weight gain; & final weights of testes, prostate, seminal vesicle, submandibular salivary gland& adrenal. Only difference due to treatment was seminal vesicle weight, which fell to 60% compared to control.
Mori et al. (1981)	Rat/11 male, 11 female per	96%	0, 0.15, 0.75 or 3	2,000	Males given stevioside dose in diet for 60 days before & during mating with females who received

	group (44 total)		% of feed/60 days		same diet (as mated male) 14 days before mating & 7 days during gestation. No effect due to treatment on fertility or mating performance & no effect of fetal development. Rats of each sex had slightly decreased body weight gain at highest dose with non-significant increase in number of dead & resorbed fetuses at highest dose.
	Rat/14 per group (28 total)	Not reported (Crude stevia extract)	0 or 5% Crude stevia extract /18 days	Not reported	Extract given orally to adult female rats for 12 days, who were mated with untreated males during last 6 days. Fertility reduced to 21% of fertility in control rats & remained reduced in a 50-60 day recovery. Histological examination, weights of organs, blood analysis, urine chemistry and necropsy not discussed.

<sup>a</sup> Only abstract available. <sup>b</sup> As reported by European Commission (1999b).

## 6. Mutagenicity & Genotoxicity Studies

In a series of studies, mutagenic and genotoxic effects of various stevia extracts and various preparations of stevioside were investigated. These studies are summarized in Table 10-5. All studies were negative with the exception of a comet assay done in rats (Nunes et al., 2007a). The methodology used in this study, and the resulting conclusions, have been questioned by Geuns (2007b), Williams (2007), and Brusick (2008), and responded to by the authors (Nunes et al., 2007b; Nunes et al., 2007c).

In a recent review, Urban et al. (2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in two recent publications (Brahmachari et al., 2011; Tandel, 2011) in which the authors concluded that additional testing is necessary to adequately address the genotoxicity profile (Urban et al., 2013). The review aimed to address this matter by evaluating the specific genotoxicity studies of concern, while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in these publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust, and does not indicate that either stevioside or rebaudioside A are genotoxic. This finding, combined with lack of carcinogenic activity in several rat bioassays, establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.



**Table 10-5. Mutagenicity & Genotoxicity Studies on Stevia Extracts & Stevioside**

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
<i>In Vitro</i>						
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate <sup>a</sup> 1 mg/plate <sup>b</sup>	Negative	Matsui et al. (1996)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside	99	50 mg/plate	Negative <sup>c</sup>	Suttajit et al. (1993)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Stevioside	NS	50 mg/plate	Negative	Klongpanichpak et al. (1997)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	83	10 mg/plate	Negative <sup>c</sup>	Matsui et al. (1996)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	10 mg/plate	Negative <sup>c</sup>	Pezzuto et al. (1985)
Forward mutation	<i>S. typhimurium</i> TM677	Stevioside	NS	Not specified	Negative <sup>c</sup>	Medon et al. (1982)
Gene mutation	Mouse lymphoma L5178Y cells, TK-locus	Stevioside	NS	5 mg/mL	Negative <sup>c,d</sup>	Oh et al. (1999)
Gene mutation (umu)	<i>S. typhimurium</i> TA1535/pSK1002	Stevioside	83	5 mg/plate	Negative <sup>c</sup>	Matsui et al. (1996)
Gene mutation	<i>B. subtilis</i> H17 rec+, M45 rec-	Stevioside	83	10 mg/disk	Negative <sup>c</sup>	Matsui et al. (1996)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/mL 12 mg/mL	Negative	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/mL	Negative	Suttajit et al. (1993)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/mL	Negative <sup>a</sup>	Ishidate et al. (1984)
<i>In Vivo</i>						
DNA damage (comet assay)	Wistar rats: liver, brain and spleen	Stevioside	88.62	4 mg/L (estimated to be 80 - 500 mg/kg bw/day) in drinking water for 45 days	Positive in all tissues examined, most notably in liver	Nunes et al. (2007a)
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52; Reb A, 22	250 – 2,000 mg/kg bw	Negative <sup>e</sup>	Sekihashi et al. (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	Stevia	NS	2,000 mg/kg bw	Negative <sup>e</sup>	Sasaki et al. (2002)
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5 - 250 mg/kg bw	Negative	Oh et al. (1999)
Mutation	<i>D. melanogaster</i> Muller 5 strain	Stevioside	NS	2% in feed	Negative	Kerr et al. (1983)

NS = Not specified. <sup>a</sup> Without metabolic activation. <sup>b</sup> As calculated by Williams (2007). <sup>c</sup> With and without metabolic activation (source not specified in original monograph). <sup>d</sup> Inadequate detail available. <sup>e</sup> Sacrificed at 3 hours and 24 hours

## 7. Clinical Studies & Other Reports in Humans

In several studies, pharmacological and biochemical effects of crude extracts of stevia leaves and purified steviol glycosides have been investigated. The effects noted included glucose uptake, insulin secretion, and blood pressure (Geuns et al., 2003a). In South America, stevioside is used

as a treatment for type 2 diabetes. These effects were key concerns for JECFA. In 2006, JECFA summarized the available clinical studies of stevioside and further studies were recommended (WHO, 2006). Subsequently, several studies were conducted, and in 2009, JECFA reviewed these new studies (WHO, 2009). JECFA's summaries of the key studies are included below.

#### **a. Studies Summarized in 2006**

In a study by Curi et al. (1986), aqueous extracts of 5 grams of *S. rebaudiana* leaves were administered to 16 volunteers at 6 hour intervals for three days, and glucose tolerance tests were performed before and after the administration. Another six volunteers were given an aqueous solution of arabinose in order to eliminate possible effects of stress. The extract increased glucose tolerance and significantly decreased plasma glucose concentrations during the test and after overnight fasting in all volunteers.

In a multi-center randomized, double-blind, placebo-controlled trial of hypertensive Chinese men and women (aged 28–75 years), 60 patients were given capsules containing 250 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 750 mg of stevioside per day [equivalent to 11 mg per kg bw per day as calculated by FSANZ (2008)] and followed up at monthly intervals for one year. Forty-six patients were given a placebo. After 3 months, systolic and diastolic blood pressure in men and women receiving stevioside decreased significantly, and the effect persisted over the year. Blood biochemistry parameters, including lipids and glucose, showed no significant changes. Three patients receiving stevioside and one receiving the placebo withdrew from the study as a result of side effects (nausea, abdominal fullness, dizziness). In addition, four patients receiving stevioside experienced abdominal fullness, muscle tenderness, nausea, and asthenia within the first week of treatment. These effects subsequently resolved, and the patients remained in the study (Chan et al., 2000).

In a follow-up multi-center randomized, double-blind, placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years), 85 patients were given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1,500 mg of stevioside per day [equivalent to 21 mg per kg bw per day, as calculated by FSANZ (2008)]. Eighty-nine patients were given a placebo. During the course of study, three patients in each group withdrew. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressures were significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ( $p < 0.001$ ). Eight patients in each group reported minor side effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a randomized, double-blind trial designed, 48 hyperlipidemic volunteers were recruited to investigate the hypolipidemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside ( $73 \pm 2\%$ ), rebaudioside A ( $24 \pm 2\%$ ), and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e., 200 mg per day, equivalent to 3.3 mg per kg bw per day assuming an average body weight of 60 kg), for 3 months. One subject from placebo group and three from treatment group failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low-density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes, and glucose indicated no adverse effects. The authors questioned the subjects' compliance with the dosing regimen, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycosides extract in incremental doses of 3.25, 7.5, and 15 mg per kg bw per day for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

In a paired cross-over study, 12 patients with type 2 diabetes were given either 1 gram of stevioside (stevioside, 91%; other stevia glycosides, 9%) or 1 gram of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 minutes before, and for 240 minutes after, ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycemic or adverse effects were reported by the patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

#### **b. Studies Summarized in 2009**

In a short-term study of stevioside in healthy subjects, 4 male and 5 female healthy volunteers (aged 21–29 years) were provided with capsules containing 250 mg stevioside (97% purity) to be consumed 3 times per day for 3 days (Temme et al., 2004). Doses, expressed as steviol, were 288 mg per day, or 4.4 mg per kg bw per day for females and 3.9 mg per kg bw per day for males. Twenty-four hour urine samples were taken before dosing on day 1 and after dosing on day 3. Fasting blood samples were taken before dosing on day 1, and six samples were taken at different time points on day 3 after dosing. Fasting blood pressure measurements were taken before the first capsule and at six different time intervals after the first dose. Urine was analyzed for creatinine, sodium, potassium, calcium, and urea. Blood was analyzed for plasma glucose, plasma insulin, alkaline phosphatase, alanine transaminase (ALT), glutamic-pyruvate transaminase (GPT), creatine kinase, and lactate dehydrogenase. The clinical analyses of blood, blood pressure, and urine showed no differences between samples taken before or after dosing.

In an unpublished double-blind, placebo-controlled trial study reviewed at the 68<sup>th</sup> JECFA meeting, 250 mg of a product containing 91.7% total steviol glycosides, including 64.5% stevioside and

18.9% rebaudioside A, was administered to groups of type 1 ( $n = 8$ ) and type 2 diabetics ( $n = 15$ ), and non-diabetics ( $n = 15$ ), 3 times daily for 3 months. Control groups with the same number of subjects received a placebo. After 3 months, there were no significant changes in systolic or diastolic blood pressure, glycated hemoglobin (HbA1c), blood lipids, or renal or hepatic function. No adverse effects were reported. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Barriocanal et al., 2006; Barriocanal et al., 2008). The Committee previously noted that this product did not meet the proposed specification of “not less than 95% steviol glycosides” and that the study was conducted in a small number of subjects.

In a follow-up study, Barriocanal et al. (2008) evaluated the effects of steviol glycosides on blood glucose and blood pressure (BP) for three months in subjects with type 1 diabetes, subjects with type 2 diabetes, and subjects without diabetes and with normal/low-normal BP levels. Patients in each group received either 250 mg total dissolved solids (tds) steviol glycoside, stevioside, or placebo treatment. The purity of the steviol glycosides was  $\geq 92\%$ . Three months of follow up revealed no changes in systolic BP, diastolic BP, glucose, or glycated hemoglobin from baseline. In placebo type 1 diabetics, there was a significant difference in systolic BP and glucose. There were no adverse effects observed in either treatment group, and the authors concluded that oral steviol glycosides are well-tolerated and have no pharmacological effect.

A study of antihypertensive effects was conducted in previously untreated mild hypertensive patients with crude stevioside obtained from the leaves of *S. rebaudiana*. Patients with essential hypertension were subjected to a placebo phase for 4 weeks and then received either capsules containing placebo for 24 weeks or crude stevioside at consecutive doses of 3.75 mg per kg bw per day (7 weeks), 7.5 mg per kg bw per day (11 weeks) and 15 mg per kg bw per day (6 weeks). Comparison of patients receiving stevioside with those on placebo showed neither antihypertensive nor adverse effects of stevioside. This study was approved by the local ethics committee and met the requirements of the Declaration of Helsinki (Ferri et al., 2006). The product in this study also did not meet the proposed specification.

A placebo-controlled double-blind trial was carried out in 49 hyperlipidemic patients (aged 20–70 years, number of males and females not supplied) not undergoing treatment. The study was approved by the local ethics committee and complied with the principles of the Declaration of Helsinki. Individuals were divided into two groups, with 24 subjects receiving placebo capsules and 25 receiving capsules containing a dose of 50 mg steviol glycosides (70% stevioside, 20% Rebaudioside A), equivalent to 1.04 mg steviol per kg bw per day, using the mean body weight of the treatment group, 72.7 kg. Two capsules were taken before lunch, and two before dinner, each day for 90 days. Six subjects withdrew from the study, four in the placebo group and two in the test group. Self-reported adverse reactions were recorded, and fasting blood samples were taken at the end of the study and analyzed for alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low density lipoprotein (VLDL), and triglycerides. No effects of treatment on ALT, AST, or GGT were found. Decreases in the total cholesterol and LDL were

observed in both the stevioside group and the placebo group, which were not treatment related. No adverse effects were observed (Silva et al., 2006). The Committee noted at its 68<sup>th</sup> meeting that the product used in this study did not meet the proposed specification.

In a long-term, randomized, double blinded, placebo-controlled study, Jeppesen et al. (2006) investigated the efficacy and tolerability of oral stevioside in patients with type 2 diabetes. In this study, 55 subjects received 500 mg stevioside (purity unspecified), or placebo (maize starch), 3 times daily for 3 months. Compared with the placebo, stevioside did not reduce the incremental area under the glucose response curve and maintained the insulin response, HbA1c, and fasting blood glucose levels. HbA1c is an indicator of mean glucose levels and is used in identifying effects on the control of diabetes. No differences in lipids or blood pressure were observed. It is not clear whether this study was approved by the local ethics committee or met the requirements of the Declaration of Helsinki (Jeppesen et al., 2006).

## Appendix 11 Summary of the Regulatory History of Steviol Glycosides

### A. History of Traditional Medicinal and Human Food Use

Stevia has been used as a traditional medicine and sweetener by native Guarani tribes for centuries (Esen, 2016; Gerwig et al., 2016; Brusick, 2008; Brandle et al., 1998). Hawke (2003) reported that stevia is commonly used as a treatment for type 2 diabetes in South America. However, for its therapeutic effects, elevated doses in the range of 1 gram per person per day or more were reported to be necessary (Gregersen et al., 2004).

For about 30 years, consumers in Japan and Brazil, where stevia has long been approved as a food additive, have been using stevia extracts as non-caloric sweeteners (Raintree, 2012). It was previously reported that 40% of the artificial sweetener market in Japan had been stevia based and that stevia is commonly used in processed foods in Japan (Lester, 1999). Use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and as a natural health product in Canada. It has wide use in China and Japan in food and in dietary supplements. In 2005, it was estimated that sales of stevia in the US reached \$45 million (Newsday, 2006).

More recent reports of consumption figures for stevia reveal pronounced increases in global consumption. Worldwide, Zenith International estimates stevia sales of 3,500 metric tons in 2010, which represents a 27% increase over 2009 figures. The market value is estimated to have increased to \$285 million (Zenith, 2011). In 2013, worldwide sales of stevia was reported to reach 4,100 tons which represents a 6.5% increase over 2011 figures, and this corresponds to an overall market value of \$304 million (Zenith, 2013).

In October 2014, Zenith International reported that worldwide stevia sales were on course to increase 14% to 4,670 tons, associated with a market value of \$336 million. Furthermore, it has been projected that the total market for stevia in 2017 will be 7,150 tons with an associated market value of \$578 million (Zenith, 2014).

NewHope360 reported that the global market for stevia in 2014 was \$347 million, and that is expected to increase to \$565.2 million by 2020. In addition, consumption is expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).

Most recently, Nutritional Outlook reported that Mintel data indicated a 48% increase in stevia-containing products over the last five years (Decker and Prince, 2018). According to Zion Market Research (2018), the global stevia market was valued at approximately \$416.6 million in 2017 and is expected to generate revenue of approximately \$721 million by the end of 2024.

## B. Summary of Regulatory History of Enzyme Modified Steviol Glycosides

Stevia-derived sweeteners are permitted as food additives in South America and in several countries in Asia, including China, Japan, and Korea. In recent years, these sweeteners have received food usage approvals in Mexico, Australia, New Zealand, Switzerland, France, Peru, Uruguay, Colombia, Senegal, Russia, Malaysia, Turkey, Taiwan, Thailand, Israel, Canada, and Hong Kong (EFSA, 2010; Watson, 2010; Health Canada, 2012). In the US, steviol glycosides have been used as a dietary supplement since 1995 (Geuns, 2003).

### 1. U.S. Regulatory History

Based on available information from FDA’s GRAS Notice Inventory website (FDA, 2018) as of October 8, 2018, FDA has issued 54 “no questions” letters on GRAS notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme treatment. A summary of these filings is presented in Table 11-1.

**Table 11-1. FDA’s GRAS Notice Inventory on Various Steviol Glycosides Preparations<sup>a,c</sup>**

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
1. Merisant	GRN 252	High-Purity Reb A $\geq 95\%$	Variety of food categories & table top sweetener
2. Cargill Inc.	GRN 253	High-Purity Reb A $\geq 97\%$	General-purpose sweetener, excluding meat & poultry products
3. McNeil Nutritionals LLC	GRN 275	Purified Steviol Glycosides – Reb A Principal Component	Table top sweetener
4. Blue California	GRN 278	High-Purity Reb A $\geq 97\%$	General-purpose & table top sweetener
5. Sweet Green Fields LLC	GRN 282	High-Purity Reb A $\geq 97\%$	General-purpose sweetener, excluding meat & poultry products
6. Wisdom Natural Brands	GRN 287	Purified Steviol Glycosides $>95\%$ - Reb A and Stevioside Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas
7. Sunwin USA LLC & WILD Flavors	GRN 303	High-Purity Reb A $\geq 95\%$ / $\geq 98\%$	General-purpose sweetener, excluding meat, poultry products & infant formulas
8. Sunwin USA LLC & WILD Flavors	GRN 304	Purified Steviol Glycosides $>95\%$ - Reb A and Stevioside Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas
9. Pyure Brands, LLC	GRN 318	High-Purity Reb A 95%/ 98%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
10. PureCircle USA Inc	GRN 323	Purified Steviol Glycosides – Reb A Principal Component	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
11. GLG Life Tech Ltd <sup>c</sup>	GRN 329	High-Purity Reb A $\geq 97\%$	General-purpose sweetener, excluding meat & poultry products
12. NOW Foods	GRN 337	Enzyme Modified Steviol Glycosides Preparation (EMSGP)	General-purpose sweetener in foods, excluding meat & poultry products, at

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
			levels determined by good manufacturing practices
13. GLG Life Tech Ltd <sup>c</sup>	GRN 348	High-Purity Stevioside $\geq 95\%$	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
14. GLG Life Tech Ltd <sup>c</sup>	GRN 349	High-Purity Steviol Glycosides $\geq 97\%$	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
15. Guilin Layn Natural Ingredients, Corp.	GRN 354	High-Purity Reb A $\geq 97\%$	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
16. BrazTek International Inc.	GRN 365	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
17. Sinochem Qingdao Co. Ltd.	GRN 367	High-Purity Steviol Glycosides $\geq 95\%$	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
18. Shanghai Freeman Americas LLC	GRN 369	Purified Reb A	General-purpose sweetener, excluding meat & poultry products
19. Toyo Sugar Refining Co., Ltd. & Nippon Paper Chemicals Co., Ltd.	GRN 375	Enzyme Modified Steviol Glycosides	General-purpose sweetener in foods, excluding meat and poultry products, at levels determined by good manufacturing practices
20. GLG Life Tech Ltd <sup>b</sup>	GRN 380	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
21. Chengdu Wagott Pharmaceutical	GRN 388	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
22. Chengdu Wagott Pharmaceutical	GRN 389	Steviol Glycosides with Stevioside as the Principal Component	General purpose & table top sweetener, excluding meat & poultry products
23. Daepyeong Co., Ltd.	GRN 393	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
24. Daepyeong Co., Ltd.	GRN 395	Steviol Glycosides with Reb A and Stevioside as the Principal Components	General purpose & table top sweetener, excluding meat & poultry products
25. MiniStar International, Inc.	GRN 418	Purified Reb A	General-purpose sweetener, excluding meat, poultry products & infant formulas.
26. Daepyeong Co., Ltd.	GRN 448	Enzyme Modified Steviol Glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
27. Daepyeong Co., Ltd.	GRN 452	Enzyme Modified Steviol Glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
28. PureCircle USA, Inc.	GRN 456	High-Purity Reb D $\geq 95\%$	General-purpose sweetener, excluding meat, poultry products & infant formulas.



COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
29. Almendra, Ltd.	GRN 461	High-Purity Reb A $\geq$ 97%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
30. Qufu Xiangzhou Stevia Products Co., Ltd.	GRN 467	High-Purity Reb A $\geq$ 98%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
31. PureCircle USA, Inc.	GRN 473	Purified Steviol Glycosides – Reb M (Reb X) Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
32. GLG Life Tech Corp.	GRN 493	High purity steviol glycosides $\geq$ 95%	General-purpose sweetener, excluding meat, poultry products.
33. GLG Life Tech Corp.	GRN 512	High purity Reb M $\geq$ 95%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
34. Almendra Limited	GRN 516	Steviol Glycosides with Reb A and Stevioside as the Principal Components	General-purpose sweetener, excluding meat, poultry products & infant formulas.
35. GLG Life Tech Corp.	GRN 536	High purity Reb C and Steviol glycosides with Reb C as the Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
36. GLG Life Tech Corp.	GRN 548	High purity Reb D	General-purpose sweetener, excluding meat, poultry products & infant formulas.
37. Productora Alysa SpA	GRN 555	Steviol Glycosides with Reb A as the Principal Component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
38. PureCircle, Ltd.	GRN 607	Glucosylated steviol glycosides (minimum purity 80%)	Use as a flavoring agent and flavor modifier at levels ranging from 100 to 1,000 ppm
39. PureCircle, Ltd.	GRN 619	Steviol Glycosides $\geq$ 95%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
40. Cargill, Inc.	GRN 626	Steviol glycosides (Reb M and Reb D) produced in <i>Saccharomyces cerevisiae</i>	General-purpose sweetener
41. DSM Nutritional Products, LLC.	GRN 632	Rebaudioside A from <i>Yarrowia lipolytica</i>	General-purpose sweetener, excluding meat, poultry products & infant formulas.
42. Hunan Huacheng Biotech Inc.	GRN 638	High purity steviol glycosides with Reb A as the principal component	General-purpose sweetener, excluding meat, poultry products & infant formulas.
43. GLG Life Tech Corporation	GRN 656	Enzyme-modified steviol glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
44. PureCircle USA	GRN 662	Glucosylated steviol glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
45. Blue California	GRN 667	Rebaudioside M	General-purpose sweetener, excluding meat, poultry products & infant formulas.

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
46. Xinghua GL Stevia Co., Ltd	GRN 702	Purified steviol glycosides	General-purpose sweetener
47. Blue California	GRN 715	Rebaudioside D	General-purpose sweetener, excluding meat, poultry products & infant formulas.
48. Shandong Shengjiangyuan Biotechnology	GRN 733	Purified steviol glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
49. PureCircle Limited	GRN 744	Rebaudioside M	General-purpose sweetener, excluding meat, poultry products & infant formulas.
50. PureCircle Limited	GRN 745	Rebaudioside M	General-purpose sweetener, excluding meat, poultry products & infant formulas.
51. DSM Food Specialties/DSM Nutritional Products North America	GRN 759	Steviol glycosides consisting primarily of Rebaudioside M from <i>Yarrowia lipolytica</i>	Flavor and general-purpose sweetener
52. Sichuan Ingia Biosynthetic Co., Ltd.	GRN 764	Rebaudioside D	General-purpose sweetener, excluding meat, poultry products & infant formulas
53. Cargill, Inc.	GRN 768	Stevia leaf extract	General-purpose sweetener, excluding meat, poultry products & infant formulas
54. Tate and Lyle	GRN 780	Rebaudioside M	General-purpose sweetener, excluding meat, poultry products & infant formulas

<sup>a</sup> This table was derived, in part, from McQuate (2011).

<sup>b</sup> The name of this company is now GLG Life Tech Corporation.

<sup>c</sup> GRN 790, submitted by GLG Life Tech Corporation, regarding steviol glycosides (minimum purity 95%), was filed by FDA and is presently under review; GRN 795, submitted by Steviana Bioscience (Suzhou) Inc., regarding purified steviol glycosides, was filed by FDA and is presently under review; GRN 799, submitted by Sichuan Ingia Biosynthetic Co., Ltd., was filed by FDA and is presently under review.

In addition, the Flavor and Extract Manufacturers Association (FEMA) has included several steviol glycosides preparations on their GRAS lists as shown in Table 11-2.

**Table 11-2. FEMA GRAS Status for Steviol Glycoside Preparations**

STEVIOLE GLYCOSIDES PREPARATION	FEMA NUMBER	REFERENCE
Rebaudioside A	4601	Smith et al. (2009)
Rebaudioside C; dulcoside B	4720	Leffingwell (2011)
Glucosyl steviol glycosides; enzymatically modified stevia extract	4728	Leffingwell and Leffingwell (2014); Marnett et al. (2013)

STEVIOL GLYCOSIDES PREPARATION	FEMA NUMBER	REFERENCE
Stevioside	4763	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 60%	4771	Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 80%	4772	Marnett et al. (2013)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside C 30%	4796	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, <i>Stevia rebaudiana</i> , Rebaudioside A 22%	4805	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, <i>Stevia rebaudiana</i> Rebaudioside C 22%	4806	Cohen et al. (2015a); Cohen et al. (2015b)

## 2. Canadian Regulatory History

On September 18, 2009, based on a review of the international regulation of *Stevia rebaudiana* and the clinical evidence for safety and efficacy, the Natural Health Products Directorate, Health Canada (2009) adopted the following guidelines for the use of *stevia* and steviol glycosides in Natural Health Products (NHPs) (Health Canada, 2009). The revised recommendation for the maximum limit for steviol glycosides in NHPs is in accordance with the full acceptable daily intake (ADI) of 4 mg steviol per kg bw established by JECFA (WHO, 2008).

On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012). In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (Health Canada, 2014). On January 15, 2016, Health Canada approved the use of Reb M for use as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (Health Canada, 2016).

Most recently, Health Canada’s Food Directorate has updated its List of Permitted Sweeteners to allow for the use of steviol glycosides as a sweetener in ‘unstandardized snack bars,’ including granola bars, cereal bars, fiber bars, and protein isolate-based bars (Health Canada, 2017b). Health Canada (2017a) also modified the List of Permitted Sweeteners to include “all the steviol glycosides in the *Stevia rebaudiana* Bertoni plant (stevia plant).”

## 3. European Regulatory History

The Joint Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 51<sup>st</sup>, 63<sup>rd</sup>, 68<sup>th</sup> and 73<sup>rd</sup> meetings. In 2000, JECFA published the original review on steviol glycosides (WHO, 2000). JECFA established a temporary ADI (acceptable daily intake) of 0-2 mg per kg (on a

steviol basis) at its 63<sup>rd</sup> meeting (WHO, 2006). Additionally, JECFA finalized food grade specifications (FAO, 2007b), although they were subsequently updated in 2008 (FAO, 2008) and 2010 (FAO, 2010) (see below). At the 69<sup>th</sup> meeting, the temporary status of the ADI was removed, and the ADI was raised to 0-4 mg per kg bw per day (on a steviol basis) as a result of the JECFA review of more recently completed clinical studies with steviol glycosides (WHO, 2008). In 2009, JECFA published a final monograph addendum on steviol glycosides (WHO, 2009).

In early 2009, a number of parties, including the government of Australia and the Calorie Control Council, submitted a request to the Codex Committee on Food Additives in which it was proposed that the JECFA specifications for steviol glycosides should be modified to allow inclusion of rebaudioside D and rebaudioside F as specifically named acceptable glycosides that would be considered as part of the minimum 95% steviol glycosides composition (CCFA, 2009). This proposed modification was endorsed by the Codex Alimentarius Committee in July 2009; it was on the agenda for discussion at the JECFA Meeting in June, 2010 (FAO/WHO, 2009), and JECFA subsequently took final action in approving the modified steviol glycosides specifications to include rebaudioside D and rebaudioside F (FAO, 2010).

In 2008, Switzerland's Federal Office for Public Health approved the use of stevia as a sweetener citing the favorable actions of JECFA (Health, 2008). Subsequently, France published its approval for the food uses of rebaudioside A with a purity of 97% (AFSSA, 2009a; AFSSA, 2009b).

In light of JECFA's 2008 findings, and in response to a June 2008 request by the European Commission for European Food Safety Authority (EFSA) to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from three petitioners, EFSA reexamined the safety of steviol glycosides (EFSA, 2010). After considering all the data on stability, degradation products, metabolism and toxicology, the EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per bw per day, which is similar to JECFA's determination.<sup>11</sup> In addition, on May 25, 2011, EFSA published a determination that the daily dietary intake for use of rebaudioside A as a flavoring substance in a variety of foods would be less than the ADI for steviol glycosides (EFSA, 2011a). In 2014, EFSA evaluated extending the use of steviol glycosides as ingredients in food categories to include coffee, tea, and herbal and fruit infusions (assessed at 10 mg per L steviol glycosides). Exposure estimates were lower than those determined by the Panel in 2011 due to available data, and remained below the ADI of 4 mg per kg bw per day, with the exception of toddlers from one country at the 95<sup>th</sup> percentile exposure level of 4.3 mg per kg bw per day (EFSA, 2014). More recently, exposure estimates, based on maximum permitted levels (MPLs) and proposed use

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<sup>11</sup> From a historical perspective, it is noted that the UK's Advisory Committee on Novel Foods and Processes for the Ministry of Agriculture, Fisheries and Food on September 24, 1998 rejected an application for use of steviol glycosides as a sweetener in herbal teas because "the applicant had not provided all of the information necessary to enable an assessment to be made" (MAFF, 1998)). In 1999, the Scientific Committee on Food for the European Commission concluded that "there are no satisfactory data to support the safe use of these stevia plants and leaves" (EuropeanCommission, 1999a) In another opinion also dated June 17, 1999, the Committee also reiterated "its earlier opinion that stevioside is not acceptable as a sweetener on the presently available data" (EuropeanCommission, 1999b)

levels increased to 29 mg per L steviol glycosides, were found to have a “negligible” impact on dietary intake for all population groups, with the mean exposure estimate below the ADI of 4 mg per kg bw per day, with the exception of toddlers from one country at the 95<sup>th</sup> percentile exposure level of 4.3 mg per kg bw per day. The EFSA panel concluded that “dietary exposure to steviol glycosides (E 960) is similar to the exposure estimated in 2014 and therefore does not change the outcome of the safety assessment” (EFSA, 2015).

The appropriate European regulatory bodies, including the joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA), have now agreed that steviol glycosides are safe for all populations to consume and are a suitable sweetening option for diabetics. Effective December 2, 2011, the EU approved their use as food additives (EU, 2011). In March 2016, the EU approved the use of steviol glycosides in mustard (Michail, 2016).

Most recently, an amendment to the EU food additives regulation 231/2012, which became active on November 3, 2016, removed the previous requirement for stevia blends to contain at least 75% Reb A or stevioside. In addition, the updated regulation ---(EU) 2016/1814---now permits the following steviol glycosides in stevia blends: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside, and dulcoside (Searby, 2016).

The EFSA Panel of Food Additives and Nutrient Sources reviewed an application for glucosylated steviol glycoside preparations for use as a new food additive. The Panel concluded that the data supplied by the applicant were “insufficient to assess the safety” of the glucosylated steviol glycosides preparation. It should be noted that no safety concerns were raised by the EFSA Panel, and that their decision was based on the “limited” data provided in the dossier submitted by the applicant (EFSA, 2018).

#### **4. Asian Regulatory History**

As of May 2010, the government of Hong Kong amended its food regulations to allow the use of steviol glycosides as a permitted sweetener in foods (Hong Kong Centre for Food Safety, 2010). This action followed in the aftermath of the detailed safety evaluation and favorable findings as reported by JECFA.

The international community continued to exhibit much interest in the food uses of steviol glycosides, with additional advances reported in early July 2011. The Codex Alimentarius Commission has adopted proposed maximum use levels for steviol glycosides in all major food and beverage categories, and this action was expected to favorably influence authorizations of stevia uses in India, Indonesia, Thailand, and the Philippines (FoodNavigator, 2011). An article published online by FoodNavigator (2013) states the following: “with approvals now in Vietnam, the Philippines, Malaysia, Singapore and Thailand, Indonesia is the only [Southeast Asian nation] where stevia hasn’t been given the rubber stamp” (Whitehead, 2013). Furthermore, the International Alliance of Dietary/Food Supplement Associations (IADSA) reported that the Codex

Alimentarius Commission agreed to adopt the use of steviol glycosides for addition to chewable food supplements as had been requested by IADSA (NewHope360, 2011).

The Food Safety and Standards Authority of India (FSSAI) convened on September 20, 2012, at which time they approved the use of steviol glycosides as a non-nutritive sweetener in a variety of foods. The FSSAI specified that: the steviol glycosides must meet the specifications and purity as established by JECFA; table top sweetener tablets may contain 7 mg of steviol equivalents per 100 mg carrier/filler, as well as established maximum use levels specific to 11 distinct food categories including dairy, beverage, and chewing gum applications (FSSAI, 2012).

Since December 10, 2012, over thirty registrations have been granted by FDA Philippines to stand-alone steviol glycosides sweeteners or foods containing steviol glycosides as ingredients, including: FR-104390, Steviten Light Brand Steviol Glycosides 95% Sweetener Powder; FR-109427, Del Monte Pineapple Chunks in Extra Light Syrup Reduced Calorie with Steviol Glycosides from Stevia; FR-101120, Diebetamil Zero Calorie Sweetener with Stevia (stick pack); and FR-102127, Sawayaka Stevia Sweetener (1 g sticks) (Philippines, 2014).

Steviol glycosides are also listed under INS number 960 in the Food Additives Permitted Under the Singapore Food Regulations document prepared by the Agri-Food & Veterinary Authority (AVA) of Singapore (AVA, 2014).

## **5. Other Regulatory History**

In 2008, the Food Standards Australia New Zealand (FSANZ) completed its evaluation of an application for use of steviol glycosides in foods. FSANZ recommended that the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels as requested since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages, and flavored soy beverages up to 200 mg per kg, and in plain soy beverages up to 100 mg per kg (FSANZ, 2011). In a recent risk assessment, FSANZ concluded that the use of Reb M does not pose any “public health and safety issues” (FSANZ, 2015b). In addition, FSANZ proposed to add Reb M to the list of permitted steviol glycosides (FSANZ, 2015a). On January 14, 2016, Reb M was approved for use “as a food additive in accordance with the current permissions for steviol glycosides” (FSANZ, 2016a).

Most recently, FSANZ called for submissions on permitting all minor steviol glycosides extracted from stevia leaf to be included in the definition of steviol glycosides in the Food Standards Code, noting that “[no] evidence was found to suggest that the proposed changes pose any public health and safety concerns.” The submission period ended on December 19, 2016 (FSANZ, 2016b).

Subsequently, on February 8, 2017, FSANZ approved a draft variation of the definition of steviol glycosides to include all steviol glycosides present in the *Stevia rebaudiana* leaf (FSANZ, 2017).

On July 20, 2018, FSANZ called for comments on the production of Reb M using enzymes derived from genetically modified yeast. A risk assessment by FSANZ found no health and safety concerns with the manufacturing method. The comment period closed on August 31, 2018 (FSANZ, 2018a).

On October 9, 2018, FSANZ called for comments on the addition of steviol glycosides to fruit drinks at a maximum permitted level of 200 mg per kg steviol equivalents. A risk assessment by FSANZ found no health and safety concerns with regard to the proposed levels of use in fruit drinks, and noted that “the impact...on total dietary exposure was determined to be small.” The deadline for submissions is November 6, 2018 (FSANZ, 2018b).

On September 10, 2012, the South African Department of Health issued an amendment to labeling regulations indicating: “in the case of the sweetener steviol glycosides, it shall be described as ‘Steviol Glycosides’ or ‘Steviol Extract.’” On the same date, steviol glycosides were added to the List of Permissible Sweeteners.

## Appendix 12 Summary of Published Safety Reviews

### 1. Summary of JECFA Reviews

At an early review during its 51<sup>st</sup> meeting, JECFA (WHO, 2000) expressed the following reservations about the safety data available at that time for steviol glycosides:

The Committee noted several shortcomings in the information available on stevioside. In some studies, the material tested (stevioside or steviol) was poorly specified or of variable quality, and no information was available on other constituents or contaminants. Furthermore, no studies of human metabolism of stevioside and steviol were available. In addition, data on long-term toxicity and carcinogenicity were available for stevioside in only one species. The mutagenic potential of steviol has been tested sufficiently only *in vitro*.

In view of the absence of information for the elaboration of specifications for stevioside and since the evaluation of the available toxicological data revealed several limitations, the Committee was unable to relate the results of the toxicological investigations to the commercial product and could not allocate an ADI to stevioside.

Before reviewing stevioside again, the Committee considered that it would be necessary to develop specifications to ensure that the material tested was representative of the commercial product. Further information on the nature of the substance that was tested, data on the metabolism of stevioside in humans and the results of suitable *in vivo* genotoxicity studies with steviol would also be necessary.

Subsequently, additional data were generated on the metabolism of steviol glycosides and submitted to JECFA. This information suggested that the common steviol glycosides are converted to steviol by intestinal bacteria and then rapidly converted to glucuronides that are excreted. The committee now had a molecular basis to become comfortable with new toxicology studies on test materials that consisted of variable composition but were relatively high purity mixtures of the common steviol glycosides. The new information also revealed that in *in vitro* studies, steviol is mutagenic, while in *in vivo* conditions, it is not mutagenic. The committee became convinced that purified steviol glycosides did not impair reproductive performance, as did crude preparations of stevia, and that there were sufficient chronic studies in rats with adequate no observed effect levels (NOEL) that could support a reasonable ADI in the range of doses that would be encountered by the use of steviol glycosides as a sugar substitute. However, JECFA wanted more clinical data to rule out pharmacological effects at the expected doses. The following excerpt was taken from the report of the 63<sup>rd</sup> meeting (WHO, 2006):

The Committee noted that most of the data requested at its fifty-first meeting, e.g., data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available. The Committee concluded that stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives *in vitro* is not expressed *in vivo*.



The NOEL for stevioside was 970 mg per kg bw per day in a long-term study (Toyoda et al., 1997) evaluated by the Committee at its fifty-first meeting. The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg per kg bw per day (equivalent to 5–10 mg per kg bw per day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes).

The Committee therefore decided to allocate a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0–2 mg per kg bw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg per kg bw per day (or 383 mg per kg bw per day, expressed as steviol) in the 2-year study in rats and a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intra-species differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.

The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

In 2007, at its 68<sup>th</sup> meeting, JECFA (WHO, 2007) concluded that sufficient progress had been made on the clinical studies and extended the temporary ADI until 2008. Subsequently, sufficient data had been received by JECFA to revise and finalize food additive specifications for steviol glycosides. The Chemical and Technical Assessment report, written after the 2007 meeting, explained the Committee's thinking, which resulted in flexibility in the identity specifications (FAO, 2007b; FAO, 2007a).

In response to the call for data on "stevioside" for the 63rd meeting of the Committee, submissions from several countries showed that the main components of the commercially available extracts of stevia are stevioside and rebaudioside A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. The information indicated that most commercial products contained more than 90% steviol glycosides with the two main steviol glycosides comprising about 80% of the material. The 63rd JECFA required that the summed content of stevioside and rebaudioside A was not less than 70% and established a minimum purity of 95% total steviol glycosides. Analytical data showed that most of the remaining 5% could be accounted for by saccharides other than those associated with the individual steviol glycosides.

Noting that the additive could be produced with high purity (at least 95%) and that all the steviol glycosides hydrolyze upon ingestion to steviol, on which the temporary ADI is based, the 68<sup>th</sup> JECFA decided it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content. The Committee recognized that the newly revised specifications would cover a range of compositions that could include, on the dried basis, product that was at least 95% stevioside or at least 95% rebaudioside A.

In 2008, based on additional clinical studies, at its 69<sup>th</sup> meeting, JECFA finalized the evaluation of steviol glycosides (WHO, 2008), raised the ADI to 0 – 4 mg per kg bw per day, and removed the “temporary” designation. The summary of the Committee’s key conclusions in the final toxicology monograph addendum (WHO, 2009) were stated as follows:

From a long-term study with stevioside, which had already been discussed by the Committee at its fifty-first meeting, a NOEL of 970 mg per kg bw per day was identified. At its sixty-third meeting, the Committee set a temporary ADI of 0–2 mg per kg bw for steviol glycosides, expressed as steviol, on the basis of this NOEL for stevioside of 970 mg per kg bw per day (383 mg per kg bw per day expressed as steviol) and a safety factor of 200, pending further information. The further information was required because the Committee had noted that stevioside had shown some evidence of pharmacological effects in patients with hypertension or with type 2 diabetes at doses corresponding to about 12.5–25.0 mg per kg bw per day (5–10 mg per kg bw per day expressed as steviol).

The results of the new studies presented to the Committee at its present meeting have shown no adverse effects of steviol glycosides when taken at doses of about 4 mg per kg bw per day, expressed as steviol, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. The Committee concluded that the new data were sufficient to allow the additional safety factor of 2 and the temporary designation to be removed and established an ADI for steviol glycosides of 0–4 mg per kg bw expressed as steviol.

The Committee noted that some estimates of high-percentile dietary exposure to steviol glycosides exceeded the ADI, particularly when assuming complete replacement of caloric sweeteners with steviol glycosides, but recognized that these estimates were highly conservative and that actual intakes were likely to be within the ADI range.

## **2. Summary of FSANZ Review of Steviol Glycosides**

In 2008, FSANZ completed a review of the safety of steviol glycosides for use as a sweetener in foods. FSANZ concluded that steviol glycosides are well tolerated and unlikely to have adverse effects on blood pressure, blood glucose, or other parameters in normal, hypotensive, or diabetic subjects at doses up to 11 mg per kg bw per day. FSANZ agreed with JECFA in setting an ADI of 4 mg steviol equivalents per kg bw per day, which was derived by applying a 100-fold safety factor to the NOEL of 970 mg per kg bw per day established by a 2-year rat study (Toyoda et al., 1997). The FSANZ review discussed the adequacy of the existing database and several new studies, including the clinical studies reviewed by JECFA in the summer of 2007, most notably the work of Barriocanal et al. (2008), which was later published in 2008.

In their draft document, FSANZ also indicated that the new data in humans provides a basis for revising the uncertainty factors that were used by JECFA to derive the temporary ADI for steviol glycosides in 2005. In particular, the evidence surrounding the pharmacological effects of steviol glycosides on blood pressure and blood glucose has been strengthened so that the additional 2-fold safety factor for uncertainty related to effects in normotensive or diabetic individuals is no longer required. Therefore, FSANZ established an ADI of 4 mg per kg bw per day for steviol glycosides as steviol equivalents, derived by applying a 100-fold safety factor to the NOEL of 970

mg per kg bw per day (equivalent to 383 mg per kg bw per day steviol) in a 2-year rat study (FSANZ, 2008). In December 2010, FSANZ recommended accepting the increased usage levels since no public health and safety issues were identified (FSANZ, 2010). Subsequently, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

### **3. Summary of EFSA Review of Steviol Glycosides**

On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides (mixtures that comprise not less than 95% of stevioside and/or rebaudioside A) as a food additive. Earlier--- in 1984, 1989 and 1999---the Scientific Committee for Food (SCF) evaluated stevioside as a sweetener. At the time, the SCF concluded that the use of stevioside was “toxicologically not acceptable” due to insufficient available data to assess its safety. However, in light of JECFA’s 2008 findings, and in response to a June 2008 request by the European Commission, EFSA reevaluated the safety of steviol glycosides as a sweetener.

As both rebaudioside A and stevioside are metabolized and excreted by similar pathways, with steviol being the common metabolite for both glycosides, the EFSA Panel agreed that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides. Considering the available safety data (*in vitro* and *in vivo* animal studies and some human tolerance studies), the EFSA Panel concluded that steviol glycosides, complying with JECFA specifications, are not carcinogenic, genotoxic, or associated with any reproductive/developmental toxicity. The EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per kg bw per day based on the application of a 100-fold uncertainty factor to the NOAEL in the 2-year carcinogenicity study in the rat when administering 2.5% stevioside in the diet. This is equal to 967 mg stevioside per kg bw per day (corresponding to approximately 388 mg steviol equivalents per kg bw per day). Conservative estimates of steviol glycosides exposures both in adults and in children suggest that the ADI could possibly be exceeded by European consumers of certain ages and geographies at the maximum proposed use levels.

Recently, EFSA (2011b) revised its exposure assessment of steviol glycosides from its uses as a food additive for children and adults, and published the reduced usage levels in 16 foods by a factor of 1.5 to 3, with no changes for 12 food groups. Additionally, 15 other foods were removed, mainly within the category of desserts and other products, while 3 new food uses were added. The mean estimated exposure to steviol glycosides (equivalents) in European children (aged 1-14 years) ranged from 0.4 to 6.4 mg per kg bw per day and from 1.7 to 16.3 mg per kg bw per day at the 95<sup>th</sup> percentile. A correction was considered to be necessary for the consumption of non-alcoholic flavored drinks (soft drinks) by children, and the corrected exposure estimate at the 95<sup>th</sup> percentile for children ranged from 1.0 to 12.7 mg per kg bw per day. For adults, the mean and 97.5<sup>th</sup> percentile intakes were estimated to range from 1.9 to 2.3 and 5.6 to 6.8 mg per kg bw per

day, respectively. Non-alcoholic flavored drinks (soft drinks) are the main contributors to the total anticipated exposure to steviol glycosides for both consumer categories. For high consumers, EFSA noted that revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent).

In addition, EFSA (2011a) recently accepted rebaudioside A as a flavoring agent in a variety of foods. EFSA reviewed the available safety data on rebaudioside A and agreed that the ADI of 4 mg per kg bw per day established for steviol glycosides applied also to rebaudioside A in a purified form. The dietary intake for use as a flavoring agent was calculated by two different methods, and EFSA determined that the worst-case exposure would be 10,888 microgram per person per day, which is equivalent to 181 microgram rebaudioside A per kg bw per day, for a person weighing 60 kg. This corresponds to a daily intake of 60 microgram steviol per kg bw per day, using a conversion factor of 0.33 for converting the amount of rebaudioside A into steviol equivalents.

#### **4. Other Published Reviews**

Stevia and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002). Four additional reviews have appeared on the toxicology and biological activity of stevia extracts and steviol glycosides (Yadav and Guleria, 2012; Brown and Rother, 2012; Brahmachari et al., 2011; Chatsudthipong and Muanprasat, 2009). In reviewing these studies, caution is warranted since these reviews do not differentiate well between studies on crude stevia extract and purified steviol glycosides. In addition, many of the reviewed studies on biological activity used routes of administration other than oral, and they may have used doses that are much higher than expected dietary exposures of steviol glycosides as a sweetener. In a letter to the editor of the *Journal of Pharmacology and Therapeutics*, Roberts and Munro (2009) criticized the Chatsudthipong and Muanprasat (2009) review with some important points that are applicable in general to these four reviews. Important excerpts from this letter are as follows:

“It is well established that some stevia extracts are crude mixtures that contain multiple components of the stevia leaf, including those components that do not provide a sweet taste. These mixtures also vary considerably in quality, purity, and composition. Therefore, it is not surprising that sometimes these crude and uncharacterized materials may contain substances that possess some degree of pharmacologic activity but any such effects cannot be attributed specifically to the steviol glycosides. In contrast to studies conducted with less pure steviol glycoside preparations, studies conducted with purified preparations do not indicate any evidence of pharmacological effects.”

“The authors consistently cite pharmacological, toxicological, and biochemical effects from in vitro studies or from studies in which animals were dosed intravenously (e.g., Melis, 1992 a,b,c). Steviol glycosides are hydrolyzed completely by the gut microflora to steviol prior to absorption, with no systemic absorption of the glycone form following oral exposure. Therefore, the results of in vitro and intravenous, intraperitoneal, or subcutaneous dosing studies of the glycone form are not relevant to the safety of steviol glycosides consumed orally.”

“Collectively, the report of Chatsudthipong and Muanprasat (2009) is incomplete and lacking discussion of key studies of the safety of stevioside and rebaudioside A. It focuses on alleged effects of stevia and steviol glycosides of low or unknown purity, fails to consider the route of exposure in relation to metabolism and safety assessment and does not include recent opinions expressed by world wide regulatory authorities affirming the safety of purified forms of stevioside and rebaudioside A as a food ingredient.”

Most recently, Urban et al. (2015) reviewed the potential allergenicity of steviol glycosides. The authors noted that: “hypersensitivity reactions to stevia in any form are rare” and concluded that current data do not support claims that steviol glycosides are allergenic. In addition, the authors stated that there is “little substantiated scientific evidence” to warrant consumer warning labels for highly purified stevia extracts (Urban et al., 2015).

## Appendix 13 Summary of Studies on Steviol Glycosides Preparations That Are Primarily Rebaudioside A

### Safety Data on Rebaudioside A<sup>12</sup>

Since 2008, several well-designed toxicology studies that followed the current regulatory and scientific guidelines for such studies have been reported on purified rebaudioside A, although it is uncertain whether or not these studies were considered by JECFA during its 2008 deliberations. These recent investigations included additional subchronic studies in rats and one in dogs, mutagenicity studies, reproduction and developmental studies in rats, and comparative pharmacokinetic studies with stevioside in rats and humans, as well as additional clinical studies. These studies confirm that rebaudioside A is metabolized similarly to other steviol glycosides, and they exhibited an absence of toxicological effects in the key studies reviewed by JECFA. It should be noted that rebaudioside A, as the steviol glycoside with high sweetness intensity and relatively high prevalence in the stevia leaves, remains an active topic of scientific research. For example, a study found in a recent literature search examined the anti-hyperglycemic activity of rebaudioside A in diabetic rats (Saravanan and Ramachandran, 2012). These investigators found that the effects of streptozotocin-induced diabetes on glucose and insulin levels were at least partially reversed in a dose-dependent manner with oral administration of rebaudioside A at doses in the range of 50-200 mg per kg bw. The doses used are 10-40 times higher than expected from the use of rebaudioside A as a sweetener. The known anti-hyperglycemic activity of steviol glycosides led JECFA to require clinical studies at reasonably high doses to show that—at levels used in food—there would be no effect on glucose homeostasis or blood pressure in human consumers. The clinical studies described below on rebaudioside A (Maki et al., 2008a; Maki et al., 2008b) the lack of these pharmacological effects of rebaudioside A at expected levels of consumption.

#### 1. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

Studies investigating the ADME of extracts from stevia are available on stevioside, Reb A, and other steviol glycosides. Data evaluating the absorption and fate of these extracts from various animal species and humans indicate that one can extrapolate these results from rats to humans. Stevioside is metabolized to steviol *via* intestinal microflora, and the absorption of stevioside after oral administration has been shown to be very low (Koyama et al., 2003b; Geuns et al., 2003b; Geuns et al., 2003a).

Studies investigating the hydrolysis of steviol glycosides by intestinal microflora have demonstrated that both stevioside and Reb A are hydrolyzed to steviol following *in vitro* incubation with various

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<sup>12</sup> Questions about the safety of rebaudioside A were previously raised by Huxtable (2002), and Kobylewski and Eckhart (2008). Their respective concerns, as well as opposing views supporting the safety of designated food uses of rebaudioside A expressed by Expert Panels, have been outlined in other GRAS notifications that were submitted to FDA. A more detailed account can be found in GRAS notifications 278, 287, 303, and 304.

cecal microflora (Wingard Jr et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Geuns et al., 2003a). In addition, the *in vitro* hydrolysis of Reb A to steviol was found to be slower than that of stevioside (Koyama et al., 2003b), which is thought to be partly due to the presence of one additional glucose moiety and to differences in structural complexities. Koyama et al. (2003b) suggest that the major pathway for Reb A is conversion to stevioside with a minor pathway of conversion to Reb B prior to being ultimately converted to steviol. Stevioside is further converted to steviolbioside, steviolmonosides, and finally steviol, with glucose being released with each subsequent hydrolysis.

In three recently completed studies, absorption and fate of rebaudioside A were systematically investigated in rats and humans.

For comparative purposes to determine whether toxicological studies conducted previously with stevioside would be applicable to the structurally-related glycoside, rebaudioside A, toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol were examined in rats (Roberts and Renwick, 2008). Orally administered single doses of the radiolabeled compounds were extensively and rapidly absorbed with plasma concentration-time profiles following similar patterns for stevioside and rebaudioside A.

Roberts and Renwick (2008) identified free steviol (82 to 86%), steviol glucuronide (10 to 12%), and two unidentified metabolites (5-6%) in rat plasma following treatment with either stevioside or Reb A eight hours post-oral administration. A comparable pharmacokinetic profile was noted following oral treatment of rats with radiolabeled Reb A or stevioside, with the time of maximum plasma concentration ( $T_{max}$ ) for radioactivity ranging between 2 and 8 hours. In comparison, steviol  $T_{max}$  for plasma was noted within 30 minutes of oral administration. All plasma samples had similar metabolite profiles; the predominant radioactive component in all samples was steviol, with lower amounts of steviol glucuronide(s) and low levels of one or two unidentified metabolites. It is believed that this delay between the occurrence of radioactivity in the plasma and time of administration of steviol glycosides is due to the fact that the Reb A and stevioside are first cleaved to steviol before absorption.

Within 72 hours of administration, elimination of radioactivity from plasma was essentially complete. Following elimination in the bile, steviol is available to be released again from its conjugated form by microflora activity and may enter enterohepatic circulation. Consequently, free and conjugated steviol are secreted in the feces along with any unhydrolyzed fraction of the administered glycosides. Following Reb A treatment, significant amounts of unchanged rebaudioside A (29% in males and 19% in females) and stevioside (3% in males and 4% in females) were excreted in the feces. Following oral stevioside administration, unchanged stevioside was excreted in rat feces. Other unidentified metabolites are also present in fecal samples of rats treated with either glycoside. Rebaudioside A, stevioside, and steviol were metabolized and excreted rapidly, with ~60% of the radioactivity eliminated in the feces within 48 hours. Urinary excretion accounted for less than 2% of the administered dose for all compounds in both intact and bile duct-cannulated rats, and the majority of the absorbed dose was excreted *via*

the bile. After administration of the compounds to intact and bile duct-cannulated rats, radioactivity in the feces was present primarily as steviol. The predominant radioactive compound detected in the bile of all cannulated rats was steviol glucuronide (Roberts and Renwick, 2008).

In summary, Roberts and Renwick (2008) found that steviol was the predominant component found in plasma samples after oral administration of Reb A, stevioside, and steviol in rats. Lower amounts of steviol glucuronide(s) and one or two unidentified metabolites were also found. The majority of all samples were found to be excreted rapidly---primarily in the feces---within 48 hours. This is in agreement with the previous *in vitro* hydrolysis data that indicated that both Reb A and stevioside are metabolized to steviol by intestinal microflora. The predominant compound detected in the bile was steviol glucuronide, while the prominent material in the intestine was steviol, which the authors suggest indicates that deconjugation occurs in the lower intestine. The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.

In a randomized, double blind, cross-over study in healthy male subjects, Wheeler et al. (2008) assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside. Following administration of rebaudioside A or stevioside, steviol glucuronide appeared in the plasma of all subjects, with median  $T_{max}$  values of 12.0 and 8.00 hours post-dose, respectively. Steviol glucuronide was eliminated from the plasma, with similar  $t_{1/2}$  values of approximately 14 hours for each compound. Administration of rebaudioside A resulted in a significantly (~22%) lower steviol glucuronide geometric mean  $C_{max}$  value (1,472 ng per mL) than administration of stevioside (1,886 ng per mL). The geometric mean  $AUC_{0-t}$  value for steviol glucuronide after administration of rebaudioside A (30,788 ng\*hr per mL) was approximately 10% lower than after administration of stevioside (34,090 ng\*hr per mL). Steviol glucuronide was excreted primarily in the urine of the subjects during the 72-hour collection period, accounting for 59% and 62% of the rebaudioside A and stevioside doses, respectively. No steviol glucuronide was detected in feces. Pharmacokinetic analysis indicated that both rebaudioside A and stevioside were hydrolyzed to steviol in the gastrointestinal tract prior to absorption. The majority of circulatory steviol was in the form of steviol glucuronide, indicating rapid first-pass conjugation prior to urinary excretion. Only a small amount of steviol was detected in urine (rebaudioside A: 0.04%; stevioside: 0.02%). The investigators concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans, with steviol glucuronide excreted primarily in the urine and steviol in the feces. No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety, or vital signs (Wheeler et al., 2008).

Another pharmacokinetic investigation was done as a toxicokinetic (TK) phase of a dietary study to determine the potential of rebaudioside A toxicity in rats at levels up to 2,000 mg per kg bw per day (Sloter, 2008a). Extremely low levels of rebaudioside A and total steviol were detected in peripheral blood of rats during daily administration of 2,000 mg per kg bw per day of rebaudioside A, with mean plasma concentrations of approximately 0.6 and 12  $\mu$ g per mL, respectively. Estimates of absorbed dose for rebaudioside A and total steviol were approximately 0.02% and



0.06%, respectively, based on the amounts measured in urine collected over 24 hours in comparison to daily administered dietary dose to rats. Mean fecal rebaudioside A and measured hydrolysis products, expressed as Total Rebaudioside A Equivalents, compared to daily administered dose results in an estimated dose recovery of approximately 84%.

## 2. Subchronic Toxicity Studies

Curry and Roberts (2008) reported the results of two repeat dose studies of rebaudioside A in Wistar rats. The results of these investigations suggest that administration of rebaudioside A to Han Wistar rats at dietary concentrations of up to 100,000 ppm (9,938 and 11,728 mg per kg bw per day for males and females, respectively) for 4 weeks, or 50,000 ppm (4,161 and 4,645 mg per kg bw per day for males and females, respectively) for 13 weeks, did not present any evidence of systemic toxicity. In the 4-week study, rebaudioside A (97% purity) was administered at dietary concentrations of 0, 25,000, 50,000, 75,000, and 100,000 ppm to male and female rats. The NOAEL, including an evaluation of testes histopathology, was determined to be 100,000 ppm. In the 13-week study, Wistar rats were fed diets containing rebaudioside A at dietary concentrations of 0, 12,500, 25,000, and 50,000 ppm. In high-dose male and females groups, reductions in body weight gain attributable to initial taste aversion and lower caloric density of the feed were observed. Inconsistent reductions in serum bile acids and cholesterol were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A *via* the liver. All other hepatic function test results and liver histopathology were within normal limits. No significant changes in other clinical pathology results, organ weights, and functional observational battery test results were noted. Macroscopic and microscopic examinations of all organs were unremarkable with respect to treatment-related findings. The NOAEL in the 13-week toxicity study was considered to be 50,000 ppm, or approximately 4,161 and 4,645 mg per kg bw per day in male and female rats, respectively (Curry and Roberts, 2008).

In another 90-day dietary admix toxicity study, effects of rebaudioside A (99.5% purity) at target exposure levels of 500, 1,000, and 2,000 mg per kg bw per day were tested in Crl:CD(SD) rats (Nikiforov and Eapen, 2008; Eapen, 2007). Each group consisted of 20 animals per sex. No treatment related effects on clinical observations, food consumption, and functional observational or locomotor activity parameters were noted. There were no treatment-related macroscopic, organ weight or microscopic findings. Significantly lower body weight gains were noted in the 2,000 mg per kg bw per day group in males but not females. At the end of the dosing period, the body weight in males was 9.1% lower than the control group. Due to the small magnitude of difference from the control group value, the investigators did not consider this result to be adverse. The decrease was most likely due to the large proportion of the diet represented by the test material. The NOAEL was determined as  $\geq 2,000$  mg per kg bw per day.

A 6-month dietary toxicity study in Beagle dogs (4 per sex per group) was conducted to investigate the potential adverse effects of rebaudioside A (97.5% purity) at dosage levels of 0, 500, 1,000, or 2,000 mg per kg bw per day (Eapen, 2008). There were no unscheduled deaths during the course of the study. No treatment-related clinical observations were noted. Administration of rebaudioside

A did not affect home cage, open field observations and functional observations and measurements. No differences in hematology findings, serum chemistry findings, or urinalysis findings between the groups were noted. Additionally, no treatment related gross necropsy observations, alterations in final body weight, alterations in organ weights, or histological changes were noted. The investigators concluded that no systemic toxicity of rebaudioside A was observed at dosage levels up to 2,000 mg per kg bw per day and the assigned NOAEL was  $\geq$  2,000 mg per kg bw per day.

In addition, a 90-day subchronic toxicity study was conducted in Sprague-Dawley rats using fermentation-derived Rebaudioside A, where no systemic or local toxicity was observed in rats dosed at 500 to 2,000 mg per kg bw per day. All test animals survived to scheduled necropsy (Rumelhard et al., 2016).

### 3. Mutagenicity Studies

In a set of *in vitro* and *in vivo* genotoxicity assays covering mutation, chromosome damage, and deoxyribonucleic acid (DNA) strand breakage, rebaudioside A consistently and uniformly revealed negative results (Pezzuto et al., 1985; Nakajima, 2000a; Nakajima, 2000b; Sekihashi et al., 2002). These studies were critically reviewed by Brusick (2008). JECFA also reviewed an unpublished chromosome aberration assay of rebaudioside A in cultured mammalian cells (Nakajima, 2000a) and did not find increases in chromosome aberrations.

Additionally, FDA also reviewed three unpublished studies on rebaudioside A, including a bacterial mutagenicity study (Wagner and Van Dyke, 2006), a mouse lymphoma study (Clarke, 2006), and a mouse micronucleus study (Krsmanovic and Huston, 2006), submitted by Merisant as part of the GRAS Notification. All three studies demonstrated lack of mutagenic or genotoxic activity. Furthermore, Williams and Burdock (2009) also reported lack of genotoxicity in another set of published studies that included *in vitro* mutagenicity assays with *Salmonella*, *E. coli*, and mouse lymphoma cells. These investigators also reported lack of *in vitro* clastogenic effects in Chinese hamster V79 cells, and the absence of *in vivo* effects in a mouse micronucleus assay and a rat study for unscheduled DNA synthesis.

The recent evaluation of fermentation-derived rebaudioside A demonstrated a similar safety profile to plant-derived rebaudioside A. Rumelhard et al. (2016) reported that fermentation-derived rebaudioside A was not mutagenic in the bacterial reverse mutation assay, nor was it found to be clastogenic or aneugenic in the *in vitro* micronucleus assay. The similarity of the safety profile observed between plant-derived and fermentation-derived rebaudioside A further supports the applicability of the safety assessments to other steviol glycoside preparations.

The key mutagenicity testing results for rebaudioside A are summarized in Table 13-1.

**Table 13-1. Mutagenicity & Genotoxicity Studies on Rebaudioside A**

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Bacterial Mutagenicity	5 <i>Salmonella</i> strains with & without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1,500 & 5,000 $\mu\text{g}$ per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	4 <i>Salmonella</i> strains & 1 <i>E. coli</i> strain with & without exogenous metabolic activation system	Reb A	95.6	Up to 5,000 $\mu\text{g}$ per plate	No mutagenic response	Williams and Burdock (2009)
Bacterial Mutagenicity	4 <i>Salmonella</i> strains & 1 <i>E. coli</i> strain with and without exogenous metabolic activation system	Fermentation-derived Reb A	$\geq 95\%$	Up to 5,000 $\mu\text{g}$ per plate	No mutagenic response	Rumelhard et al. (2016)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	99.5	Cloning conc. of 500, 1,000, 2,000, 3,000, 4,000 & 5,000 $\mu\text{g}/\text{mL}$	No mutagenic or clastogenic response	Clarke (2006)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5,000 $\mu\text{g}/\text{mL}$	No mutagenic or clastogenic response	Williams and Burdock (2009)
Human Lymphocytes	Human lymphocytes in absence & presence of exogenous activation system	Fermentation-derived Reb A	$\geq 95\%$	Up to 5,000 $\mu\text{g}/\text{mL}$	Not clastogenic or aneugenic	Rumelhard et al. (2016)
Chromosome Aberration	Human lymphocytes in absence & presence of exogenous metabolic activation system	Reb A	95.6	Up to 5,000 $\mu\text{g}/\text{mL}$	No mutagenic or clastogenic response	Williams and Burdock (2009)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female ICR mice	Reb A	99.5	500, 1,000 & 2,000 mg/kg bw	No increase in micronuclei formation	Krsmanovic and Huston (2006)
Mouse Micronucleus	Micronucleus study in groups of 5 male & 5 female NMRI mice	Reb A	95.6	Up to 750 mg/kg bw	No increase in micronuclei formation	Williams and Burdock (2009)
Unscheduled DNA Synthesis	Unscheduled DNA synthesis in one group of 4 Wistar rats	Reb A	95.6	Up to 2,000 mg/kg bw	No increase in unscheduled DNA synthesis	Williams and Burdock (2009)
DNA damage	Male BDF1 mouse	Stevia extract	Stevio-	250 – 2,000	Negative <sup>a</sup>	Sekihashi et al.

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
(comet assay)	stomach, colon, liver		side, 52%; Reb A, 22%	mg/kg bw		(2002)
Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Reb A	NS	1.2 - 55 mg/mL	Negative <sup>b</sup>	Nakajima (2000a)
Micronucleus formation	BDF1 mouse bone marrow	Reb A	NS	500-2,000 mg/kg bw/ day for 2 days	Negative <sup>c</sup>	Nakajima (2000b)
Forward mutation	<i>S. typhimurium</i> TM677	Reb A	NS	10 mg/plate	Negative <sup>b</sup>	Pezzuto et al. (1985)

NS = Not specified.

<sup>a</sup> Sacrificed at 3 hours and 24 hours.

<sup>b</sup> With or without metabolic activation (source not specified in original monograph).

<sup>c</sup> Sacrificed at 30 hours after 2nd administration.

#### 4. Reproductive & Developmental Toxicity Studies

In a two-generation reproductive toxicity study, rebaudioside A (97% purity) at 0, 7,500, 12,500, and 25,000 ppm was administered in diet to male and female Han Wistar rats (Curry et al., 2008). Administration of rebaudioside A was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. Similarly, administration of rebaudioside A did not affect reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology in either the F<sub>0</sub> or F<sub>1</sub> generations. The survival and general condition of the F<sub>1</sub> and F<sub>2</sub> offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected by rebaudioside A treatment. The NOAEL for reproductive effects was 25,000 ppm, and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm, or 2,048 to 2273 mg per kg bw per day (the highest dose tested).

The results from two unpublished studies with rebaudioside A (Sloter, 2008a; Sloter, 2008b) further support the above described findings from published studies. In a two-generation dietary reproduction study, four groups of male and female CrI:CD(SD) rats (30 per sex per group) were fed either basal diet or the diet containing rebaudioside A (purity 95.7%) for at least 70 consecutive days prior to mating (Sloter, 2008a). For the F<sub>0</sub> and F<sub>1</sub> generations, rebaudioside A doses were 0, 500, 1,000, and 2,000 mg per kg per day. At initiation of study, F<sub>0</sub> animals were approximately 7 weeks of age. The test diet was offered to the offspring selected to become the F<sub>1</sub> generation following weaning [beginning on postnatal day (PND) 21]. The F<sub>0</sub> and F<sub>1</sub> males continued to receive rebaudioside A throughout mating, continuing through the day of euthanasia. The F<sub>0</sub> and F<sub>1</sub> females continued to receive rebaudioside A throughout mating, gestation and lactation until day of

euthanasia. The authors concluded that there were no effects on reproduction in males or females as evaluated by estrus cycles, mating, fertility, conception or copulation indices, number of days between pairing and coitus, gestation length, and spermatogenic endpoints. Both for parental systemic and reproductive toxicity, a dose level  $\geq 2,000$  mg per kg bw per day (highest dose administered) was assigned to be the NOAEL.

In an embryo/fetal developmental toxicity study in rats (Sloter, 2008b), effects of rebaudioside A administered *via* gavage were investigated. Rebaudioside A administration did not affect intrauterine growth and survival, and there were no test article-related fetal malformations or developmental variations at any dosage level. In the absence of maternal or developmental toxicity, a dose level  $\geq 2,000$  mg per kg bw per day (highest dose administered) was considered to be the NOAEL for maternal and embryo/fetal developmental toxicity.

## 5. Clinical Studies on Rebaudioside A

In a four week randomized, double-blind, placebo controlled trial, hemodynamic effects of rebaudioside A, at a dose of 1,000 mg per day rebaudioside A (97% purity) or placebo in 100 individuals with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP), were investigated (Maki et al., 2008a). Subjects were predominantly female (76% rebaudioside A and 82% placebo) with a mean age of  $\sim 41$  (range 18 to 73) years. At baseline, mean resting, seated SBP/DBP was 110.0/70.3 mm Hg and 110.7/71.2 mm Hg for the rebaudioside A and placebo groups, respectively. Compared with placebo, administration of rebaudioside A did not significantly alter resting, seated SBP, DBP, mean arterial pressure (MAP), heart rate (HR) or 24-hour ambulatory blood pressure responses. The investigators concluded that consumption of 1,000 mg per day of rebaudioside A produced no clinically important changes in blood pressure in healthy adults with normal and low-normal blood pressure.

In another trial, effects of 16 weeks of consumption of 1,000 mg per person per day rebaudioside A (97% purity,  $n = 60$ ) were compared to placebo ( $n = 62$ ) in men and women (33-75 years of age) with type 2 diabetes mellitus (Maki et al., 2008b). Changes in glycosylated hemoglobin levels did not differ significantly between the rebaudioside A ( $0.11 \pm 0.06\%$ , mean  $\pm$  standard error) and placebo ( $0.09 \pm 0.05\%$ ;  $p = 0.355$ ) groups. Similarly, no significant ( $p > 0.05$  for all) changes from baseline for rebaudioside A and placebo, respectively, in fasting glucose ( $7.5 \pm 3.7$  mg per dL and  $11.2 \pm 4.5$  mg per dL), insulin ( $1.0 \pm 0.64$   $\mu$ U per mL and  $3.3 \pm 1.5$   $\mu$ U per mL), and C-peptide ( $0.13 \pm 0.09$  ng per mL and  $0.42 \pm 0.14$  ng per mL) were noted. No treatment related changes in blood pressure, body weight, and fasting lipids were noted. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo. Based on these results, the investigators suggested that chronic use of 1,000 mg per person per day rebaudioside A does not alter glucose homeostasis or blood pressure in individuals with type 2 diabetes mellitus.

## 6. Safety of Rebaudioside A

There have been a significant number of studies regarding the safety and toxicity of rebaudioside A, including many that have been published since the two initial GRAS notifications were submitted to FDA by Cargill (GRN 253) and Merisant (GRN 252). These, and some other unpublished studies, formed the basis of the two initial GRAS notifications to FDA by Cargill (GRN 253) and Merisant (GRN 252). Prior to this, a limited number of toxicology studies specifically on rebaudioside A were conducted. Even before these new studies were completed, and as noted in the previous section, JECFA concluded that 7 (which was later expanded to 9) common steviol glycosides are deemed to be safe for use as sweetener preparations when present in any combination, as long as a combined purity of 95% or more was established.

Since a majority of the previous pharmacokinetic research was conducted with steviol glycosides, the presumed strategy adopted for the more recent research on rebaudioside A was to conduct a limited number of well-designed and executed toxicology studies on rebaudioside A itself, and to demonstrate that rebaudioside A is handled pharmacokinetically similarly to stevioside in rats and humans. This approach appears to have been undertaken to justify the JECFA-generated ADI without having to conduct a chronic study in rats with rebaudioside A. Additionally, the Merisant group conducted three mutagenicity assays on rebaudioside A that FDA generally considers to be most predictive for carcinogenicity potential. The Cargill group conducted two clinical studies to assure that rebaudioside A does not have potentially problematic pharmacological effects on blood glucose and blood pressure.

In a review article, Carakostas et al. (2008) summarized the most recent Cargill research program findings on rebaudioside A, as follows:

- Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro*.
- In well-conducted *in vivo* assays, steviol glycosides, rebaudioside A, and stevioside have not been found to be genotoxic.
- A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed (Nunes et al., 2007a) and was improperly interpreted as a positive response.
- Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
- The primary evidence for steviol genotoxicity is derived from very specific bacterial tests or purified plasmid DNA that lack DNA repair capabilities.
- Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- While studies with Reb A indicated slight gastrointestinal (GI) absorption of the glycoside *per se*, the predominant metabolic pathway is comparable to that of stevioside and the use of the ADI established by JECFA, which was determined on studies employing stevioside as the main component, can be used as the ADI for rebaudioside A.

- The dietary levels expected from consumption of rebaudioside A as a total replacement of sugar (Renwick, 2008) are less than the ADI and, therefore, there is no safety concern for consumers.

The consumption estimates described by JECFA, Renwick (2008), and the GRN 252 and GRN 253 Expert Panels very conservatively represent a potential high user of Rebaudioside A if this non-nutritive sweetener becomes widely available in food.

Regarding the available aggregate safety information, multiple qualified entities have concluded that JECFA has critically and extensively evaluated the use of steviol glycosides in foods and agrees that, at the present time, the ADI for steviol glycosides of adequate purity, as defined by JECFA specifications, has been properly determined to be 4 mg per kg bw per person as steviol equivalents, which corresponds to 12 mg per kg bw per day for rebaudioside A, on a dry weight basis. Unwanted pharmacological effects are not likely to occur at this level and, moreover, high consumers of rebaudioside A are not likely to exceed this level. Therefore, the JECFA-derived ADI was adopted as a safe exposure for rebaudioside A and the corresponding food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be generally recognized as safe (GRAS).

JECFA---which is composed of dozens of scientists that are internationally known experts on food ingredient safety---has established ADIs for food ingredients over the last 40 years. Both Merisant and Cargill took rather rigorous scientific approaches to demonstrate the safety of rebaudioside A. The studies were equally well conducted. The safety profiles compiled by Merisant and Cargill differ somewhat, yet the results are complementary and are mutually reinforcing of rebaudioside A safety.

The studies conducted by Cargill provided significant insight into the pharmacokinetics of rebaudioside A, while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food. The Merisant notification augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems. Additional mutagenicity and genotoxicity studies have been published on rebaudioside A (Williams and Burdock, 2009). Merisant added a subchronic study in dogs and a teratology study in rats. Both Cargill and Merisant relied on the JECFA ADI for steviol glycosides as determined largely by published chronic studies in rat. Both groups justified the use of the ADI on pharmacokinetic arguments showing the similarity of stevioside and rebaudioside A metabolism and excretion.

## Appendix 14 Studies on Principal Metabolite: Steviol

### Studies on Principal Metabolite: Steviol

In a number of studies, steviol, the principal mammalian metabolite of stevioside, has been investigated for its safety. The results of these studies are summarized below.

#### 1. Acute Toxicity Studies

The oral LD<sub>50</sub> of steviol (purity, 90%) in male and female mice and rats was reported to be > 15 grams per kg bw. In this study, only one of 15 animals died within 14 days of administration. The LD<sub>50</sub> values in hamsters given steviol orally were 5.2 grams per kg bw in males and 6.1 grams per kg bw in females. Histopathological examination of the kidneys revealed severe degeneration of the proximal tubular cells, and these structural alterations were correlated with increased serum blood urea nitrogen and creatinine. The authors concluded that the cause of death was acute renal failure (Toskulkac et al., 1997).

#### 2. Developmental Toxicity Studies

Groups of 20 pregnant golden hamsters were given steviol (purity, 90%) at doses of 0, 250, 500, 750, or 1,000 mg per kg bw per day (only 12 animals at the highest dose) by gavage in corn oil on days 6 - 10 of gestation. A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses, and the number of live fetuses per litter and mean fetal weight decreased in parallel. Histopathological examination of the maternal kidneys showed a dose-dependent increase in the severity of effects on the convoluted tubules (dilatation, hyaline droplets). However, no dose-dependent teratogenic effects were seen. The NOEL was 250 mg per kg bw per day for both maternal and developmental toxicity (Wasuntarawat et al., 1998).

#### 3. Mutagenicity & Genotoxicity Studies

In a number of studies mutagenicity and genotoxicity of steviol has been investigated. These studies reviewed by JECFA are summarized in Table 14-1.



**Table 14-1. Mutagenicity & Genotoxicity Studies on Steviol**

REFERENCE	IN VIVO / IN VITRO	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Sekihashi et al. (2002) <sup>a</sup>	<i>In Vivo/In Vitro</i>	Comet Assay	Not reported	Negative	In <i>in vitro</i> study, steviol at 62.5, 125, 250 and 500 µg/ml did not damage DNA of TK6 and WTK1 cells in presence or absence of S9 mix. In <i>in vivo</i> study, mice sacrificed 3 or 24 hours after one-time oral administration of 250, 500, 1,000 or 2,000 mg/kg of steviol. Stomach, colon, kidneys, testis and liver DNA not damaged. An identical <i>in vivo</i> experiment with stevia extract performed, which also gave negative results.
Oh et al. (1999) <sup>b</sup>	<i>In Vivo?</i>	Cell Mutation and DNA damage	Not reported	Negative	Steviol gave negative results for cell mutation and DNA damage in cultured cells.
Matsui et al. (1996) <sup>c</sup>	<i>In Vivo?</i>	Mutagenicity and Chromosome aberration (Chinese hamster lung fibroblasts)	Not reported	Positive	Gene mutation and chromosomal aberration found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.
Terai et al. (2002) <sup>a</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Steviol found to be mutagenic in Aroclor-induced rat liver S9 fraction. 15-oxo-steviol found to be mutagenic at 10% level of steviol. Specific mutagenicity of lactone derivative in presence of S9 mixture 10x lower than that of derivative without S9 mixture.
Temcharoen et al. (1998) <sup>c</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Mutagenic effects of steviol and/or metabolites found in <i>S. typhimurium</i> TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. Magnitude of increase of these mutations over the control not reported.
Klongpanich pak et al. (1997) <sup>c</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	Steviol and stevioside inactive in TA strains of <i>S. typhimurium</i> , <i>E. coli</i> WP2, <i>uvrA/PKM101</i> and rec assay using <i>B. subtilis</i> even when microsomal activated fraction present. Magnitude of increase of these mutations over the control not reported.
Matsui et al. (1996) <sup>a</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	Testing of Southern Blot technique with probe for gpt gene DNA of <i>E. coli</i> . The chromosomal DNA of TM677 and steviol-induced TM677 mutants digested by restriction enzymes and probed. No significant differences found in fragment length between wild-type and mutant DNA.
Matsui et al. (1996) <sup>a</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Both	Steviol weakly positive in umu test, either with or without metabolic activation. Steviol negative in reverse mutation and other bacterial assays even in presence of S9 activation.

REFERENCE	IN VIVO / IN VITRO	SYSTEM	TEST SAMPLE PURITY	AUTHOR CONCLUSION	RESULTS AND REMARKS
Procinska et al. (1991) <sup>c</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Negative	The direct mutagenic activity of 15-oxo-steviol was refuted.
Compadre et al. (1988) <sup>a</sup>	<i>In Vitro</i>	Bacterial Mutagenicity, Mass Spec	Not Reported	Positive	Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be direct-acting mutagen. Magnitude of increase over control in assay not discussed.
Pezzuto et al. (1985) <sup>d</sup>	<i>In Vitro</i>	Bacterial Mutagenicity	Not Reported	Positive	Using <i>S. typhimurium</i> TM677 strain, steviol found to be highly mutagenic in presence of 9000 x g supernatant from livers of Aroclor 1254-pretreated rats. This mutagenicity dependent on pretreatment of rats with Aroclor and NADPH addition, as unmetabolized steviol was inactive. None of other metabolites tested was mutagenic. Authors concluded that structural features of requisite importance for the expression of mutagenic activity may include a hydroxy group at position 13 and an unsaturated bond joining the carbon atoms at positions 16 and 17.
Temcharoen et al. (2000) <sup>c</sup>	<i>In Vivo</i>	Micronucleus (rat)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Temcharoen et al. (2000) <sup>c</sup>	<i>In Vivo</i>	Micronucleus (mouse)	90%	Negative	Very high doses (8 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.
Matsui et al. (1996) <sup>a</sup>	<i>In Vivo</i>	Micronucleus (mouse)	Not Reported	Negative	Steviol did not increase number of micronuclei observed in this study.
Temcharoen et al. (2000) <sup>c</sup>	<i>In Vivo</i>	Micronucleus (hamster)	90%	Negative	Very high doses (4 g/kg bw) given to rats did not induce micronucleus in bone marrow erythrocytes in male and female animals.

<sup>a</sup> Abstract only. <sup>b</sup> As reported in WHO (2006). <sup>c</sup> As reviewed by Geuns (2003). <sup>d</sup> Full article.

#### 4. Endocrine Disruption Studies

Shannon et al. (2016) investigated the endocrine disrupting potential of stevioside, rebaudioside A, and steviol in a series of *in vitro* bioassays. Steviol was reported to 1) antagonize progesterone nuclear receptor transcriptional activity; 2) increase progesterone production; and 3) induce an agonistic response on the progesterone receptor of sperm cells (Catsper). While the authors concluded that *Stevia* might not qualify as a safer alternative to sugar or synthetic sweeteners, it is important to note that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at the receptor level. Furthermore, no adverse effects were observed in the reproductive studies.

## Appendix 15 GRAS Associates Expert Panel Report

### **The Generally Recognized as Safe (GRAS) Status of the Proposed Uses of BESTEVIA® Rebaudioside E**

November 7, 2018

#### **Foreword**

An independent panel of experts (“Expert Panel”) was convened by GRAS Associates, LLC on behalf of their client, Blue California, to evaluate the safety and Generally Recognized as Safe (GRAS) status Blue California’s proposed uses of BESTEVIA® Rebaudioside E in conventional foods. The members of this Expert Panel<sup>†</sup> are qualified to serve in this capacity by qualification of scientific training and experience in the safety of food and food ingredients.

#### **Discussion**

A significant amount of safety information related to the consumption of steviol glycosides is generally available, and has been discussed in Part 6, as well as Appendices 9-14, of Blue California’s dossier. First, there is a history of safe consumption of steviol glycosides when used as an ingredient in food products in the U.S., Canada, South America, Europe, Asia, and Australia and New Zealand. Second, a number of experimental studies have investigated the safety of steviol glycosides. The composite evidence from historical safe consumption and experimental studies together demonstrate the safety of BESTEVIA® Rebaudioside E preparations for human food consumption.

The majority of the studies reviewed on steviol glycosides and steviol have been discussed in detail in previous GRAS notifications (GRNs), including GRN 278, GRN 667, and GRN 715, which were submitted by Blue California.

With regard to the safety documentation, the key pharmacokinetic data establish that steviol glycosides are not absorbed through the gastrointestinal (GI) tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but is rapidly glucosylated and excreted in the urine and feces. It has been well-established experimentally from various published studies that the steviol glycoside molecules are not absorbed from the GI tract (Gardana et al., 2003; Koyama et al., 2003b). The action of bacteria in the large intestine is directly

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<sup>†</sup> Dr. Emmel, Chair of the Expert Panel, is a chemist with substantial food safety experience in addressing steviol glycosides and other food ingredients. Dr. Kapp is a toxicologist with over 35 years of experience. He is a Fellow of the Academy of Toxicological Sciences, a Fellow of the Royal Society of Biology, and a European Registered Toxicologist. Dr. Lewis is a biologist with more than 10 years of experience preparing GRAS dossiers. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in deliberations of GRAS Expert Panels.

supported by the published study that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003a; Renwick and Tarka, 2008). Geuns et al. (2006) measured blood, urine, and fecal metabolites in human subjects who received purified stevioside over 3 days and found steviol glucuronide in blood, urine, and feces samples. The authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide. Furthermore, Purkayastha et al. (2015; 2016) reported that rebaudioside E and rebaudioside A are metabolized to steviol in a similar, concentration-dependent manner in an *in vitro* fecal homogenate study.

The acceptable daily intake (ADI) for steviol glycosides has been set largely based on a published chronic study in rats (Toyoda et al., 1997) and several published clinical studies show that there are no pharmacological effects in humans at doses several fold higher than the ADI (Barriocanal et al., 2006; Barriocanal et al., 2008; Wheeler et al., 2008). Recently, Roberts et al. (2016) noted in a persuasive argument using a chemical-specific adjustment factor (CSAF) that the ADI could be higher. The toxicity of the metabolite steviol has been well-reviewed in the published literature (Geuns, 2003; WHO, 2006; Urban et al., 2013). In addition, FDA has issued “no questions” letters to 54 GRN submissions for steviol glycosides preparations as of October 15, 2018.

The Expert Panel notes that Blue California’s manufacturing process for BESTEVIA® Rebaudioside E is similar to the processes described for other GRAS steviol glycosides materials synthesized from *Stevia rebaudiana* extract by genetically-modified yeast, as described in GRN 667 and GRN 715. The Expert Panel notes that while some of the representative lots appear to be sequential in numbering, Blue California states that the lots are non-consecutive in their report.

The GRAS Associates Expert Panel convened on behalf of Blue California has reviewed the proposed uses for BESTEVIA® Rebaudioside E. The highest 90<sup>th</sup> percentile consumption by any population subgroup of BESTEVIA® Rebaudioside E was calculated to be approximately 7.23 mg per kg body weight (bw) per day, which is equivalent to 2.38 mg per kg bw per day steviol equivalents (calculated by a weighted sum estimate) for any population group, on a worst-case scenario basis. This estimated intake value is well below the Joint FAO/WHO Expert Committee on Food Additives (JECFA) ADI of 4 mg per kg bw per day expressed as steviol equivalents. Therefore, BESTEVIA® Rebaudioside E is expected to be safe within established allowable limits.

A compelling case can be made that scientific consensus exists regarding the safety of steviol glycosides when of sufficiently high purity. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which the various steviol glycosides molecules are metabolized and eliminated from the body. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as evidenced by several publications (Carakostas, 2012; Geuns, 2007a; Urban et al., 2013; Waddell, 2011; Williams, 2007; Brusick, 2008) that refute safety concerns expressed by a minority of scientists. In addition, Roberts et al. (2016) suggest that the ADI for steviol glycosides

could be as high as 6-16 mg per kg bw per day, which is higher than has been previously accepted by the scientific community, providing the potential for an even more robust safety profile.

In summary, sufficient qualitative and quantitative scientific evidence in the composite is available to support the safety-in-use of Blue California’s BESTEVIA® Rebaudioside E preparation (≥95% total steviol glycosides comprised mostly of rebaudioside E) given the following conditions:

- Blue California’s BESTEVIA® Rebaudioside E preparation continues to meet the designated specifications;
- the minimum sweetness intensity for BESTEVIA® Rebaudioside E remains unchanged; and
- BESTEVIA® Rebaudioside E is produced in accordance with Current Good Manufacturing Practices (CGMPs).

### Conclusion

The Expert Panel critically reviewed the data provided by Blue California for their BESTEVIA® Rebaudioside E preparation, as well as publicly available published information obtained from peer reviewed journals and other safety assessments prepared by other Expert Panels and well-respected international regulatory bodies.

The ingestion of Blue California’s BESTEVIA® Rebaudioside E from the intended uses results in intakes that are safe within the limits of established historical use and published safety studies and the widely accepted ADI of 4 mg per kg bw per day steviol equivalents.

The Expert Panel unanimously concluded that the proposed uses of Blue California’s BESTEVIA® Rebaudioside E preparation, manufactured as described in Part 2.B. of their dossier, and declared within the subject notification meet the FDA definition of safety in that there is “reasonable certainty of no harm under the intended conditions of use” as described herein, and Blue California’s BESTEVIA® Rebaudioside E preparation is generally recognized as safe (GRAS).

(b) (6)



Robert W. Kapp, Jr., Ph.D.  
Fellow ATS, FRSB, & ERT(UK)

(b) (6)



Kara Lewis, Ph.D.

(b) (6)



Katrina Emmel, Ph.D.  
Panel Chair

**END**

## Bonnette, Richard

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**From:** Katrina Emmel <emmel@gras-associates.com>  
**Sent:** Tuesday, December 04, 2018 2:33 PM  
**To:** Bonnette, Richard  
**Cc:** William J. Rowe; Amy Mozingo GRAS Associates  
**Subject:** Submission to the FDA GRAS notification for Dihydroquercetin and Reb E on behalf of Blue California

Hello Mr. Bonnette,

We can confirm that no confidential information is included in either the Dihydroquercetin or Rebaudioside E notifications, and the pages are releasable under FOIA.

Thank you,

Katrina

Katrina Emmel, Ph.D.  
Senior Scientist/Project Manager/Associate  
GRAS Associates, LLC.

[emmel@gras-associates.com](mailto:emmel@gras-associates.com)

**From:** Bonnette, Richard <[Richard.Bonnette@fda.hhs.gov](mailto:Richard.Bonnette@fda.hhs.gov)>  
**Sent:** December-04-18 1:23 PM  
**To:** William J. Rowe <[wrowe@nutrasource.ca](mailto:wrowe@nutrasource.ca)>  
**Subject:** submission to the FDA GRAS notification program for Reb E

Mr. Rowe,

Similar to my email from earlier, we noted some pages labelled as confidential in the rebaudioside E submission dated November 15, 2018 (on behalf of Blue California). The pages confidential notes are 48-49, and 62-66. Can you confirm that these pages are releasable under FOIA?

Thanks,  
Richard

**From:** Bonnette, Richard  
**Sent:** Tuesday, December 04, 2018 9:50 AM  
**To:** [wrowe@nutrasource.ca](mailto:wrowe@nutrasource.ca)  
**Subject:** submission to the FDA GRAS notification program for dihydroquercetin

Dear Mr. Rowe,

We've completed a pre-filing evaluation of the submission dated November 5, 2018 regarding GRAS uses of dihydroquercetin in food and had a quick detail to clarify before we move forward with filing. We see that in Part 1 and on Form 3667 you have noted that the submission does not contain trade secret or confidential information. We note that on pages 79-83 of the submission and pages 100-103 of the appendix contain stamps or language suggesting these documents are confidential. Just wanted to confirm with you that these pages are indeed releasable. If this is the case,

I'll append your response to this email to the submission and we'll move forward with filing. Let me know if this isn't the case and we can talk about options.

Thanks,  
Richard

**Richard E. Bonnette, M.S.**  
Center for Food Safety and Applied Nutrition  
Office of Food Additive Safety  
U.S. Food and Drug Administration  
Tel: 240-402-1235  
[richard.bonnette@fda.hhs.gov](mailto:richard.bonnette@fda.hhs.gov)



**From:** [Katrina Emmel](#)  
**To:** [Zhang, Janet](#)  
**Cc:** [William J. Rowe](#); [Amy Mozingo](#)  
**Subject:** Question for GRN 000823 Rebaudioside E  
**Date:** Wednesday, April 3, 2019 6:10:22 PM  
**Attachments:** [FDA Response Ltr BC Reb E GRN 823 4-3-19.pdf](#)

---

Hello Dr. Zhang,

Attached you will find a response letter addressing the questions provided in your email on March 25, 2019 regarding GRN 823. Please let me know if you have any further questions.

Thank you,

Katrina

Katrina Emmel, Ph.D.  
Senior Scientist/Project Manager/Associate  
GRAS Associates, LLC.

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April 3, 2019

Food and Drug Administration  
Center for Food Safety & Applied Nutrition  
Office of Food Additive Safety (HFS-255)  
5001 Campus Drive  
College Park, MD 20740-3835

Attention: Dr. Janet Zhang  
Re: GRN 823 – Rebaudioside E – Response to Questions Posed in an Email Dated 3/25/19

Dear Dr. Zhang:

GRAS Associates, LLC, acting as the agent for Blue California, is providing a response regarding the following question posed by FDA in your email dated March 25, 2019, in which Blue California was asked about the following:

*Based on information on page 10 of your notice, Blue California uses a starting material that is  $\geq 95\%$  steviol glycosides and derived from *Stevia rebaudiana* leaves through extraction with a 70% ethanol/30% water solution, as well as additional steps. However, on page 48 and 49 (i.e., Appendices 2.1 and 2.2), the manufacturing flow chart includes a hot water extraction step instead of aqueous ethanol and the specifications do not include a limit for residual ethanol. Please clarify.*

Blue California would like to clarify that two extraction techniques can be used to obtain equivalent preparations of  $\geq 95\%$  steviol glycosides starting material: a hot water extraction process; or an aqueous ethanol extraction process. The specifications for both raw material steviol glycosides extracts are provided in the table below.

The specifications and manufacturing flow charts for the  $\geq 95\%$  steviol glycosides starting material obtained by hot water extraction and aqueous ethanol extraction are provided in Appendix A and Appendix B, respectively.



PHYSICAL & CHEMICAL PARAMETERS	Blue California's Specifications for Steviol Glycosides Starting Material (Hot Water Extraction) Item# ST0301245	Blue California's Specifications for Steviol Glycosides Starting Material (Aqueous Ethanol Extraction) Item# ST0301238
Appearance Form	Powder	Powder
Appearance Color	White	White
Solubility in Water	Soluble	Soluble
Assay <sup>a</sup>	≥ 95% steviol glycosides	≥ 95% steviol glycosides
Residual Ethanol	< 5,000 mg/kg	< 5,000 mg/kg
Residual Methanol	NS	< 200 mg/kg
Loss on Drying	≤ 6%	≤ 6%
pH, 1% Solution	4.5-7.0	4.5-7.0
Total Ash	< 1%	< 1%
Arsenic	< 1 ppm	< 1 ppm
Lead	< 1 ppm	< 1 ppm
Cadmium	< 1 ppm	< 1 ppm
Mercury	< 1 ppm	< 1 ppm
Total Plate Count	< 1,000 cfu/g	< 1,000 cfu/g
Total Coliform	< 10 cfu/g	< 10 cfu/g
Yeast & Mold	< 100 cfu/g	< 100 cfu/g
<i>Salmonella</i>	Negative	Negative
<i>Escherichia coli</i>	Negative	Negative

NS = not specified; NMT = not more than; ppm = parts per million; cfu = colony forming units.

<sup>a</sup> Total steviol glycosides as the sum of all compounds containing a steviol backbone conjugated to any number, combination, or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose, and xylose) occurring in the leaves of *Steviana rebaudiana* Bertoni.

Furthermore, Blue California has adopted product specifications for its BESTEVIA® Rebaudioside E preparation manufactured with either of the starting raw materials described herein, that meets or exceeds JECFA recommendations, while also complying with Food Chemicals Codex specifications for rebaudioside A as a consumable human food substance, regardless of which raw material steviol



glycosides preparation is used in the manufacturing process, as described in more detail in Part 2.C.2 of GRN 823.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via email.

We look forward to your feedback.

(b) (6)

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Katrina Emmel, Ph.D.  
Senior Scientist/Project Manager/Associate

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Bonita Springs, FL 34134

**List of appendices:**

**Appendix A: Specifications and Manufacturing Flow Chart for Steviol Glycosides Raw Material Obtained by Hot Water Extraction**

**Appendix B: Specifications and Manufacturing Flow Chart for Steviol Glycosides Raw Material Obtained by Aqueous Ethanol Extraction**

**Appendix A: Specifications and Manufacturing Flow Chart for Steviol Glycosides Raw Material Obtained by Hot Water Extraction**



*Blue California*<sup>®</sup>

30111 Tomas  
Rancho Santa Margarita, CA 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**PRODUCT SPECIFICATION**

**Product: Steviol Glycosides 95% (*Stevia rebaudiana*, leaves)**  
**Item# ST0301245**

<b>Country of Origin:</b>	<b>China</b>	<b>Shelf life:</b>	<b>2 Years</b>
<b>Grade:</b>	<b>Food</b>	<b>Extraction Solvent:</b>	<b>Water</b>
<b>ATTRIBUTES</b>	<b>SPECIFICATION</b>	<b>METHODS</b>	
APPEARANCE	WHITE POWDER	VISUAL	
FOREIGN MATTER	ABSENT	VISUAL	
ODOR	CHARACTERISTIC	OLFACTORY	
TASTE	CHARACTERISTIC	GUSTATORY	
STEVIOL GLYCOSIDES	≥ 95%	HPLC	
SOLUBILITY IN WATER	SOLUBLE	USP	
LOSS ON DRYING	≤ 6%	USP	
HEAVY METALS	< 10 ppm	USP	
LEAD	< 1 ppm	ICP-MS	
ARSENIC	< 1 ppm	ICP-MS	
CADMIUM	< 1 ppm	ICP-MS	
MERCURY	< 1 ppm	ICP-MS	
Ethanol	< 5000 mg/kg	USP	
pH	4.5-7.0	USP	
ASH	< 1%	USP	
BULK DENSITY	> 0.2 g/ml	USP	
TAP DENSITY	≥ 0.3 g/ml	USP	
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP	
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	
TOTAL COLIFORM	< 10 cfu/gm	AOAC	
YEAST AND MOLDS	< 100 cfu/gm	AOAC	
E. COLI:	NEGATIVE	AOAC	
SALMONELLA	NEGATIVE	AOAC	

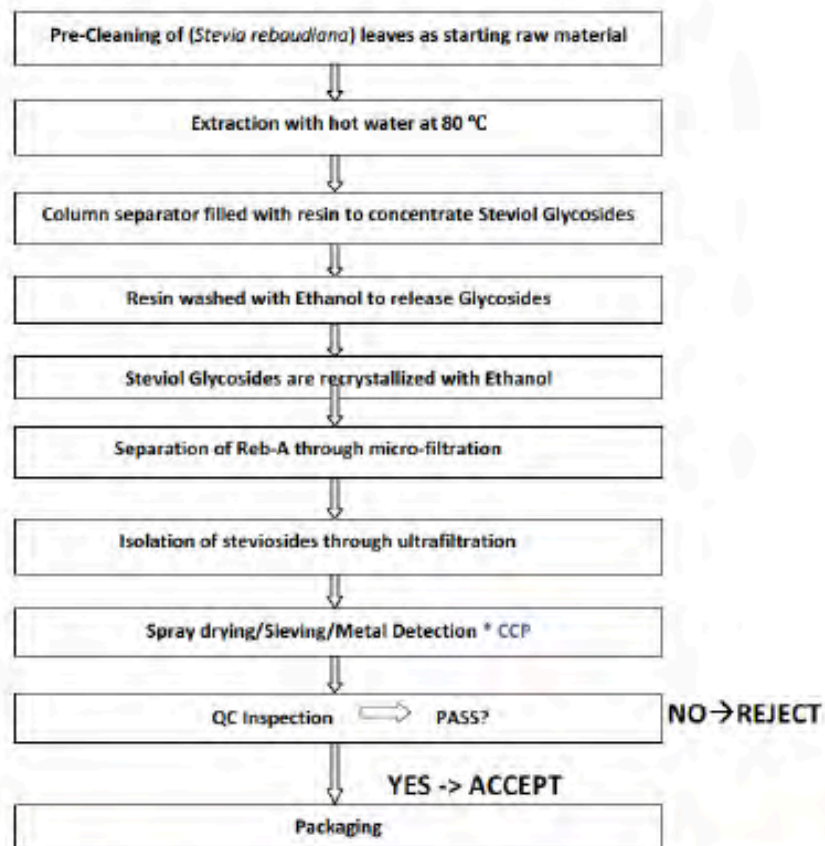
Approved by: X.Y.Mao (QC Manager) Revision date: 10-15-2018



## A Perfect Blend of Science and Nature

Product Name: Steviol Glycosides 95%

### PROCESS DIAGRAM



\* CCP: Metal detection. Physical Hazard: Metal impurities. Criterion: Metal equipment used in production line, such as mesh screens. In order to prevent chronic intoxication by metal impurities, product has to go through magnet and metal detector.

✦ THIS DOCUMENT IS INTENDED ONLY FOR THE USE OF PARTY TO WHOM IT IS ADDRESSED AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL, AND PROTECTED FROM DISCLOSURE UNDER APPLICABLE LAW.

Corporate Headquarters  
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Website: [www.bluecal-ingredients.com](http://www.bluecal-ingredients.com)

**Appendix B: Specifications and Manufacturing Flow Chart for Steviol Glycosides Raw Material Obtained by Aqueous Ethanol Extraction**



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30111 Tomas  
Rancho Santa Margarita, CA 92688  
Tel: 949.635.1990  
Fax: 949.635.1988

**PRODUCT SPECIFICATION**

**Product: Stevia Extract 95% (*Stevia rebaudiana*, leaves)**

**Item# ST0301238**

Country of Origin:	China	Shelf life:	2 Years
Grade:	Food		
ATTRIBUTES	SPECIFICATION	METHODS	
APPEARANCE	WHITE POWDER	VISUAL	
FOREIGN MATTER	ABSENT	VISUAL	
ODOR	CHARACTERISTIC	OLFACTORY	
TASTE	CHARACTERISTIC	GUSTATORY	
STEVIOLE GLYCOSIDES	> 95%	HPLC	
SOLUBILITY IN WATER	SOLUBLE	USP	
LOSS ON DRYING	≤ 6%	USP	
HEAVY METALS	< 10 ppm	USP	
LEAD	< 1 ppm	ICP-MS	
ARSENIC	< 1 ppm	ICP-MS	
CADMIUM	< 1 ppm	ICP-MS	
MERCURY	< 1 ppm	ICP-MS	
Methanol	< 200 mg/kg	GC	
Ethanol	< 5000 mg/kg	GC	
pH	4.5-7.0	USP	
ASH	< 1%	USP	
BULK DENSITY	> 0.2 g/ml	USP	
TAP DENSITY	≥ 0.3 g/ml	USP	
PARTICLE SIZE:	> 95% through Mesh #80 Sieve	USP	
TOTAL PLATE COUNT	< 1,000 cfu/gm	AOAC	
TOTAL COLIFORM	< 10 cfu/gm	AOAC	
YEAST AND MOLDS	< 100 cfu/gm	AOAC	
E. COLI:	NEGATIVE	AOAC	
SALMONELLA	NEGATIVE	AOAC	

Approved by: X.Y.Mao (QC Manager) Revision date: 11-20-2018

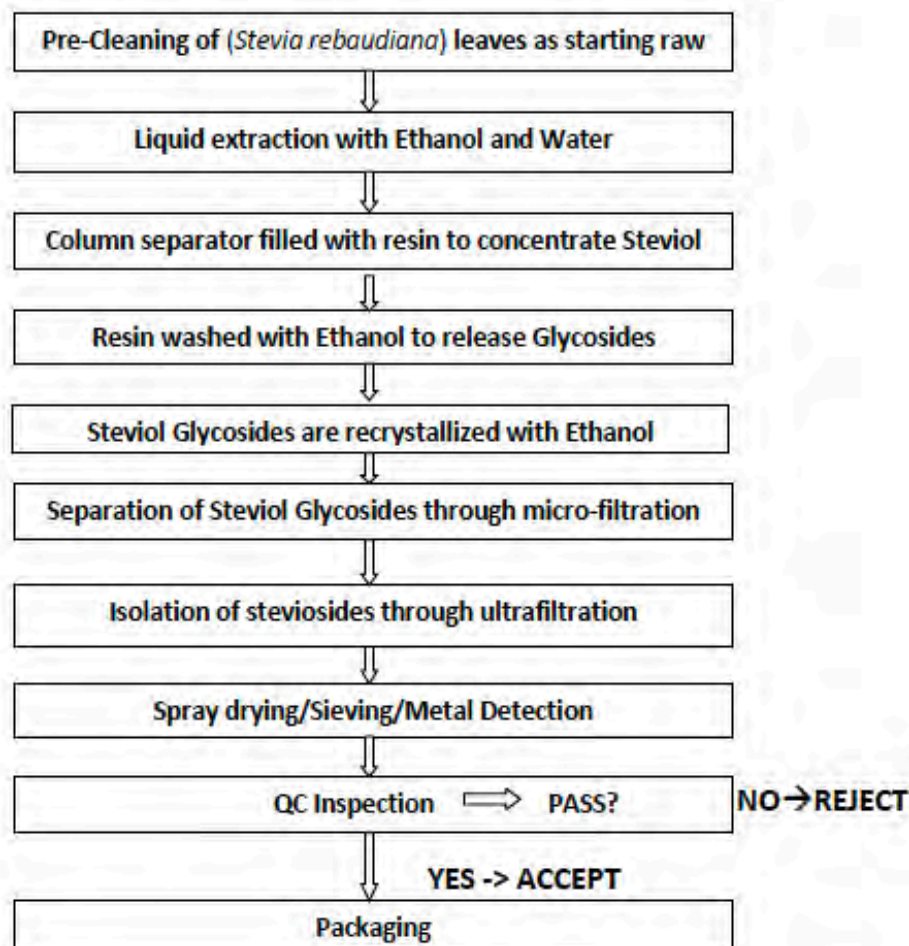


*Blue California*  
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## A Perfect Blend of Science and Nature

Product Name: **Stevia Extract 95%**

### PROCESS DIAGRAM



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