GRAS ASSOCIATES, LLC

GRAS Notification

of

Rebaudioside D
(≥ 95%)

Food Usage Conditions for General Recognition of Safety

on behalf of

Blue California
Rancho Santa Margarita, California

6/26/17

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FOREWORD

At the request of Blue California, GRAS Associates, LLC ("GA") has undertaken an independent safety evaluation of Blue California's high purity rebaudioside D product, referred to as "Reb D" and "BESTEVIA™ Rebaudioside D 95%." The purpose of the evaluation is to confirm Blue California's conclusion that the intended food uses of high purity rebaudioside D as a non-nutritive sweetener 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 that GRAS Associates act as agent for the submission of this GRAS notification.

Blue California based its GRAS assessment on a large body of information that addressed the safety/toxicity of purified steviol glycosides, history of use of purified steviol glycosides and similar compounds, and compositional details, specifications, and method of preparation of the subject ingredient.

Safety/toxicity studies performed with animals and human clinical trials were noted to have value. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

In addition to the product specifications, chemical properties, manufacturing, and safety-related information, Blue California also provided consumption/exposure information, along with other related documentation. This was augmented with an independent search of the scientific and regulatory literature extending through June 17, 2017. A GRAS assessment based primarily on the composite safety information, i.e., based on scientific procedures, was undertaken by Blue California in concert with an Expert Panel review by GRAS Associates. Those references that were deemed pertinent to the objective at hand are listed in Part 7.

PART 1. SIGNED STATEMENTS AND CERTIFICATION

A. Basis of Exclusion from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)[1]

Blue California has concluded that its high purity rebaudioside D, also referred to as "Reb D" and "BESTEVIA™ Rebaudioside D 95%," and which meet the specifications described below, is Generally Recognized As Safe (GRAS) in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic (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 D (≥95%) preparation.

[1] See 81 FR 54960, 17 August 2016. Accessible at: https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf (Accessed 5/24/17).

Signed:

(b) (6)

Agent for Blue California

Steven Overgaard
President
GRAS Associates, LLC
27499 Riverview Center Blvd.
Suite 212
Bonita Springs, FL 34134

Date: 6/26/17

B. Name and Address of Responsible Party

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 its high purity rebaudioside D (≥ 95%) preparation, which is also referred to as "Reb D" and "BESTEVIA™ Rebaudioside D 95%," as described in the subject safety evaluation; consequently, the purified steviol glycosides preparations having purities no less than 95% rebaudioside D which meet the conditions described herein, are not subject to premarket approval requirements for food ingredients.

C. Common Name and Identity of Notified Substance

High purity rebaudioside D, abbreviated as Reb D or reb D, is the common name for the notified substance.

D. Conditions of Intended Use in Food

Blue California's BESTEVIA™ Rebaudioside D 95% preparation (≥ 95% reb D) is 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.

E. Basis for GRAS Conclusion

Pursuant to 21 CFR 170.30(a) and (b), Blue California's BESTEVIA™ Rebaudioside D 95% (≥ 95% Reb D) preparation 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 and GRAS Associates certify, 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 and GRAS Associates, LLC certify 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

High purity rebaudioside D is the common or usual name of the non-nutritive sweetener synthesized from an extract of *Stevia rebaudiana* Bertoni by genetically-modified yeast. The compositional features of the subject high purity rebaudioside D are described in more detail elsewhere in Part 2.

In the scientific literature, steviol glycosides have been referred to as stevia, stevioside, steviol glycosides, and stevia glycoside. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) adopted the term "steviol glycosides" for the family of steviol derivatives with sweetness properties that are derived from the stevia plant. Presently, the term "stevia" is used more narrowly to describe the plant or crude extracts of the plant, while Reb D–like stevioside–is the common name for another one of the specific glycosides that is extracted from stevia leaves.

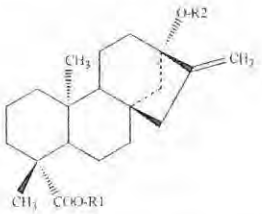
1. Chemistry of Steviol Glycosides

At its 51st meeting, JECFA reviewed the safety related information on steviol glycosides, including the identity and chemistry of these compounds. The following chemistry related description of steviol glycosides is taken from the original JECFA monograph (WHO, 2000).

> Stevioside is a glycoside of the diterpene derivative steviol (ent-13-hydroxykaur-16-en-19-oic acid). Steviol glycosides are natural constituents of the plant *Stevia rebaudiana Bertoni*, belonging to the Compositae family. The leaves of *S. rebaudiana Bertoni* contain eight different steviol glycosides, the major constituent being stevioside (triglucosylated steviol), constituting about 5-10% in dry leaves. Other main constituents are rebaudioside A (tetraglucosylated steviol), rebaudioside C, and dulcoside A. *S. rebaudiana* is native to South America and has been used to sweeten beverages and food for several centuries. The plant has also been distributed to Southeast Asia. Stevioside has a sweetening potency 250-300 times that of sucrose and is stable to heat. In a 62-year-old sample from a herbarium, the intense sweetness of *S. rebaudiana* was conserved, indicating the stability of stevioside to drying, preservation, and storage (Hanson and De Oliveira, 1993; Soejarto et al., 1982).

In the Chemical and Technical Assessment (FAO, 2007a), JECFA identified the sweetener components. They updated the list of common glycosides and their chemical structures, which are slightly different from compounds depicted in older publications (Nanayakkara et al., 1987; Suttajit et al., 1993). They are shown in Figure 1.

Figure 1. Chemical Structures of Various Steviol Glycosides[a,b]



	Compound name	C.A.S. No.	R1	R2
1	Steviol	471-80-7	H	H
2	Steviolbioside	41093-60-1	H	β-Glc-β-Glc(2→1)
3	Stevioside	57817-89-7	β-Glc	β-Glc-β-Glc(2→1)
4	Rebaudioside A	58543-16-1	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
5	Rebaudioside B	58543-17-2	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
6	Rebaudioside C (dulcoside B)	63550-99-2	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
7	Rebaudioside D	63279-13-0	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
8	Rebaudioside E	63279-14-1	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
9	Rebaudioside F	438045-89-7	β-Glc	β-Glc-β-Xyl(2→1) | β-Glc(3→1)
10	Rubusoside	63849-39-4	β-Glc	β-Glc
11	dulcoside A	64432-06-0	β-Glc	β-Glc-α-Rha(2→1)

[a] From FAO, 2007b.

[b] The indicated CAS No. for Rubusoside as reported in the cited reference is incorrect and should be 64849-39-4.

In a number of reviews by different authors (Geuns, 2003; Kennelly, 2002; Kinghorn, 2002; Kinghorn and Soejarto, 1989), the structures of the components of steviol glycosides have been described. Through a series of chemical reactions and analyses, the structures, stereochemistry, and absolute configurations of steviol and isosteviol were established over a 20-year period after the seminal work of Bridel and Lavielle (1931) in France (Bridel and Lavielle, 1931). The work by Ogawa et al. [1980, cited in (Brandle et al., 1998)] on synthetic transformation of steviol into stevioside supported the proposed structures. Two other sweet glycosides, rebaudioside A (Reb A) and rebaudioside B (Reb B), were obtained from methanol extracts of stevia leaves, along with the major sweet principal constituent, stevioside, and a minor constituent steviolbioside, which was first prepared from stevioside by alkaline hydrolysis by Wood et al. [1955, cited in (Brandle et al., 1998)]. Subsequently, it was suggested that Reb B was an artifact formed from Reb A during isolation (Brandle et al., 1998; Kennelly, 2002). In addition, stevioside can be converted both chemically and enzymatically to Reb A. Further fractionation led to the isolation and identification of three other sweet glycosides, respectively named rebaudioside C (Reb C), rebaudioside D, and

rebaudioside E (Reb E). It was reported that Reb A and Reb D could be converted to Reb B by alkaline hydrolysis showing that only the ester functionality differed (Brandle et al., 1998). Dulcosides A and B were also described (Kobayashi et al., 1977). Later, dulcoside B and Reb C were shown to be structurally identical.

More recently, Chaturvedula et al. (2013) reported isolating the minor-component steviol glycoside, rebaudioside M (Reb M), from commercially available *Stevia rebaudiana* Bertoni extracts (Chaturvedula et al., 2013).

There are no known toxicants that have been identified in stevia.

A recent publication by Kumari et al. (2016) addressed the content and distribution of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Antioxidant Capacity (TAC) of different portions of *Stevia rebaudiana.* TPC, TFC, and TAC were observed in all portions of the stevia plant, with the highest levels noted in the leaves; the relative TPC, TFC, and TAC amounts reflect the following order: leaf > flower > stem > branch > root. The higher levels in the leaves were attributed to an increased content of phenolics, flavonoids, and pigments in the stevia leaf. The authors also reported that TPC, TFC, and TAC amounts decreased with leaf maturity.

2. Chemistry of Rebaudioside D

Rebaudioside D is a minor naturally occurring steviol glycoside obtained from the leaves of *Stevia rebaudiana* Bertoni; it is reported to be 250-400 times sweeter than sugar (Kinghorn, 2002). Similar to the other steviol glycosides, Reb D is an *ent*-kaurane diterpene glycoside with a steviol backbone.

Chemical name: 13-[(O-ß-D-Glucopyranosyl—3-O-ß-D-glucosylpyranosyl-ß-D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid, 2-O-ß-D-glucopyranosyl- ß-D-glucopyranosyl ester.

Synonyms: Rebaudioside D, Reb D

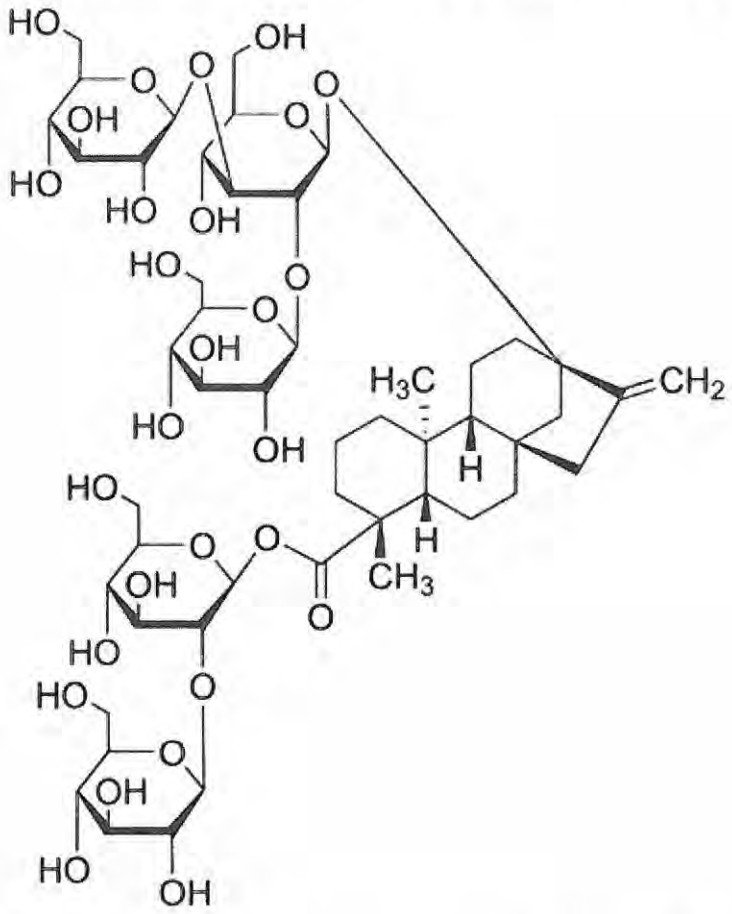
Chemical formula: $C_{50}H_{80}O_{28}$

Molecular weight: 1129.15 daltons

CAS Number: 63279-13-0

The chemical structure of rebaudioside D is presented in Figure 2.

Figure 2. Chemical Structure of Rebaudioside D[a]



[a] From Sigma-Aldrich www.sigmaaldrich.com (Accessed 5/24/17).

3. Chemistry of the Yeast Vector

Blue California's manufacturing process for its high purity Reb D preparation uses a set of enzymes to carry out catalytic bio-conversion. The enzymes are produced by a nonpathogenic and nontoxigenic 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,

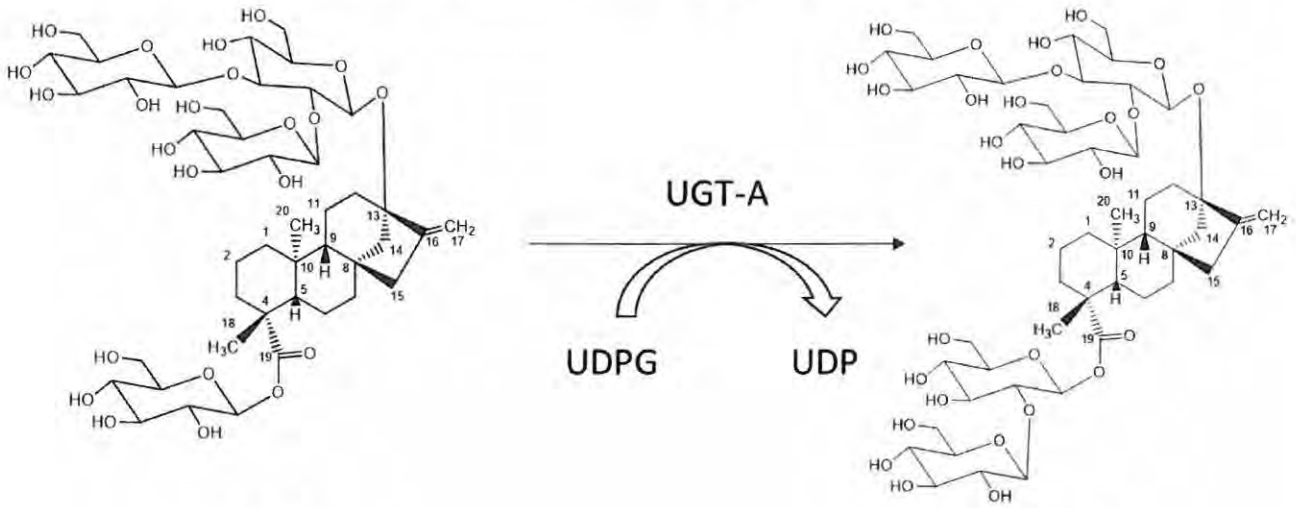
inexpensive medium. Its morphological, physiological, and growth conditions have been widely studied and reported. The detailed transformation protocol and plasmid information has been reported in Blue California's published patents, which are listed in Appendix 1.

UGT-A enzyme is a member of the 5'-diphosphouridine-glucosyltransferase (UGT) family, which was identified from *Hordeum vulgare subsp. Vulgare.* UGT-A produces Reb D from Reb A *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 rebaudioside A (Figure 3).

Sucrose synthases (SUS) catalyze the conversion of the 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 and SUS genes into yeast cells to produce both enzymes simultaneously.

Figure 3. Biosynthesis Pathway from Reb A to Reb D



B. Manufacturing Process

Blue California manufactures its high purity rebaudioside D preparation in a process similar to that described for rebaudioside M in GRN 667. The multi-step biosynthesis pathway process to manufacture BESTEVIA Rebaudioside D 95% uses enzymes produced by a strain of *Pichia*

pastoris yeast that contains uridine 5'-diphosphouridine-glucosyltransferase (UGT) enzymes that facilitate the transfer of glucose to small molecules *via* a glycosidic bond.

1. Scientific & Patent Literature

In general, steviol glycosides are typically obtained by extracting leaves of *Stevia rebaudiana* Bertoni with hot water or alcohols (ethanol or methanol). This extract is a dark particulate solution containing all the active principles, plus leaf pigments, soluble polysaccharides, and other impurities. Some processes remove the "grease" from the leaves before extraction by employing solvents such as chloroform or hexane (Kinghorn, 2002). There are several extraction patents for the isolation of steviol glycosides. Kinghorn (2002) has categorized the extraction patents into those based on solvent, solvent plus a decolorizing agent, adsorption and column chromatography, ion exchange resin, and selective precipitation of individual glycosides. In recent patents, methods such as ultrafiltration, use of metallic ions, supercritical fluid extraction with CO_2, and extract clarification with zeolite have been employed.

At the 68th JECFA meeting, steviol glycosides were defined as the products obtained from the leaves of *Stevia rebaudiana* Bertoni. As described by JECFA, the typical manufacturing process starts with extracting leaves with hot water, and the aqueous extract is then passed through an adsorption resin to trap and concentrate the component steviol glycosides. The resin is then washed with methanol to release the steviol glycosides, and the product is recrystallized with methanol. Ion-exchange resins may be used in the purification process. The final product is commonly spray-dried.

More recently, novel processes for conversion of steviol glycosides to particular glycosides have been described in the scientific literature. The use of UGT enzymes generated from yeast in the Saccharomycetaceae family has been described for a number of processes to chemically incorporate glucose molecules into a variety of substances.

2. Manufacturing Process for Rebaudioside D

a. Catalytic Bioconversion Process

To produce the enzymes used in the bio-conversion, the glycerol stock of Yeast Cell A (carrying UGT-A and SUS enzyme genes) are 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[2] read OD_{600} = 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

[2] Blue California uses older, larger cells to perform the measurement.

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 D, 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.

b. Extraction & Purification

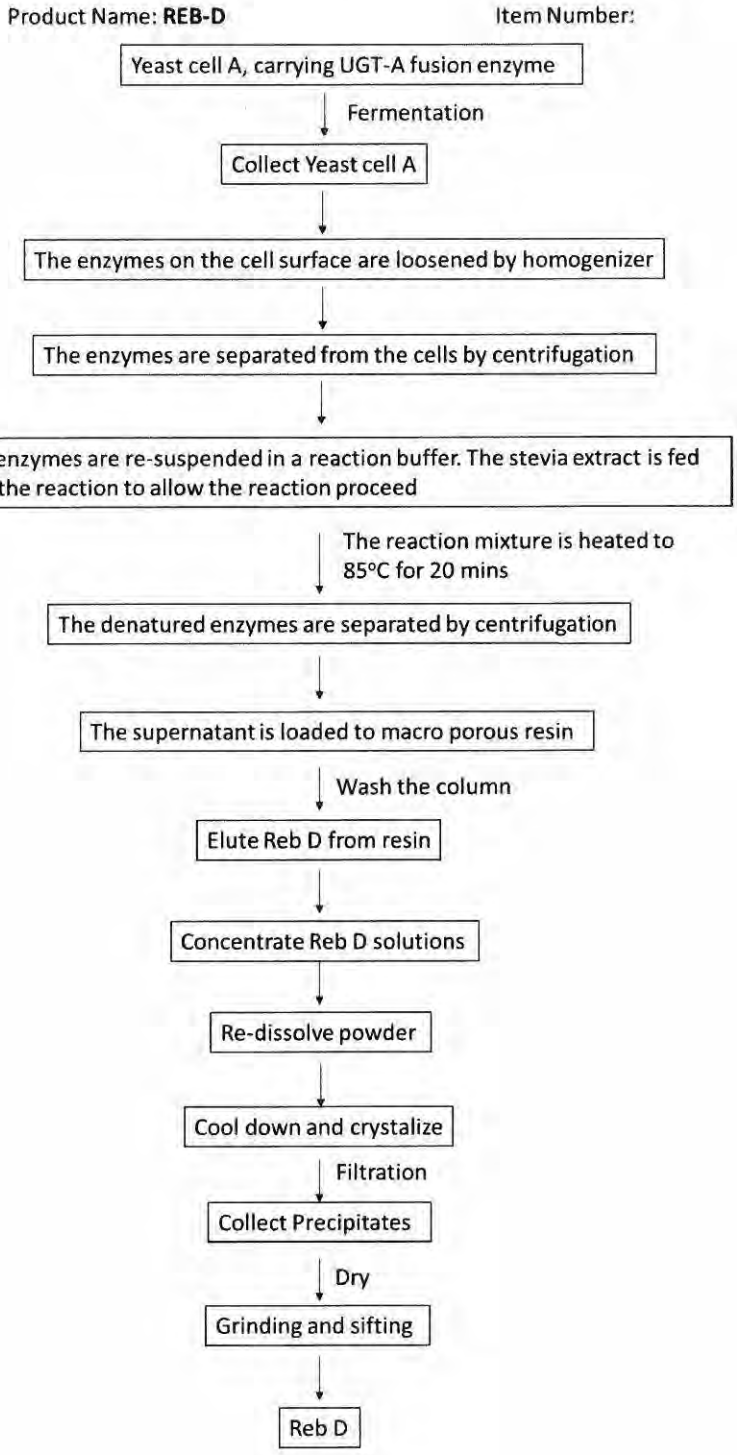
The supernatant from the Catalytic Bioconversion Process, described above, is filtered to remove any remaining debris. The supernatant is then loaded onto large columns containing a macro-porous resin. The supernatant flows through the column by gravity and is bound to the resin. The column is then rinsed with a series of buffers. Reb D 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 Red D remains in aqueous solution.

The condensate is chilled to allow Reb D 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 D, while removing impurities, including other steviol glycosides that exhibit higher solubilities than Reb D. Consequently, the other steviol glycosides (i.e. impurities) will remain in solution while Red D precipitates out first, allowing Blue California to produce a higher purity Reb D product. Activated charcoal is used to purify the final product by adsorbing the non-steviol glycosides impurities. The resulting Reb D product is re-crystallized, dried, and processed to the final BESTEVIA™ Rebaudioside D 95% product.

The manufacturing process is summarized in a flow chart provided in Figure 4.

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

Figure 4. Flow Chart of Manufacturing Process for Blue California's BESTEVIA™ Rebaudioside D 95%



C. Product Specifications

1. JECFA Specifications for Steviol Glycosides

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

In 2007, JECFA recommended that the method of assay should include a minimum requirement of 95% of the total of 7 specific steviol glycosides on a dried weight basis, and JECFA finalized food grade specifications at the 68th JECFA meeting with publication in the Food and Agriculture Organization of the United Nations (FAO) JECFA Monograph 4 (FAO, 2007a). Stevioside and rebaudioside A (Reb A) are the major component glycosides of interest because of their sweetening property. The five other associated glycosides found in preparations of steviol glycosides accepted by the JECFA specifications with the 95% requirement are Reb C, dulcoside A, rubusoside, steviolbioside, and Reb B. These, however, are typically found at much lower levels than stevioside or rebaudioside A. JECFA updated the specifications for steviol glycosides in 2008 (FAO, 2008), and then again in 2010, when the specifications were expanded to include the original seven specific steviol glycosides plus Reb D and rebaudioside F (Reb F) (FAO, 2010). Recently, Reb M has garnered interest as an additional naturally-occurring sweet steviol glycoside.

JECFA describes steviol glycosides as a white to yellow powder, odorless to having a slight characteristic odor, and exhibiting a sweetness that is 200-300 times greater than sucrose. The ingredient must consist of a minimum of 95% of nine specific steviol glycosides. The steviol glycosides are freely soluble in water and ethanol, 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 ppm, and ethanol residues should not exceed 5,000 ppm. Arsenic levels should not exceed 1 ppm as determined by the atomic absorption hydride technique. Lead levels should not exceed 1 ppm.

2. Specifications for Blue California's Rebaudioside D Preparation and Supporting Methods

Blue California has adopted product specifications for its BESTEVIA™ Rebaudioside D 95% 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 lots of Blue California's BESTEVIA™ Rebaudioside D 95% are compared to the JECFA and FCC specifications in Table 1.

Table 1. Specifications for Blue California's Rebaudioside D Preparations

Physical & Chemical Parameters	JECFA[a] Specifications Steviol Glycosides	FCC[b] Specifications Rebaudioside A	Blue California's Specifications for BESTEVIA™ Rebaudioside D 95%	BESTEVIA™ Rebaudioside D 95% Representative Lots				
				Lot D195-160113	Lot D195-160126	Lot D195-160265	Lot D195-160324	Lot D195-160425
Appearance Form	Powder	Crystal, granule or powder	Powder	Pass	Pass	Pass	Pass	Pass
Appearance Color	White to light Yellow	White to off-white	Off-white to white	Pass	Pass	Pass	Pass	Pass
Solubility[d]	Freely soluble in water	Freely soluble in water:ethanol (50:50)	NS	Very slightly soluble in water	Very slightly soluble in water	Very slightly soluble in water	Very slightly soluble in water	Very slightly soluble in water
Purity (HPLC Area)	NS	≥ 95%	≥ 95% Reb D	97.4% Reb D	96.2% Reb D	96.1% Reb D	97.2% Reb D	96.8% Reb D
Residual Ethanol	NMT 5,000 mg/kg	NMT 0.5%	< 1,000 ppm	< 20 ppm	< 20 ppm	< 20 ppm	< 20 ppm	< 20 ppm
Residual Methanol	NMT 200 mg/kg	NMT 0.02%	< 200 ppm	< 50 ppm	< 100 ppm	< 100 ppm	< 50 ppm	< 80 ppm
Loss on Drying (%)	NMT 6.0%	NMT 6.0%	≤ 5%	0.53%	0.85%	0.86%	0.55%	0.70%
pH, 1% Solution	4.5-7.0	4.5-7.0	5-7	6.05	5.95	5.95	5.95	6.05
Total Ash (%)	NMT 1%	NMT 1%	≤ 1%	0.12%	0.132%	0.157%	0.114%	0.071%
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	< 0.25 ppm	< 0.25 ppm	< 0.25 ppm	< 0.25 ppm	< 0.25 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	NA	NA	< 3,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g
Total coliform	NA	NA	< 100 cfu/g	< 3 cfu/g	< 3 cfu/g	< 3 cfu/g	< 3 cfu/g	< 3 cfu/g
Yeast & Mold	NA	NA	< 100 cfu/g	< 50 cfu/g	< 50 cfu/g	< 50 cfu/g	< 50 cfu/g	< 50 cfu/g
Salmonella spp	NA	NA	Negative	ND	ND	ND	ND	ND
***E. coli* (mpn/g)**	NA	NA	Negative	ND	ND	ND	ND	ND

[a] Prepared at 73rd JECFA, 2010.

[b] 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™ Reb D 95% 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 2 for comparison with generally accepted purity standards, certificates of analysis for five representative lots of BESTEVIA™ Reb D 95% are provided in Appendix 5. The chromatograms for representative BESTEVIA™ Reb D 95% are provided in Appendix 6. Test reports for analysis of pesticide residues in representative lots of BESTEVIA™ Reb D 95% 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 has determined the relative sweetness of BESTEVIA™ Reb D 95% by organoleptic comparison to sucrose following the method presented in Appendix 8. The relative sweetness of Blue California's BESTEVIA™ Reb D 95% was found to be approximately 202 times sweeter than sucrose, which is somewhat less than previously reported in the literature (GLG, 2014; Kinghorn, 2002; PureCircle, 2013).

E. Stability

1. Stability Data on Steviol Glycosides

Steviol glycosides have been reported to be stable over the pH range 3-9 and can be heated at 100°C for 1 hour, but, at pH levels greater than 9, it rapidly decomposes (Kinghorn, 2002). At pH 10, steviolbioside would be the major decomposition product produced from stevioside by alkaline hydrolysis (Wood et al., 1955). Chang and Cook (1983) investigated the stability of pure stevioside and Reb A in carbonated phosphoric and citric acidified beverages. Some degradation of each sweetening component after 2 months of storage at 37°C was noted. However, no significant change at room temperature or below, following 5 months of storage of stevioside and 3 months of storage of Reb A, was observed. Exposure to one week of sunlight did not affect stevioside, but approximately 20% loss of rebaudioside A was detected. Heating at 60°C for 6 days resulted in 0-6% loss of rebaudioside A.

Merisant (2008) conducted stability testing on rebaudioside A (1) as a powder, (2) as a pure sweetener in solution, and (3) on both cola-type and citrus carbonated beverages. In these investigations, no degradation was detected when the powder was stored at 105°C for 96 hours. It was concluded that the powder was stable when stored for 26 weeks at 40±2°C with relative humidity of 75±5%. Both published and unpublished testing results from Merisant revealed that rebaudioside A in carbonated citric acid beverages and phosphoric acid beverages did not significantly degrade during prolonged storage at refrigeration, normal ambient, or elevated ambient temperatures. Minimal loss of rebaudioside A was detected after storage at 60°C with considerable degradation noted after 13 hours at 100°C for carbonated beverage solutions and pure sweetener solutions (Merisant, 2008).

Cargill (2008) also conducted extensive stability testing on rebaudioside A as a powder under various storage conditions and under a range of pH and temperatures. Additionally, Cargill also investigated rebaudioside A stability in several representative food matrices at room temperature and elevated temperatures. Stability profiles were created for table top sweetener applications, mock beverages including cola, root beer and lemon-lime, thermally processed beverages, yogurt, and white cake. The results of stability testing revealed some degradation products that had not been detected in bulk rebaudioside A. These degradation products were structurally related to the steviol glycosides that are extracted from the leaves of *Stevia rebaudiana* Bertoni. All the degradation products were found to share the same steviol aglycone backbone structure as found in stevioside and rebaudioside A, but they differ by virtue of the glucose moieties present. The results of stability testing revealed that rebaudioside A is stable in various food matrices following several days or weeks of storage. The extent and rate of degradation is dependent on pH, temperature, and time. When placed in beverages, rebaudioside A is more stable in the pH range 4 to 6 and at temperatures from 5˚C to 25˚C (Cargill, 2008).

Photostability studies of the dry powder and mock beverages were performed to ascertain rebaudioside A behavior under defined conditions of fluorescent and near UV light exposure. Rebaudioside A was found to be photostable under the defined conditions of analysis (Clos et al., 2008).

In addition to the above described stability reports for purified rebaudioside A, in a GRAS notification by Sunwin and WILD Flavors (2010)---regarding purified steviol glycosides with rebaudioside A and stevioside as the principal components---stability was investigated using a 0.04% solution of Reb A 80% in acidic solutions between pH 2.81 and 4.18. In this study, the solutions were stored at 32°C for 4 weeks, and the Reb A content was determined at 1, 2, and 4 weeks. Reb A 80% was found to be very stable at pH 3.17 and above. At pH 2.81, after 4 weeks of storage under accelerated conditions, only a 7% loss of Reb A was noted. Sunwin and WILD Flavors also studied the stability of Reb A 80% in simulated beverages using 0.1% citric acid (pH 3.2). The solutions were pasteurized and stored for 8 weeks at 4°C and 32 °C, and little difference in sweetness perception was found under these conditions.

2. Stability Data for Blue California's BESTEVIA™ Reb D 95 Preparation

Blue California conducted a 6-month stability study of five lots of Reb D 95%. The samples were stored at 40°C ± 2°C at a relative humidity of 75% ± 5%. Reb D 95% was observed to be stable over the course of the accelerated stability study, as demonstrated in Table 2.

Table 2. Rebaudioside D Storage Stability Data

Reb-D 95% Lot# D195-160113			
Duration	**Appearance**	**Moisture (%)**	**Rebaudioside D (HPLC %)**
t=0	White Powder	1.03	97.4
1 month	White Powder	1.15	96.9
2 months	White Powder	1.48	97.6
3 months	White Powder	1.32	97.2
4 months	White Powder	1.26	97.4
5 months	White Powder	1.30	97.3
6 months	White Powder	1.41	96.4
Reb-D 95% Lot# D195-160265			
Duration	**Appearance**	**Moisture (%)**	**Rebaudioside D (HPLC %)**
t=0	White Powder	2.15	96.1
1 month	White Powder	2.03	96.6
2 months	White Powder	2.22	96.5
3 months	White Powder	2.14	96.4
4 months	White Powder	2.21	96.4
5 months	White Powder	2.18	96.5
6 months	White Powder	2.31	96.4
Reb-D 95% Lot#D195-160126			
Duration	**Appearance**	**Moisture (%)**	**Rebaudioside D (HPLC %)**
t=0	White Powder	1.82	96.2
1 month	White Powder	1.63	96.7
2 months	White Powder	1.95	96.4
3 months	White Powder	2.02	96.5
4 months	White Powder	2.15	96.6
5 months	White Powder	2.09	96.5
6 months	White Powder	2.20	96.4
Reb-D 95% Lot# D195-160324			
Duration	**Appearance**	**Moisture (%)**	**Rebaudioside D (HPLC %)**
t=0	White Powder	1.56	97.2
1 month	White Powder	1.72	97.5
2 months	White Powder	1.78	97.5
3 months	White Powder	1.73	97.4
4 months	White Powder	1.77	97.4
5 months	White Powder	1.70	97.3

6 months	White Powder	1.77	97.4
Reb-D 95% Lot# D195-160425			
Duration	**Appearance**	**Moisture (%)**	**Rebaudioside D (HPLC %)**
t=0	White Powder	1.36	96.8
1 month	White Powder	1.53	96.6
2 months	White Powder	1.49	96.6
3 months	White Powder	1.62	96.5
4 months	White Powder	1.70	96.4
5 months	White Powder	1.68	96.5
6 months	White Powder	1.77	96.8

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™ Reb D 95% preparation is well-suited for the intended food uses.

PART 3. DIETARY EXPOSURE

The subject Blue California BESTEVIA™ Reb D 95% preparation, containing rebaudioside D as the principal component (≥95%), 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).[3] 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 Reb D 95% 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.[4]

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; Renwick, 2008; WHO, 2003) or submitted to FDA (Merisant, 2008). These are summarized in Appendix 9. 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

[3] Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
[4] See 21 CFR 182.1(b)(1).

sweetener (BioVittoria, 2009). As summarized in GRN 301, the 90th 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™ Reb D 95% preparation is approximately 202-fold that of sucrose (Part 2.D). Therefore, the highest 90th percentile consumption by any population subgroup of Blue California's Reb D ≥ 95% preparation would consume approximately 4.90 mg per kg steviol glycosides bw per day. Based on an estimate that Reb D preparations consist of approximately 28% steviol equivalents,[5] the consumption would be less than 1.38 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™ Reb D 95%

Population Group	Intakes of Sweeteners (mg sucrose/kg bw/day)[a]		Calculated Intake of BESTEVIA™ Reb D 95% (mg/kg bw/day)[b]		Calculated Intake of BESTEVIA™ Reb D 95% as Steviol Equivalents (mg/kg bw/day)[c]	
	Low	High	Low	High	Low	High
Healthy Population	255	675	1.26	3.34	0.36	0.94
Diabetic Adults	280	897	1.39	4.44	0.39	1.25
Healthy Children	425	990	2.10	4.90	0.59	1.38
Diabetic Children	672	908	3.33	4.50	0.94	1.27

[a] From Renwick (2008).
[b] Calculated by dividing the sucrose intake by the minimum average relative sweetness value of 202 for Blue California's Reb D.
[c] Calculated based on the ratio of molecular weights of Reb D and steviol.

The values in Table 3 assume that Blue California's BESTEVIA™ Reb D 95% preparation constitute 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 3.34 mg per kg bw per day, or 0.94 mg per kg steviol equivalents. For healthy children, the estimated maximal intake is 4.90 mg per kg bw per day, or 1.38 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.

[5] Calculated by Expert Panel as percent of molecular weight of steviol to molecular weight of rebaudioside D.

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™ Reb D 95% product.

C. Dietary Exposure to Contaminants or Byproducts

While a recent publication by Kumari et al. (2016) has demonstrated the presence of TPC, TFC, and 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 the highly purified stevia extract, of which ≥ 95% consists of the most familiar steviol glycosides. These contaminants, if present, are in low amounts and were likely similarly present in purified test materials that were used in the toxicology studies.

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 (Brandle et al., 1998; Carakostas et al., 2008; Gerwig et al., 2016; Gupta et al., 2016; Kochikyan et al., 2006; Prakash et al., 2008). 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 (Brandle et al., 1998; Brusick, 2008; Esen, 2016; Gerwig et al., 2016).

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 were 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).

Even more recently, 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).

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).

B. Summary of Regulatory History of Rebaudioside D

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; HealthCanada, 2012; Watson, 2010). 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, 2017) as of June 17, 2017, FDA has issued 45 "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 4.

Table 4. FDA's GRAS Notice Inventory on Rebaudioside & Steviol Glycosides Preparations[a]

Company	FDA GRAS Identifier	Material Identity	Intended Food Uses
1. Merisant	GRN 252	High-Purity Reb A ≥95%	Variety of food categories & table top sweetener
2. Cargill Inc.	GRN 253	High-Purity Reb A ≥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 ≥97%	General-purpose & table top sweetener
5. Sweet Green Fields LLC	GRN 282	High-Purity Reb A ≥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 ≥95%/ ≥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[c]	GRN 329	High-Purity Reb A ≥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 levels determined by good manufacturing practices
13. GLG Life Tech Ltd[c]	GRN 348	High-Purity Stevioside ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
14. GLG Life Tech Ltd[c]	GRN 349	High-Purity Steviol Glycosides ≥97%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
15. Guilin Layn Natural Ingredients, Corp.	GRN 354	High-Purity Reb A ≥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 ≥95%	General-purpose & table top sweetener, excluding meat, poultry products & infant formulas
18. Shanghai Freemen 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[b]	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. Daepyung Co., Ltd.	GRN 393	Purified Reb A	General purpose & table top sweetener, excluding meat & poultry products
24. Daepyung 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. Daepyung Co., Ltd.	GRN 448	Enzyme Modified Steviol Glycosides	General-purpose sweetener, excluding meat, poultry products & infant formulas.
27. Daepyung 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 ≥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 ≥97%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
30. Qufu Xiangzhou Stevia Products Co., Ltd.	GRN 467	High-Purity Reb A ≥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 ≥95%	General-purpose sweetener, excluding meat, poultry products.
33. GLG Life Tech Corp.	GRN 512	High purity Reb M ≥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 ≥95%	General-purpose sweetener, excluding meat, poultry products & infant formulas.
40. Cargill, Inc.	GRN 626	Steviol glycosides (Reb M and Reb D) produced in *Saccharomyces cerevisiae*	General-purpose sweetener
41. DSM Nutritional Products, LLC.	GRN 632	Rebaudioside A from *Yarrowia lipolytica*	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.

Company	FDA GRAS Identifier	Material Identity	Intended Food Uses
45. Blue California	GRN 667	Rebaudioside M	General-purpose sweetener, excluding meat, poultry products & infant formulas.

[a] This table was derived, in part, from McQuate (2011).
[b] The name of this company is now GLG Life Tech Corporation.

In addition, the Flavor and Extract Manufacturers Association (FEMA) has included several steviol glycosides preparations on their GRAS lists as shown in Table 5.

Table 5. FEMA GRAS Status for Steviol Glycoside Preparations

Steviol 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)
Stevioside	4763	(Leffingwell and Leffingwell, 2014; Marnett et al., 2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 60%	4771	(Marnett et al., 2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 80%	4772	(Marnett et al., 2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside C 30%	4796	(Cohen et al., 2015a; Cohen et al., 2015b)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 22%	4805	(Cohen et al., 2015a; Cohen et al., 2015b)
Steviol glycoside extract, *Stevia rebaudiana* 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) (HealthCanada, 2009). The revised recommendation for the maximum limit for steviol glycosides in NHPs is in accordance with the full 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 (HealthCanada, 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 (HealthCanada, 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 (HealthCanada, 2016).

3. European Regulatory History

The Joint Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 51st, 63rd, 68th and 73rd meetings. In 2000, JECFA published the original review on steviol glycosides (WHO, 2000). JECFA established a temporary ADI of 0-2 mg per kg (on a steviol basis) at its 63rd 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 69th 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; b).

In light of JECFA's 2008 findings, and in response to a June 2008 request by the 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.[6] In addition, on

[6] 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) Advisory Committee on Novel Foods and Processes for the Ministry of Agriculture, Fisheries and Food.). 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) Opinion on Stevia Rebaudiana Bertoni plants and leaves. Scientific Committee on Food (CS/NF/STEV/3 Final, 17 June 1999). In another opinion also dated June 17, 1999, the Committee also reiterated "its earlier opinion that stevioside is not acceptable as a sweetener

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 95th 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 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 95th 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).

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 (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

on the presently available data" EuropeanCommission (1999b) Opinion on stevioside as a sweetener. Scientific Committee on Food (CS/ADD/EDUL/167Final, 17 June 1999).

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, 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 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 (RSADH, 2012a; b).

PART 6. NARRATIVE

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."[7]

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."[8]

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:[9]

- 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

[7] 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 4/15/17).
[8] See 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 4/15/17).
[9] See Footnote 1.

- 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 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. Blue California's Findings on the Safety of Rebaudioside D

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; 2006; 2007; 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 9.

Since the JECFA evaluation (WHO, 2008), over forty GRAS notifications for steviol glycosides or enzyme modified steviol glycosides were submitted to FDA, and all of the safety reviews that have been completed by FDA were determined to be 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 12. To date, 45 of the submitted notifications have had "no questions" letters of response from FDA including 2 notifications on purified rebaudioside D (see Table 4).

Blue California has also reviewed the complete literature available on the safety of steviol glycosides. The literature on steviol glycosides preparations that primarily consist of stevioside and

reb A is summarized in Appendix 11, the literature on purified preparations of reb A is summarized in Appendix 12, and studies on the primary metabolite steviol are summarized in Appendix 13. These appendices also note information that may be inconsistent with a conclusion of GRAS status. Blue California has thoroughly reviewed this information and as explained in the appendices and confirmed by the Expert Panel (See Part 6.C) has put this information in context: in addressing the totality of safety information cited, the finding that steviol glycosides are GRAS remains in place.

There is a high presumption of safety of rebaudioside D because it is a naturally occurring steviol glycoside obtained from *Stevia rebaudiana* Bertoni that is isolated in a manner similar to the other well-recognized steviol glycosides, including rebaudioside A. In a June 17, 2017 search of the Toxnet database, no studies with any significant impact on the safety of rebaudioside D have been conducted since the last GRAS notice was submitted to FDA (GLG, 2014).

The metabolism of rebaudioside D has been studied by *in vitro* methods (Nikiforov et al., 2013; Purkayastha et al., 2014) similar to those used in previous studies with enzyme treated stevia extract (Koyama et al., 2003a; NOWFoods, 2010).

Rebaudioside D was found to be resistant to metabolic processes in simulated extracts of the stomach and small intestine and with rat liver extract (Nikiforov et al., 2013). Reb D was also observed to be converted to steviol by rat cecal extracts. No metabolism was observed.

Another study compared the anaerobic *in vitro* metabolism of rebaudiosides A, B, D, and M with human fecal homogenates (Purkayastha et al., 2014). In all cases, the rebaudiosides were hydrolyzed to steviol within 24 hours, with the majority of metabolism occurring within the first 8 hours. Metabolism of rebaudiosides took longer at higher concentrations (2.0 mg per mL vs. 0.2 mg per mL). There were no marked differences in rate or extent of hydrolysis observed between male and female fecal homogenates or the individual rebaudiosides.

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 α(1-2), β-1, β(1-2), β(1-3), and β(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 difference in metabolism were observed between male and female, or different ethnicity, fecal homogenates.

In addition, the study by Nikiforov et al. (2013) examined the *in vivo* metabolism and toxicity of reb D in comparison to reb A in a dietary study in Sprague-Dawley rats. Plasma concentrations of Reb

D, Reb A, and/or the final hydrolysis product of each compound, free/conjugated steviol, were consistent between animals administered either Reb D or Reb A in the diet. There were no treatment related effects seen at exposure levels of 500, 1,000, and 2,000 mg per kg bw per day to Sprague-Dawley rats for 28 days as compared to Reb A administered at a target exposure level of 2,000 mg per kg bw per day.

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

Blue California's BESTEVIA™ Reb D 95% preparation contains not less than 95% steviol glycosides, and it is principally composed of rebaudioside D. 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 has a direct bearing on the present safety assessment for Blue California's BESTEVIA™ Reb D 95% preparation. This is further supported by over a decade and a half of scientific studies on the safety of these structurally related 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---noting that 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 11-13.

Blue California has reviewed this safety information and has concluded that their BESTEVIA™ Reb D 95% preparation (≥ 95% total steviol glycosides) is generally recognized as safe for the proposed uses.

C. Expert Panel[10] Findings on the Safety of Rebaudioside D

Because of their sweetness characteristics, steviol glycosides have viable uses as a non-nutritive sweetener in foods.[11] Periodic reviews by JECFA over the years indicate the progression of

[10] The detailed educational and professional credentials for two of the individuals serving on the Expert Panel can be found on the GRAS Associates website at www.gras-associates.com. Drs. Kraska and McQuate worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in their careers, and subsequently continued working within this area in the private sector. Dr. Emmel has substantial food safety experience in addressing steviol glycosides and other food ingredients. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in the deliberations of GRAS Expert Panels. Dr. Kraska served as Chair of the Panel.

[11] 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. Chatsudthipong V and Muanprasat C (2009) Stevioside and related compounds: therapeutic benefits beyond sweetness. *Pharmacology & therapeutics* **121**:41-54., 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 more a more comprehensive discussion of this subject, see Section 7 of Appendix 13.

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 10 and elsewhere, at its 51st meeting, JECFA determined that there were adequate chronic studies in rats, particularly the study by Toyoda et al. (1997) to establish a temporary ADI of 0 - 2 mg per kg bw per day with an adequate margin of safety. 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). The GRAS Expert Panel critically reviewed the JECFA assessment and agrees with the calculation of the ADI for steviol glycosides.

Several published and unpublished studies (summarized in Appendix 12 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; Sloter, 2008a) and developmental effects (Sloter, 2008b) in rats. 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 comparable to the exposures expected in food. 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. The Panel concludes that these studies on rebaudioside A strengthen the argument that all steviol glycosides that follow the same metabolic pathway are safe at the JECFA established ADI.

The Panel has reviewed the findings from human clinical studies. The Panel noted that, regarding 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 that 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). It is possible that JECFA may also have reviewed the preliminary results associated with the published clinical studies on rebaudioside A (Maki et al., 2008a; Maki et al., 2008b). The Panel concludes that there will be no effects on blood pressure and glucose metabolism in humans at the doses of rebaudioside A expected from its use in food as a non-nutritive sweetener.

Two previously published studies summarized in Appendix 11 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 (Brusick, 2008; Geuns, 2007a; Williams, 2007). The Panel has reviewed the cited publications, along with the responses made by the authors (Nunes et al., 2007b; c), 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, tartrate-resistant alkaline phosphatase (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 finding, combined with a lack of evidence for neoplasm development in rat bioassays,

establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential.

In addition, a recent paper by Shannon et al. (2016) raises a possible concern of endocrine disruption by steviol. The Panel has 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. The Panel concludes that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at receptors and that no adverse effects were observed in reproductive studies. Therefore, this study does not alter the opinion of the Expert Panel that steviol glycosides preparations, with rebaudioside A and stevioside as the main components, are generally recognized as safe. A summary of this study is provided in Appendix 11.

The Expert Panel has reviewed the studies conducted on Reb D (Nikiforov et al., 2013; Purkayastha et al., 2016; Purkayastha et al., 2014), and agree with Blue California's conclusion that the two studies support the presumption of safety of rebaudioside D given its metabolic and toxicological similarity with other steviol glycosides. Nikiforov et al. (2013) observed Reb D to be resistant to metabolic processes in simulated stomach and small intestine extracts and with rat liver extract. In addition, Reb D was found to be converted to steviol by rat cecal extracts. In Sprague-Dawley rats, the plasma concentrations of Reb D, Reb A, and/or the free/conjugated steviol were consistent between animals administered either Reb D or Reb A in the diet. In the 28-day toxicology study, no differences in treatment related effects were observed at exposure levels of up to 2,000 mg per kg bw per day Reb D or Reb A.

In two *in vitro* metabolism studies using human fecal homogenates, Purkayastha et al. (2016; 2014) found that a number of steviol glycosides, including rebaudiosides A and D, 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 8 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).

The Expert Panel agrees with the safety conclusions of the 45 GRAS Expert Panels in the notifications for steviol glycosides previously submitted to FDA that resulted in "no questions" responses from FDA (as summarized in Table 4), JECFA (WHO, 2006; 2008), and Renwick (2008) that a sufficient number of good quality health and safety studies exist to 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.

The Panel 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™ Reb D 95% preparation (≥ 95% total steviol glycosides). Therefore, with the steviol equivalence values shown in Table 3, the Panel concludes that, for the general population, the estimated maximum daily

intake of Blue California's BESTEVIA™ Reb D 95% preparation is 4.90 mg per kg bw or 1.38 mg per kg expressed as steviol equivalents. Based upon these calculations, the intake of all of Blue California's BESTEVIA™ Reb D 95% safely aligns with the 4 mg per kg bw per day ADI expressed as steviol equivalents as determined by JECFA.

In addition, Blue California affirms that its BESTEVIA™ Reb D 95% 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 and consistent with other steviol glycosides on the market---and has demonstrated batch-to-batch consistency against these specifications.

The Panel notes that the relative sweetness intensity of Blue California's Reb D (≥95%) preparation was determined to be somewhat lower than values reported elsewhere (202 vs. 250-400); however, the use levels needed to provide adequate sweetness in food are still within the ADI.

Therefore, a compelling case can be made that a scientific consensus exists regarding the safety of Blue California's Reb D (≥95%) preparation in support of a GRAS conclusion under the conditions of its intended use.

D. 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 must 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 11, 12, and 13. 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. In addition, most of the unpublished studies relied on by JECFA were subsequently published in the scientific literature.

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. It has been well-established experimentally from various published studies that the steviol glycosides 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 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., 2003b; Renwick and Tarka, 2008). Three publications confirm that the metabolism of reb D follows the same pathway as other steviol glycosides (Nikiforov et al., 2013; Purkayastha et al., 2014, 2016). In addition, one published rat study found no adverse effects of reb D in the diet (Nikiforov et al., 2013).

The ADI for steviol glycosides has been set largely based on published chronic study in rats (Toyoda et al., 1997) in concert with several published clinical studies that demonstrate that no pharmacological effects were observed 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 that the ADI could be higher than the 4 mg per kg bw per day figure (on a steviol equivalency basis) as established by JECFA based on evidence that glucuronidation of absorbed steviol is faster in humans than rats. The toxicity of the metabolite steviol has been well reviewed in the published literature (Geuns, 2003; Urban et al., 2013; WHO, 2006). 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.

The Panel maintains that well-qualified scientists would conclude that Blue California's BESTEVIA™ Reb D 95% preparation is not absorbed from the GI tract, *per se*. By virtue of fundamental principles of pharmacokinetics, the majority of scientists would support this determination, and they would likewise concur that the subject Blue California's BESTEVIA™ Reb D 95% undergoes a conversion to steviol as is known to be the case with naturally occurring steviol glycosides.

A number of well-respected regulatory agencies, including JECFA, EFSA, FSANZ, the Switzerland Office of Public Health, and Health Canada, as well as numerous well-respected individual scientists, have indicated that highly purified steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (EFSA, 2010; FAO, 2010; FSANZ, 2008; Geuns, 2003; Health, 2008; HealthCanada, 2012; Toyoda et al., 1997; Williams, 2007; Xili et al., 1992). We also note that, since December 2008, over forty GRAS notifications have been submitted to FDA for highly purified stevia-derived sweetener products, and FDA 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. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, Blue California and the Expert Panel believe that a wide consensus does exist in the scientific community to support a

GRAS conclusion as evidenced by several publications (Brusick, 2008; Carakostas, 2012; Geuns, 2007a; Urban et al., 2013; Waddell, 2011; Williams, 2007) that refute safety concerns expressed by a limited number of individuals. Furthermore, FDA has reviewed 45 notifications regarding purified steviol glycosides preparations that yielded "no questions" letters, and these actions further support a scientific consensus of safety for steviol glycosides.

E. Conclusion

In consideration of the aggregate safety information available on naturally occurring steviol glycosides, Blue California and the designated Expert Panel conclude that the purified Reb D preparation (≥ 95% total steviol glycosides) 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™ Reb D 95% 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 estimated intakes of Blue California's BESTEVIA™ Reb D 95% preparation for several population groups summarized in Table 3 are no greater than 1.38 mg per kg steviol equivalents per bw per day, which is well below the ADI of 4 mg per kg bw expressed as steviol equivalents as established by JECFA. The Panel agrees that the dietary levels from anticipated food consumption will not exceed the ADI when purified steviol glycosides (≥95% total steviol glycosides) are used as a general non-nutritive sweetener.

Blue California also finds that the minimum ≥ 95% purity specification for Blue California's BESTEVIA™ Reb D 95% preparation is sufficient in view of the accepted JECFA specification for 95% purity for naturally occurring steviol glycosides. The Panel concludes that Blue California's BESTEVIA™ Reb D 95% preparation (≥ 95% total steviol glycosides), as manufactured by Blue California, is an appropriate food grade ingredient and that adverse pharmacological effects are not likely to occur at this designated ADI level. Furthermore, even high consumers of steviol glycosides are unlikely to exceed this specified ADI. Therefore, Blue California and the Expert Panel conclude that Blue California's BESTEVIA™ Reb D 95% preparation (≥ 95% total steviol glycosides), when consumed in foods as described within this GRAS notification, is generally recognized as safe within the meaning of the Food, Drug, and Cosmetic Act.

Blue California's BESTEVIA™ Reb D 95% preparation (≥ 95% total steviol glycosides), when produced in accordance with FDA Good Manufacturing Practices requirements and when meeting at a minimum the JECFA purity specifications for steviol glycosides and those specifications presented by Blue California in Table 1, is Generally Recognized As Safe when consumed as a non-nutritive sweetener within the JECFA ADI of 4 mg/kg bw/day. In order to remain within the designated ADI, it is important to observe good manufacturing practices principles in that the quantity of a substance added to food should not exceed the amount reasonably required to accomplish its intended technical effect.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

(b) (6)

Richard Kraska, Ph.D., DABT

Chair

(b) (6)

Robert S. McQuate, Ph.D.

(b) (6)

Katrina V. Emmel, Ph.D.

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

A. List of Acronyms

ADI	Acceptable Daily Intake
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AUC	Area under the plasma-concentration time curve

AVA	Agri-Food & Veterinary Authority
BMI	Body Mass Index
BP	Blood pressure
bw	Body weight
CAS	Chemical Abstract Service
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
cGMP	Current good manufacturing practices
C_{max}	Maximum (or peak) serum concentration of a substance is observed
Co	Company
CO_2	Carbon dioxide
CSAF	Chemical-specific adjustment factor
DABT	Diplomat of the American Board of Toxicology
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EMSGP	Enzyme modified steviol glycosides preparation
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FD&C	Federal Food, Drug, and Cosmetic Act
FEMA	Flavor and Extract Manufacturers Association
FOIA	Freedom of Information Act
FSANZ	Food Standards Australia New Zealand

FSSAI	Food Safety and Standards Authority of India
g	Gram
GA	GRAS Associates
GGT	Gamma-glutamyltransferase
GI	Gastrointestinal
GMP	Good Manufacturing Process
GPT	Glutamic-pyruvate transaminase
GRAS	Generally Recognized as Safe
GRN	GRAS Notification
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
Hg	Mercury
HPLC	High Performance Liquid Chromatography
h	Hour
HR	Heart rate
IADSA	International Alliance of Dietary/Food Supplement Associations
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
L	Liter
LD_{50}	Lethal Dose, 50%
LDL	Low-density lipoprotein
LLC	Limited Liability Corporation
Ltd.	Limited
MAP	Mean arterial pressure
mg	Milligram
min.	Minimum

mL	Milliliter
mm	Millimeter
MPL	Maximum permitted level
n	Number
NA	Not applicable
ND	Not detected
NHANES	National Health and Nutrition Examination Survey
NHPs	Natural Health Products
NMT	Not more than
No.	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No observed effect level
NS	Not Specified
OD	Optical density
PCV	Packed cell volume
Ph.D.	Doctor of Philosophy
PND	postnatal day
ppm	parts per million
RBC	Red blood cells
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	Scientific Committee for Food
SUS	Sucrose synthases
t	Time
TAC	Total antioxidant capacity
t.d.s.	Total dissolved solids
TFC	Total flavonoid content
TK	Toxicokinetic
T_{max}	Time at which maximum (peak) plasma concentration (C_{max}) of a substance is observed
TPC	Total phenolic content
TRAP	Tartrate-resistant alkaline phosphatase
UDP	Uridine diphosphate
UDPG	Uridine diphosphate-glucose
μg	Microgram
UGT-A	Uridine 5'-diphosphouridine-glucosyltransferase
US	United States
UK	United Kingdom
UV	Ultraviolet
VLDL	Very low density lipoprotein
WBC	White blood cells
WHO	World Health Organization

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C. Appendices

Appendix 1List of Scientific Publications Regarding the Synthesis of Rebaudiside D

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 200 (2016) 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).

6. WO2013/176738A1. High-purity steviol glycosides. Purecircle and coca-cola company. (Identify UGTs involve in biosynthesis of D and X (M)).

7. WO 2015/007748A1. Diterpene production. DSM. (De novo biosynthesis of Reb M).

Appendix 2 Steviol Glycosides Raw Material Flow Chart and Specifications

Appendix 2.1 Steviol Glycosides Raw Material Flow Chart

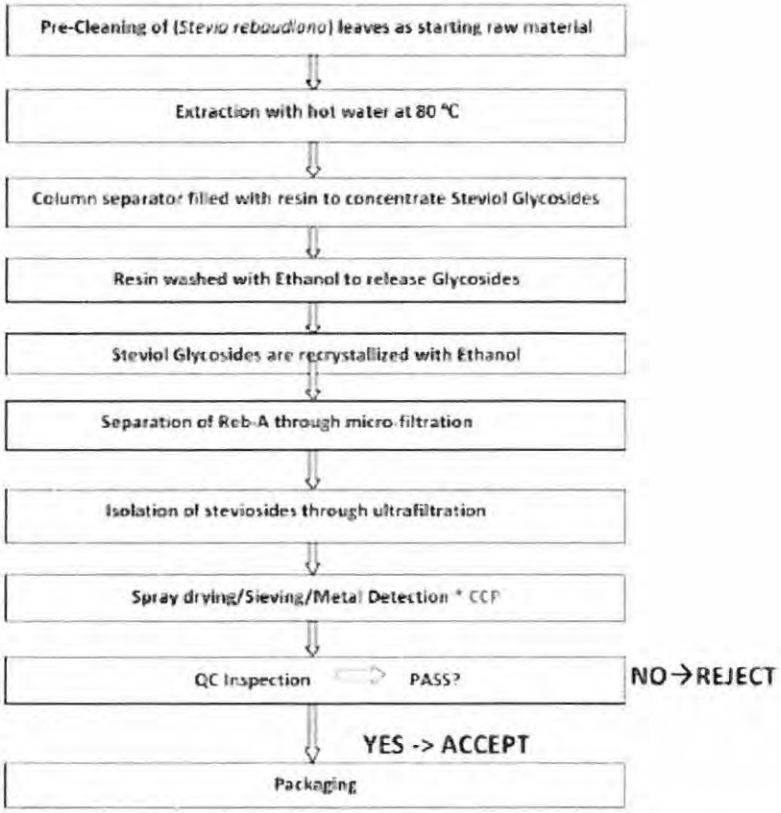
Appendix 2.2 Steviol Glycosides Raw Material Specifications

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.

Corporate Headquarters
30111 Tomas, Rancho Santa Margarita, CA 92688 Tel: 949-635-1990 Fax: 949-635-1984
Website: www.bluecal ingredients.com

Appendix 2.2 Steviol Glycosides Raw Material Specifications

Blue California

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**	**Extraction Solvent:**	**Not Applicable**
Grade:	**68th JECFA 2007**	**Shelf life:**	**2 Years**

ATTRIBUTES	SPECIFICATION	METHODS
APPEARANCE	WHITE POWDER	VISUAL
FOREIGN MATTER	ABSENT	VISUAL
ODOR	CHARACTERISTIC	OLFACTORY
TASTE	CHARACTERISTIC	GUSTATORY
STEVIOL GLYCOSIDES	**≥ 95%**	**HPLC**
SOLUBILITY IN WATER	**Excellent**	**USP**
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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Appendix 3 Regulatory Status of Raw Materials and Processing Aids Used in the Manufacture of BESTEVIA™ Reb D 95%

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 Method of Analysis for BESTEVIA™ Reb D 95%

eurofins

METHOD

Title	Number	Effective Date
HPLC Determination of Rebaudioside A and Related Steviol Glycosides – modified JECFA 2010 KK262	LC-KK262.02	10/20/2014 Replaces: LC-KK262.01

Purpose

This method is for the determination of rebaudioside A (Reb A), stevioside, dulcoside A, steviolbioside, rebaudioside C (Reb C), rebaudioside D (Reb D), rebaudioside F (Reb F), rubusoside and rebaudioside B (Reb B) by high performance liquid chromatography (HPLC). Quantitation of stevioside, dulcoside A, steviolbioside, Reb C, Reb D, Reb F, Rubusoside and Reb B are based on the response of stevioside reference material using the JECFA established conversion factors. The method is applicable for the determination of steviol glycosides in raw materials (purities) and finished products.

Definitions

N/A

Responsibility

Senior operations will implement this method. Only properly trained personnel may perform this method. The revision of or any deviation from this method requires written approval of supervisory personnel prior to initiation of work.

Safety

Follow all applicable safety, health, and environmental programs.

Environmental Conditions

N/A

Equipment

HPLC, Agilent 1100 HPLC or equivalent
Column, Phenomenex Luna C18, 4.6 x 250 mm, 5 micron, or equivalent
Analytical balance, 0.00001 g resolution
Microbalance, 0.000001 g resolution
pH Meter
Sonicator
Serological pipets
Class A pipettes, various sizes
Disposable glass pipets, various sizes
0.45-µm PTFE filter

Approved by:

Quality Control Supervisor	Date	Quality Manager	Date
(b) (6)	29 Sep 2014	(b) (6)	29 SEP 2014

Graduated cylinder, 1000-mL
Glass eluent bottles, 1000-mL
VOA vials, 20-mL and 40-mL
Amber autosampler vials
Disposable syringes, 5-mL

Reference Materials/Reagents

Rebaudioside A (Reb A), ChromaDex, or equivalent
Stevioside, USP reference material, or equivalent
Sodium Phosphate monobasic (NaH_2PO_4), minimum 99.0%
Phosphoric acid (H_3PO_4), HPLC grade
Acetonitrile, HPLC Grade
Milli-Q water, fresh daily

Quality Control Plan

1. A preparation solvent blank must be free from interfering peaks, and is analyzed at the start of each run, and additionally throughout the run, if deemed necessary based on the sample set.
2. When performing finished product testing, linearity must be demonstrated by a 3-point calibration of the reference material or other means. Correlation coefficient of the reference material curve must be greater than 0.999.
3. When performing a purity test for raw materials, five replicate injections of the reference material preparation are compared to ascertain precision, and for calibration purposes. The %RSD of five replicate injections at the start of the run must be less than 1.5.
4. Re-inject the reference material preparation after every five sample injections, and at the end of the run. The response of the reference material injections must agree within ±5% of the average of the initial reference material injections. Any portion which does not meet this criterion is rejected and must follow OOS procedures.
5. Every tenth sample in a set must be prepared and analyzed in duplicate. If the set is fewer than ten samples, one sample in the set must be run in duplicate. The percent difference between duplicate results must be less than ten for finished products and less than two for purity samples.
6. A laboratory control sample of known concentration of steviol glycosides, is to be analyzed with each run. Control charting is performed according to SOP QUA-003, Quality Control, Control Charting and Proficiency Testing.
7. If expected levels or specifications have been provided, the sample area count must fall within the area counts of the reference material curve.

Procedure

Preparation Solvent Preparation:

30% acetonitrile: 70% Milli-Q water

1. Using a graduated cylinder, measure 700 mL of Milli-Q water and transfer to a fresh 1000-mL glass eluent bottle.
2. Using a graduated cylinder, measure 300 mL of acetonitrile, and transfer to the eluent bottle.
3. Swirl to mix and label appropriately.

Note: This solution can be stored at 1-8°C for up to three months.

Buffer Preparation:

10mmol/L sodium phosphate buffer, pH 2.6

1. Using a graduated cylinder, measure 1000 mL of Milli-Q water and transfer to a fresh 1000-mL glass eluent bottle.
2. Using an analytical balance, accurately weigh 1.20 ± 0.05 g of sodium phosphate monobasic and transfer to the eluent bottle.
3. Sonicate for 15 ± 5 minutes to dissolve.
4. Using a pH meter, adjust the pH to 2.6 ± 0.05 with phosphoric acid.
5. Swirl to mix and label appropriately.
6. Use this buffer solution for the mobile phase preparation.

Note: This buffer solution may be stored at 1-8°C for up to two weeks.

Mobile Phase Preparation:

32:68 mixture of acetonitrile and 10mmol/L sodium phosphate buffer

1. Using a graduated cylinder, measure 1000 mL of the 10 mmol/L sodium phosphate buffer, pH 2.6, and transfer to a fresh 2000-mL eluent bottle.
2. Using a graduated cylinder, measure 470 mL of acetonitrile, and transfer to the eluent bottle.
3. Swirl to mix and label appropriately.

Note: This solution may be stored at 1-8°C for up to three months.

Reference Material Preparation:

1. Using a commercially available reference material, dry the stevioside reference material at 105 ± 1°C for two hours. After drying, allow the reference material to cool to room temperature.
2. On a microbalance, accurately weigh 5.0 ± 0.5 mg of each of the rebaudioside A and the dried stevioside reference materials, and transfer to separate 20-ml VOA vials.
3. Dilute with 5.0 mL of preparation solvent via a 5.0-mL class A volumetric pipette.
4. Sonicate for 15 ± 5 minutes to dissolve.
5. If warming during sonication has occurred, allow the solution to cool to room temperature.
6. Transfer the reference material solution to an amber autosampler vial and cap.

Note: All raw material (purity) samples need to be analyzed against a one-point calibration curve.

Title: HPLC Determination of Rebaudioside A and Related Steviol Glycosides- Modifed JECFA 2010 - KK262
Number: LC-KK262.02

Note: Correct the reference material for purity using the following calculation:

$$[\text{reference material}_{mg/mL}]_{corrected} = \frac{[\text{reference material}_{mg/mL}] \times \%\ \text{purity}}{100\%}$$

Raw Material Sample Preparation:

1. Thinly distribute approximately 1-2 g of sample in a suitably sized weigh boat and allow to sit on a workbench, to equilibrate with atmospheric moisture, for no less than 15 hours. Record the start and stop times.
2. Sample size should be based on client specifications or estimates, and prepared according to the calibration reference material levels. Accurately weigh 50.0 ± 5 mg into a 40-mL VOA vial.
3. Dilute with 40 mL of preparation solvent via a 40.0-mL class A volumetric pipette.
4. Sonicate for 30 ± 5 minutes.
5. If warming during sonication has occurred, allow the solution to cool to room temperature.
6. Filter through a 0.45-µm PTFE filter into an amber autosampler vial, cap and analyze.

Note: Ensure that the equilibrated sample is analyzed for water content using Karl Fischer titration, on the **same day** as the HPLC analysis. The water content obtained from the Karl Fischer titration must be used to calculate the dry-weight basis result of the sample.

General Sample Preparation:

1. Sample size should be based on client specifications or estimates and prepared according to the calibration reference material levels. Weigh an accurate amount into a 40-mL VOA vial.
2. Dilute with 40 mL of preparation solvent via a 40.0-mL class A volumetric pipette.
3. Sonicate for 30 ± 5 minutes.
4. If warming during sonication has occurred, allow the solution to cool to room temperature.
5. Filter through a 0.45-µm PTFE filter into an amber autosampler vial, cap and analyze.

Instrument Conditions:

Column Temperature:	40°C		
Detection:	UV 210 nm		
Flow Rate:	1.0 mL/minute		
Injection Volume:	5.0 µL, (may be modified for calibration purposes)		
Isocratic Run:	100% Mobile Phase		
Run Time:	30 minutes		
Retention Times:	rebaudioside D	~3.1	minutes
	rebaudioside A	~6.2	minutes
	stevioside	~6.6	minutes
	rebaudioside F	~7.9	minutes
	rebaudioside C	~8.6	minutes
	dulcoside A	~9.4	minutes
	rubusoside	~12.7	minutes
	rebaudioside B	~17.8	minutes
	steviolbioside	~19.3	minutes

Calculations

Determine the peak areas of rebaudioside A in the reference material chromatograms. Quantitate the other major steviol glycosides based on the average response of the stevioside reference material. The concentrations of the following steviol glycosides are calculated using the following conversion factors:

Compound	Molecular Weight	Conversion stevioside to listed compound
rebaudioside D	1129.15	1.40
rebaudioside F	936.99	1.16
rebaudioside C	951.01	1.18
dulcoside A	788.87	0.98
rubusoside	642.73	0.80
rebaudioside B	804.88	1.00
steviolbioside	642.73	0.80

$$\% \text{ glycoside} = \frac{\text{area (sample)} \times [\text{reference material}] \times 100\%}{\text{area (reference material)} \times [\text{sample}]}$$

where,

[ ] reference material/sample is in mg/ml..

Method Uncertainty

The method uncertainty for this method was obtained during in-house validation, and was calculated using the equation, MU = k × standard deviation, where, k is the coverage factor (k= 2 for 95% confidence). Results for typical samples are expressed in the following table:

Sample Type/Description:	Method Uncertainty:
98% steviol glycosides purity product	± 2.62 %wt/wt
90% steviol glycosides purity product	± 1.03 %wt/wt
90% steviol glycosides purity product	± 0.894 %wt/wt
90% steviol glycosides purity product	± 1.22 %wt/wt

With all conditions being ideal during the validation process, it is often more useful to view laboratory control sample data, which better takes into consideration the range of effects operating during normal use of the method.

References

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

Revision History

Effective Date	Version	Pages Affected	Reason/Summary of Changes
10/20/2014	02	2	Highlighted areas. Changed quality control requirements 2 and 3 to reflect raw material/purity samples and finished products.

Appendix 5 Certificates of Analysis for Multiple Batches of BESTEVIA™ Reb D 95%

Appendix 5.1 BESTEVIA™ Reb D 95% Batch D195-160113

Appendix 5.2 BESTEVIA™ Reb D 95% Batch D195-160126

Appendix 5.3 BESTEVIA™ Reb D 95% Batch D195-160265

Appendix 5.4 BESTEVIA™ Reb D 95% Batch D195-160324

Appendix 5.5 BESTEVIA™ Reb D 95% Batch D195-160425

Appendix 5.1 BESTEVIA™ Reb D 95% Batch D195-160113

Blue California

30111 Tomas
Rancho Santa Margarita, CA 92688
Tel 949.635.1990
Fax 949.635.1908

CERTIFICATE OF ANALYSIS

Product: BESTEVIA ™ Rebaudioside D 95%
Item# BE17073D1

Lot No: (b) (6) **Original Manufacturer:** Blue California Co.
Date of Manufacturing: January 07-2016 **Expiration/Re-test date:** January 07-2018
QC acceptance date: January 15-2016 **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
REBAUDIOSIDE D	**≥ 95%**	**HPLC**	**97.4%**
LOSS ON DRYING	≤ 5%	USP 34	0.53%
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	< 0.25 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 20 ppm
METHANOL	< 200 ppm	USP 34	< 50 ppm
PH	5-7	USP 34	6.05
ASH	≤ 1%	USP 34	0.12%
SOLUBILITY	IN WATER	USP 34	VERY SLIGHTLY SOLUBLE
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	< 3,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	N/D
SALMONELLA	NEGATIVE	AOAC	N/D
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 06-09-2017

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Appendix 5.2 BESTEVIA™ Reb D 95% Batch D195-160126

Blue California

30111 Tomas
Rancho Santa Margarita, CA 92688
Tel 949.635.1990
Fax 949.635.1998

CERTIFICATE OF ANALYSIS

Product: BESTEVIA ™ Rebaudioside D 95%
Item# BE17073D1

Lot No:	(b) (6)	**Original Manufacturer:**	**Blue California Co.**
Date of Manufacturing:	**January 26-2016**	**Expiration/Re-test date:**	**January 26-2018**
QC acceptance date:	**February 05-2016**	**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
REBAUDIOSIDE D	**≥ 95%**	**HPLC**	**96.2%**
LOSS ON DRYING	≤ 5%	USP 34	0.85%
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	< 0.25 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 20 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
PH	5-7	USP 34	5.95
ASH	≤ 1%	USP 34	0.132%
SOLUBILITY	IN WATER	USP 34	VERY SLIGHTLY SOLUBLE
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	< 3,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	N/D
SALMONELLA	NEGATIVE	AOAC	N/D
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 06-09-2017

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Appendix 5.3 BESTEVIA™ Reb D 95% Batch D195-160265

Blue California®

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

CERTIFICATE OF ANALYSIS

Product: BESTEVIA ™ Rebaudioside D 95%
Item# BE17073D1

Lot No: (b) (6) **Original Manufacturer: Blue California Co.**
Date of Manufacturing: January 27-2016 **Expiration/Re-test date: January 27-2018**
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
REBAUDIOSIDE D	**≥ 95%**	**HPLC**	**96.1%**
LOSS ON DRYING	≤ 5%	USP 34	0.86%
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	< 0.25 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 20 ppm
METHANOL	< 200 ppm	USP 34	< 100 ppm
PH	5-7	USP 34	5.95
ASH	≤ 1%	USP 34	0.157%
SOLUBILITY	IN WATER	USP 34	VERY SLIGHTLY SOLUBLE
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	< 3,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	N/D
SALMONELLA	NEGATIVE	AOAC	N/D
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 06-09-2017

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Appendix 5.4 BESTEVIA™ Reb D 95% Batch D195-160324

Blue California

Rancho Santa Margarita, CA 92688
Tel: 949.635.1990
Fax: 949.635.1980

CERTIFICATE OF ANALYSIS

Product: BESTEVIA ™ Rebaudioside D 95%
Item# BE17073D1

Lot No: (b) (6)
Original Manufacturer: Blue California Co.
Date of Manufacturing: March 24-2016
Expiration/Re-test date: March 24-2018
QC acceptance date: April 22-2016
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
REBAUDIOSIDE D	**≥ 95%**	**HPLC**	**97.2%**
LOSS ON DRYING	≤ 5%	USP 34	0.55%
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	< 0.25 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 20 ppm
METHANOL	< 200 ppm	USP 34	< 50 ppm
PH	5-7	USP 34	5.95
ASH	≤ 1%	USP 34	0.114%
SOLUBILITY	IN WATER	USP 34	VERY SLIGHTLY SOLUBLE
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	< 3,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI.	NEGATIVE	AOAC	N/D
SALMONELLA	NEGATIVE	AOAC	N/D
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 06-09-2017

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Appendix 5.5 BESTEVIA™ Reb D 95% Batch D195-160425

Blue California®

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

CERTIFICATE OF ANALYSIS

Product: BESTEVIA ™Rebaudioside D 95%
Item# BE17073D1

Lot No: (b) (6) **Original Manufacturer:** Blue California Co.
Date of Manufacturing: April 22-2016 **Expiration/Re-test date:** April 22-2018
QC acceptance date: May 05-2016 **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
REBAUDIOSIDE D	**≥ 95%**	**HPLC**	**96.8%**
LOSS ON DRYING	≤ 5%	USP 34	0.70%
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	< 0.25 ppm
MERCURY	< 0.5 ppm	ICP-MS	< 0.1 ppm
ETHANOL	< 1,000 ppm	USP 34	< 20 ppm
METHANOL	< 200 ppm	USP 34	< 80 ppm
PH	5-7	USP 34	6.05
ASH	≤ 1%	USP 34	0.071%
SOLUBILITY	IN WATER	USP 34	VERY SLIGHTLY SOLUBLE
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	< 3,000 cfu/gm	AOAC	< 1,000 cfu/gm
TOTAL COLIFORM	< 100 cfu/gm	AOAC	< 3 cfu/gm
YEAST AND MOLDS	< 100 cfu/gm	AOAC	< 50 cfu/gm
E. COLI:	NEGATIVE	AOAC	N/D
SALMONELLA	NEGATIVE	AOAC	N/D
SHELF LIFE	2 YEARS	HPLC	PASS

Approved by: J.H.Zhou (QC Manager) Revised date: 06-09-2017

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Appendix 6 Analytical Chromatograms for Multiple Production Batches of Rebaudioside D

Appendix 6.1 BESTEVIA™ Reb D 95% Batch D195-160113

Appendix 6.2 BESTEVIA™ Reb D 95% Batch D195-160126

Appendix 6.3 BESTEVIA™ Reb D 95% Batch D195-160265

Appendix 6.4 BESTEVIA™ Reb D 95% Batch D195-160324

Appendix 6.5 BESTEVIA™ Reb D 95% Batch D195-160425

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

(1) Eurofins sample 740-2016-12462, BESTEVIA-D, Powder, Lot (b) (6)

(2) Eurofins sample 740-2016-12463, BESTEVIA-D, Powder, Lot (b) (6)

(3) Eurofins sample 740-2016-12464, BESTEVIA-D, Powder, Lot (b) (6)

(4) Eurofins sample 740-2016-12465, BESTEVIA-D, Powder, Lot (b) (6)

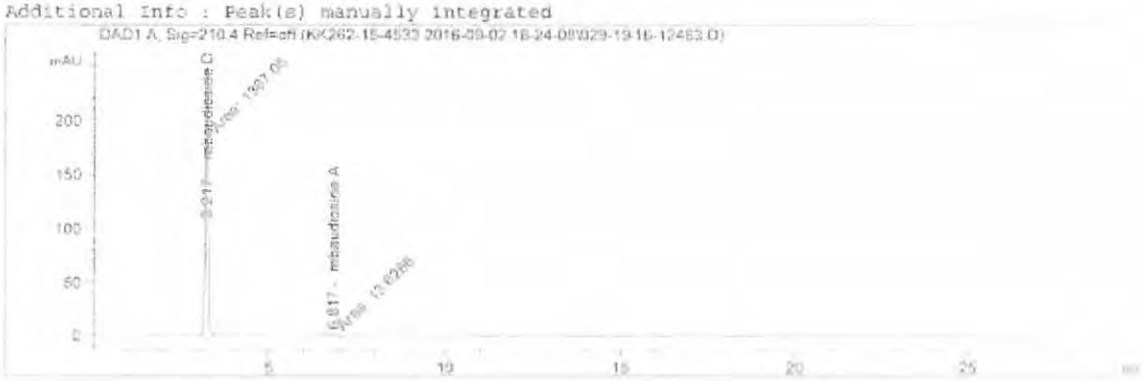
(5) Eurofins sample 740-2016-12466, BESTEVIA-D, Powder, Lot (b) (6)

Appendix 6.1 BESTEVIA™ Reb D 95% Batch D195-160113

```
Data File G:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\029-19-16 12463 D
Sample Name: 16-12463

------------------------------------------------------------------------
Acq. Operator    : Gombu Sherpa                    Seq. Line :  29
Acq. Instrument  : HPLC-05                          Location :  19
Injection Date   : 9/3/2016 10:01:40 AM               Inj :  1
                                                  Inj Volume : 5.000 µl
Acq. Method      : D:\Chem32\4\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M
Last changed     : 9/3/2016 7:53:12 AM by Gombu Sherpa
Analysis Method  : d:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M (Sequence
                   Method)
Last changed     : 9/22/2016 4:39:26 PM by Jeff Lin
Method Info      : JECFA kk262

ECM Server       : http://usc5aprvp001/ecmwg
ECM Operator     : Jeff Lin
ECM Path         : \Petaluma\LC\HPLC-05\Data\KK262-15-4533 2016-09-02 18-24-08 SC SS1zip
ECM Version      : 14 (modified after loading)
Additional Info  : Peak(s) manually integrated
```




```
------------------------------------------------------------------------
                        ESTD Percent Report
------------------------------------------------------------------------

Sorted By                :      Signal
Calib. Data Modified     :      Thursday, September 22, 2016 4:20:22 PM
Multiplier               :      1.0505
Dilution                 :      40.0000
Sample Amount:           :      51.94000  [mg/ml
Do not use Multiplier & Dilution Factor with ISTDs

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

RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [mAU*s]                   %
-------|------|----------|----------|----------|--|-----------------
  3.217 MF     1367.05078  9.14482e-4  1.39733     rebaudioside D
  6.817 MM       13.62662  8.14363e-4  0.897776    rebaudioside A
  7.102            -           -          -        stevioside
  8.561            -           -          -        rebaudioside F
  9.425            -           -          -        rebaudioside C
 10.304            -           -          -        dulcoside A
 13.605            -           -          -        rubusoside
 18.970            -           -          -        rebaudioside B
 20.431            -           -          -        steviolbioside

DA-11 9/22/2016 4:54:28 PM Jeff Lin                          Page  1 of 2
```

Data File d:\Chem32\6\Data\RR262-15-453 2016-09-02 18-24-05\023-19-16-12463.D
Sample Name: 1E-12-E1

Totals : 102.037515

1 Warnings or Errors:

Warning : Calibrated compound(s) not found

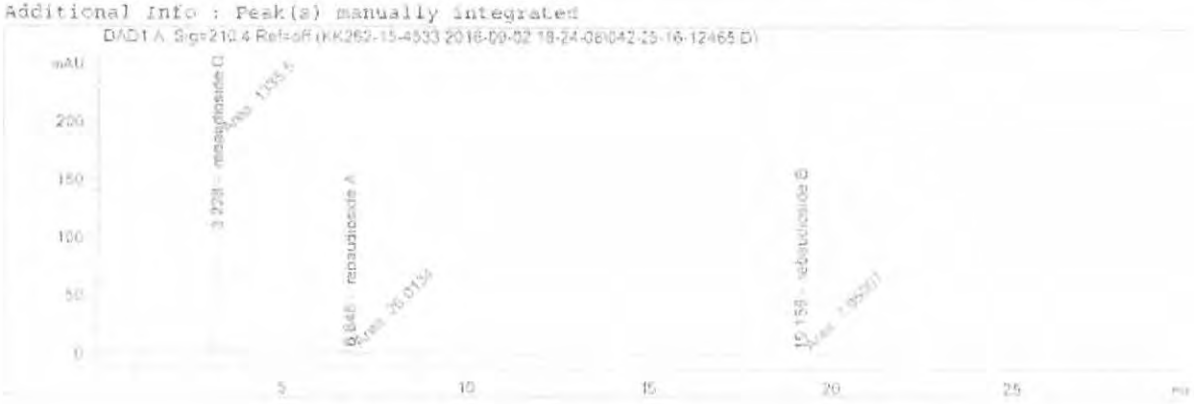
*** End of Report ***

DA-11 9/22/2016 4:54:26 PM Jeff Lin Page 2 of 2

Appendix 6.2 BESTEVIA™ Reb D 95% Batch D195-160126

Data File D:\Chem32\3\Data\KK262-15-4533 2016-09-02 18-24-08\042-25-16-12465.D
Sample Name: 16-12465

Acq. Operator	: Gombu Sherpa	Seq. Line :	42
Acq. Instrument	: HPLC-05	Location :	25
Injection Date	: 9/3/2016 4:52:53 PM	Inj :	1
		Inj Volume :	5.000 µl
Acq. Method	: D:\Chem32\4\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M		
Last changed	: 9/3/2016 7:53:12 AM by Gombu Sherpa		
Analysis Method	: d:\Chem32\3\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M (Sequence Method)		
Last changed	: 9/23/2016 5:13:47 PM by Hong You		
Method Info	: JECFA KK262		
ECM Server	: http://us05apvp001/ecmwg		
ECM Operator	: Hong You		
ECM Path	: \Petaluma\LC\HPLC-05\Data\KK262-15-4533 2016-09-02 18-24-08.SC.SSIzip		
ECM Version	: 16 (modified after loading)		
Additional Info	: Peak(s) manually integrated		



ESTD Percent Report

Sorted By	: Signal
Calib. Data Modified	: Thursday, September 22, 2016 4:20:24 PM
Multiplier	: 1.0510
Dilution	: 40.0000
Sample Amount:	: 52.26000 [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
3.228	PM	1335.49841	9.14482e-4	98.248873		rebaudioside D
6.848	MM	26.01337	8.24363e-4	1.704211		rebaudioside A
7.102		-	-	-		stevioside
8.591		-	-	-		rebaudioside F
9.425		-	-	-		rebaudioside C
10.304		-	-	-		dulcoside A
13.605		-	-	-		rubusoside
15.158	MM	7.95607	8.51201e-4	0.417760		rebaudioside B
20.421		-	-	-		steviolbioside

DR-1[Rev 7/23/2015 5:22:10 PM Hong You] Page 1 of 2

```
Data File D:\Chem32\3\Data\85262-15-4533 2016-05-02 18-24-08\042-25-16-12465.D
Sample Name: 16-12465

    Totals :                                      100.370846

    1 Warnings or Errors :

    Warning : Calibrated compound(s) not found

    ------------------------------------------------------------------------
                              *** End of Report ***
```

Appendix 6.3 BESTEVIA™ Reb D 95% Batch D195-160265

Data File d:\Chem32\3\Data\KK262-15-4533 2016-09-02 18-24-08\048-20-16-12466.D
Sample Name: 16-12366

Acq. Operator	: Gombu Sherpa	Seq. Line	: 48
Acq. Instrument	: HPLC-05	Location	: 28
Injection Date	: 9/3/2016 6:02:54 PM	Inj	: 1
		Inj Volume	: 5.000 µl

Acq. Method : D:\Chem32\4\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M
Last changed : 9/3/2016 7:53:12 AM by Gombu Sherpa
Analysis Method : d:\Chem32\3\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M (Sequence Method)
Last changed : 9/23/2016 5:13:47 PM by Hong You
Method Info : JECFA kk262

ECM Server : http://us05apvp001/ecmws
ECM Operator : Hong You
ECM Path : \Petaluma\LC\HPLC-05\Data\KK262-15-4533 2016-09-02 18-24-08.RC SSIzip
ECM Version : 18 (modified after loading)
Additional Info : Peak(s) manually integrated

ESTD Percent Report

Sorted By : Signal
Calib. Data Modified : Thursday, September 22, 2016 4:20:23 PM
Multiplier : 1.0518
Dilution : 40.0000
Sample Amount: 51.83000 [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
3.227	PM	1332.78674	9.14482e-4	98.939709		rebaudioside D
6.849	MM	25.60079	8.34363e-4	1.692379		rebaudioside A
7.102		-	-	-		stevioside
8.581		-	-	-		rebaudioside F
9.425		-	-	-		rebaudioside C
10.304		-	-	-		dulcoside A
13.605		-	-	-		rubusoside
10.135	MM	7.43192	6.53201e-4	0.396177		rebaudioside B
20.421		-	-	-		steviolbioside

DA-11Nov 9/23/2016 5:29:31 PM Hong You Page 1 of 2

```
Data File d:\chem32\3\Data\[illegible] 2016-09-02 [illegible]\[illegible]-16-12466.D
Sample Name: 16-12466

    Totals :                              101.[illegible]

    1 Warnings or Errors :

    Warning : Calibrated compound(s) not found

    ===================================================================
                        *** End of Report ***
```

Appendix 6.4 BESTEVIA™ Reb D 95% Batch D195-160324

Data File d:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\035-22-16-12464.D
Sample Name: 16-12464

Acq. Operator	: Gombu Sherpa	Seq. Line	: 35
Acq. Instrument	: HPLC-05	Location	: 22
Injection Date	: 9/3/2016 3:11:26 PM	Inj	: 1
		Inj Volume	: 5.000 µl
Acq. Method	: D:\Chem32\4\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M		
Last changed	: 9/3/2016 7:53:12 AM by Gombu Sherpa		
Analysis Method	: d:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M (Sequence Method)		
Last changed	: 9/22/2016 4:39:26 PM by Jeff Lin		
Method Info	: JECFA KK262		
ECM Server	: http://us05apvp001/ecmws		
ECM Operator	: Jeff Lin		
ECM Path	: \Petaluma\LC\HPLC-05\Data\KK262-15-4533 2016-09-02 18-24-08.SC SSI.zip		
ECM Version	: 14 (modified after loading)		
Additional Info	: Peak(s) manually integrated		

ESTD Percent Report

Sorted By : Signal
Calib. Data Modified : Thursday, September 22, 2016 4:20:23 PM
Multiplier : 1.0506
Dilution : 40.0000
Sample Amount: 52.44000 [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
3.223	PM	1389.73645	9.14482e-4	101.849593		rebaudioside D
6.833	MM	13.56328	8.14763e-4	0.885186		rebaudioside A
7.102		-	-	-		stevioside
8.581		-	-	-		rebaudioside F
9.425		-	-	-		rebaudioside C
10.304		-	-	-		dulcoside A
13.605		-	-	-		rubusoside
18.970		-	-	-		rebaudioside B
20.421		-	-	-		steviolbioside

DA-14 9/22/2016 4:54:47 PM Jeff Lin Page 3 of 4

Data File d:\Chem32\6\Data\RS262-15-4533 2016-05-02 18-24-08\035-32-16-12464.D
Sample Name: 16-12464

Totals : 102 736779

1 Warnings or Errors :

Warning : Calibrated compound(s) not found

*** End of Report ***

DA-11 9/22/2016 4:56:47 PM Jeff Lin Page: 2 of 2

Appendix 6.5 BESTEVIA™ Reb D 95% Batch D195-160425

```
Data File d:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\025-6-15-13221.D
Sample Name: 16-12467

================================================================
Acq. Operator    : Gombu Sherpa                  Seq. Line    : 26
Acq. Instrument  : HPLC-05                       Location     : 18
Injection Date   : 9/3/2016 8:26:22 AM           Inj          : 1
                                                 Inj Volume   : 5.000 µl
Acq. Method      : D:\Chem32\4\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M
Last changed     : 9/3/2016 7:53:12 AM by Gombu Sherpa
Analysis Method  : d:\Chem32\6\Data\KK262-15-4533 2016-09-02 18-24-08\LCKK262.M (Sequence
                   Method)
Last changed     : 9/22/2016 4:39:26 PM by Jeff Lin
Method Info      : JECFA kk262

ECM Server       : http://us05apvp001/ecmwg
ECM Operator     : Jeff Lin
ECM Path         : \Petaluma\LC\HPLC-05\Data\KK262-15-4533 2016-09-02 18-24-08.SC.SS1zip
ECM Version      : 14 (modified after loading)
Additional Info  : Peak(s) manually integrated
```


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

Sorted By             :      Signal
Calib. Data Modified  :      Thursday, September 22, 2016 4:20:23 PM
Multiplier            :      1.0507
Dilution              :      40.0000
Sample Amount         :      52.28000  [mg/ml]
Do not use Multiplier & Dilution Factor with ISTDs

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

RetTime  Type     Area      Amt/Area    Amount    Grp   Name
 [min]          [mAU*s]                   %
-------|------|----------|----------|----------|---|------------
  3.209 PM     1347.17761  9.14482e-4 93.037928     rebaudioside D
  6.793 MM       19.63562  8.14363e-4  1.285477     rebaudioside A
  7.102            -           -          -         stevioside
  8.581            -           -          -         rebaudioside F
  9.425                                  -         rebaudioside C
 10.304                                  -         dulcoside A
 13.605                                  -         rubusoside
 18.970                                  -         rebaudioside B
 20.421                                  -         steviolbioside

DA-13 9/22/2016 4:54:03 PM Jeff Lin                          Page  1 of 2
```

Data File d:\Chem32\6\Data\KX282 15-4533 2016-09-02 16-24-06\025-8-15-13221.D
Sample Name: 16-12463

Totals : 100.323405

1 Warnings or Errors :

Warning : Calibrated compound(s) not found

*** End of Report ***

DA-11 9/22/2016 4:54:03 PM Jeff Lin Page 2 of 2

Appendix 7 Pesticide Testing Report for Rebaudioside D

eurofins | Supplement Analysis Center

Eurofins Scientific Inc
Supplement Analysis Center
1365 Redwood Way
Petaluma, CA 94954
Tel.+1 707 792 7300
Fax.+1 707 792 7309

August 25, 2016

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

CERTIFICATE OF ANALYSIS
AR-16-KK-012703-04
Batch #: EUCAPE-00083509
This analytical report supersedes AR-16-KK-012703-03.

Sample Identification:
Sample #: 740-2016-00012463
Description: BESTEVIA-D, Powder, Lot # (b) (6)
Condition: White powder in a double ziplock bag received at room temperature
Date Received: August 12, 2016

QA12C: Pesticides - USP 561 Screen (USP 39)
Method Reference: USP 561 (Modified)
Completed: 08/24/2016

	Result	Theoretical Level
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	

eurofins Sample #: 740-2016-00012463

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Rancho Santa Margarita, CA
92688

QA12C: Pesticides - USP 561 Screen (USP 39)
Method Reference: USP 561 (Modified)
Completed: 08/24/2016

	Result	Theoretical Level
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: CVUA Stuttgart 2008 GC-MS
Completed: 08/24/2016

	Result	Theoretical Level
Bromide *[Method performed by an outsource lab.]*	<10 mg/kg	

eurofins

Sample #: 740-2016-00012463

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Rancho Santa Margarita, CA
92688

QA602: EBDCs (Dithiocarbamates) (CS2 method, GC-MS)
Method Reference: J. Agric. Food Chem. Vol. 49 pp 2152, 2001
Completed: 08/24/2016

	Result	Theoretical Level
Total Dithiocarbamates, as CS2	0.02 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)

Brian Thomas
Lab Tech

Appendix 8 Relative Sweetness Intensity Study

SWEETNESS EQUIVALENCY OF BESTEVIA REB-D

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-D, a trademarked product produced by Blue California, is made from isolating the sweetest compound of fermentation, Rebaudioside D, 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-D (Rebaudioside D) produced by Blue California in comparison to sucrose.

TEST SAMPLES:

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

EQUIPMENT & MATERIALS:

Bestevia-D

Sucrose

Purified water

Analytical Scale

100ml 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 D 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%, 2.5%, and 5.0% sucrose in bottled water (w/v) at room temperature.
3. The steviol glycoside Reb D 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 steviol glycoside sample was evaluated against several control sucrose samples at 1.0%, 2.5%, and 5.0% by a panel of thirteen 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 majority of thirteen volunteers. The result indicates that the rebaudioside D is 202 times sweeter to sucrose.

Appendix 9 Estimated Daily Intake Levels of Steviol Glycosides

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.

A. 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 90th precentile 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.

B. 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[a] (mg steviol glycosides /kg of food)	Maximum Use Level Calculated for Rebaudioside A[b] mg rebaudioside A /kg of food	Maximum Use Level Calculated for Rebaudioside A[b] 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

[a] Reproduced from WHO (2006).

[b] 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[a]

Food Uses	Reb A (ppm)
Tabletop sweeteners	30,000[b]
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

[a] Merisant (2008)

[b] 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 overestimates 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 90th 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 90th 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[a] (mg/kg bw/day)	As Rebaudioside A[b] (mg/kg bw/day)	Total Daily Intake[c] (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[d]	120 - 282
CARGILL			
		1.3 - 3.4[d]	78 - 204

[a] 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.

[b] 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.

[c] Total daily intake figures were calculated for a 60 kg adult.

[d] 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)[a]	1.3 -3.5 (for a 60 kg person)
Japan, Per Capita	0.04
Japan, Replacement Estimate[b]	3
US, Replacement Estimate[b]	5

[a] WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

[b] 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 90th 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 Summary of Published Safety Reviews

1. Summary of JECFA Reviews

At an early review during its 51st 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 63rd 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 68th 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, 2007a; b).

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 68th 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 69th 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 95th 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 95th percentile for children ranged from 1.0 to 12.7 mg per kg bw per day. For adults, the mean and 97.5th 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 (Brahmachari et al., 2011; Brown and Rother, 2012; Chatsudthipong and Muanprasat, 2009; Yadav and Guleria, 2012). In reviewing these studies, caution is warranted since these reviews do not differentiate well between studies on crude stevia extract and purified steivol 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 steviolprior 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 11 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 (Koyama et al., 2003b; Nakayama et al., 1986; Wingard Jr et al., 1980) 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 α-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 α-glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of α-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.

In a recent publication, 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. Given the similarity in the microbial metabolism of stevioside and rebaudioside A, with the formation of steviol as the single hydrolysis product that is absorbed from the intestinal tract, these investigators concluded that the toxicological data on stevioside are relevant to the risk assessment of rebaudioside C. A summary of the mutagenicity and genotoxicity studies on Reb A is provided in Table 11-1.

Table 11-1. Mutagenicity & Genotoxicity Studies on Rebaudioside A

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Bacterial Mutagenicity	5 *Salmonella* strains with and without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1,500 and 5,000 µg per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	5 *Salmonella* strains and 1 *E. coli* strain with and without exogenous metabolic activation system	Reb A		Up to 5,000 µg per plate	No mutagenic response	Williams and Burdock (2009)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A	99.5	Cloning conc. of 500, 1,000, 2,000, 3,000, 4,000 and 5,000 µg/mL	No mutagenic or clastogenic response	Clarke (2006)
Mouse Lymphoma	L5178Y/TK+/- mouse lymphoma mutagenesis assay in the absence and presence of exogenous metabolic activation system	Reb A		Up to 5,000 µg/mL	No mutagenic or clastogenic response	Williams and Burdock (2009)
Chromosome Aberration	Chinese Hamster V79 cells	Reb A		Up to 5,000 µg/mL		Williams and Burdock (2009)

END-POINT	TEST SYSTEM	MATERIAL	PURITY (%)	CONCENTRATION / DOSE	RESULT	REFERENCE
Mouse Micronucleus	Micronucleus study consisted of 7 groups, each containing 5 male and 5 female ICR mice.	Reb A	99.5	500, 1,000 and 2,000 mg/kg bw	No increase in micronuclei formation	Krsmanovic and Huston (2006)
Mouse Micronucleus		Reb A		Up to 750 mg/kg bw	No increase in micronuclei formation	Williams and Burdock (2009)
Unscheduled DNA Synthesis	*In vivo* rat	Reb A		Up to 2,000 mg/kg bw	No increase in unscheduled DNA synthesis	Williams and Burdock (2009)
DNA damage (comet assay)	*Male BDF1 mouse stomach, colon, liver*	Stevia extract	Stevio-side, 52%; Reb A, 22%	250 – 2,000 mg/kg bw	Negative[a]	Sekihashi et al. (2002)
Chromosomal aberration	*CHL/IU Chinese hamster lung fibroblasts*	Reb A	NS	1.2 - 55 mg/mL	Negative[b]	Nakajima (2000a)
Micronucleus formation	*BDF1 mouse bone marrow*	Reb A	NS	500-2,000 mg/kg bw per day for 2 days	Negative[c]	Nakajima (2000b)
Forward mutation	*S. typhimurium* TM677	Reb A	NS	10 mg/plate	Negative[b]	Pezzuto et al. (1985)

NS = Not specified. [a] Sacrificed at 3 hours and 24 hours. [b] With or without metabolic activation (source not specified in original monograph).
[c] Sacrificed at 30 hours after 2nd administration.

2. Acute Toxicity Studies

The oral LD_{50} studies of stevioside (purity, 96%) following administration of a single dose to rodents are summarized in Table 11-2. 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 11-2. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents

Species	Sex	LD_{50} (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_0, F_1, and F_2 generations. These studies are summarized in Table 11-3. 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 11-3. 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)[a]	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.
Yodyingyuad and Bunyawong (1991)[a]	Hamster/ four groups of 20 (10 male, 10 female)	Stevioside/ 90%	0, 0.5, 1.0, 2.5 g/kg bw/day/ duration unclear/ 3 months	2,500	F_0, F_1 & F_2 generations in reproductive study dosed for 90 days. Histological examination showed no effect at any dose. Weights of organs, blood analysis, urine chemistry & gross necropsy not discussed. The F_1 & F_2 hamsters continued to receive stevioside (via drinking water for one month, then at same dose as parents).
Mitsuhashi (1976)[b]	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)[b]	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, ATL and AST.

[a] Abstract only. [b] As reported by Geuns (2003).

4. Chronic Toxicity Studies

Chronic effects of stevioside have been studied in three separate studies (Table 11-4). 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 11-4. 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	*Ad libitum* 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)[a]	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 low-dose	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 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.

[a] 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 11-5.

Table 11-5. 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)[a]	Wistar Rat/4 groups of 25 or 26 pregnant rats	95.6%[b]	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_1 & F_2 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)[a]	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 ^{3}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 group (44 total)	96%	0, 0.15, 0.75 or 3 % of feed/60 days	2,000	Males given stevioside dose in diet for 60 days before & during mating with females who received 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.
Planas and Kuć (1968)	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.

[a] Only abstract available. [b] As reported by EuropeanCommission (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 11-6. 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; c).

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 11-6. Mutagenicity & Genotoxicity Studies on Stevia Extracts & Stevioside

End-Point	Test System	Material	Purity (%)	Concentration / Dose	Result	Reference
In Vitro						
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate[a] 1 mg/plate[b]	Negative	Matsui et al. (1996)
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	99	50 mg/plate	Negative[c]	Suttajit et al. (1993)
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	NS	50 mg/plate	Negative	Klongpanichpak et al. (1997)
Forward mutation	*S. typhimurium* TM677	Stevioside	83	10 mg/plate	Negative[c]	Matsui et al. (1996)
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	10 mg/plate	Negative[c]	Pezzuto et al. (1985)
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	Not specified	Negative[c]	Medon et al. (1982)
Gene mutation	Mouse lymphoma L5178Y cells, TK- locus	Stevioside	NS	5 mg/mL	Negative[c,d]	Oh et al. (1999)
Gene mutation (umu)	*S. typhimurium* TA1535/pSK1002	Stevioside	83	5 mg/plate	Negative[c]	Matsui et al. (1996)
Gene mutation	*B. subtilis* H17 rec+, M45 rec-	Stevioside	83	10 mg/disk	Negative[c]	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[a]	Ishidate et al. (1984)
In Vivo						
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[e]	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[e]	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	*D. melanogaster* Muller 5 strain	Stevioside	NS	2% in feed	Negative	Kerr et al. (1983)

NS = Not specified. [a] Without metabolic activation. [b] As calculated by Williams (2007). [c] With and without metabolic activation (source not specified in original monograph). [d] Inadequate detail available. [e] 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 (BMI) 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 ± 2%), rebaudioside A (24 ± 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 68th 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 (t.d.s.) steviol glycoside, stevioside, or placebo treatment. The purity of the steviol glycosides was ≥ 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 68th 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 12 Summary of Studies on Steviol Glycosides Preparations That Are Primarily Rebaudioside A

Safety Data on Rebaudioside A[12]

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 (Geuns et al., 2003a; Geuns et al., 2003b; Koyama et al., 2003b).

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

[12] Questions about the safety of rebaudioside A were previously raised by Huxtable RJ (2002) Pharmacology and toxicology of stevioside, rebaudioside A, and steviol. , in *Stevia: The Genus of Stevia* (Kinghorn AD, (Ed.) ed), Taylor and Francis, Inc., NY., and Kobylewski S and Eckhert CD (2008) Toxicology of Rebaudioside A: A Review, University of California at Los Angeles.. 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 (Gardana et al., 2003; Geuns et al., 2003a; Hutapea et al., 1997; Wingard Jr et al., 1980). 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 μg 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 (Eapen, 2007; Nikiforov and Eapen, 2008). 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 ≥ 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 ≥ 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 DNA strand breakage, rebaudioside A consistently and uniformly revealed negative results (Nakajima, 2000a; b; Pezzuto et al., 1985; 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* micronuleus 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 12-1.

Table 12-1. Mutagenicity & Genotoxicity Studies on Rebaudioside A

End-Point	Test System	Material	Purity (%)	Concentration / Dose	Result	Reference
Bacterial Mutagenicity	5 *Salmonella* strains with & without exogenous metabolic activation system	Reb A	99.5	1.5, 5.0, 15, 50, 150, 500, 1,500 & 5,000 µg per plate	No mutagenic response	Wagner and Van Dyke (2006)
Bacterial Mutagenicity	4 *Salmonella* strains & 1 *E. coli* strain with & without exogenous metabolic activation system	Reb A	95.6	Up to 5,000 µg per plate	No mutagenic response	Williams and Burdock (2009)
Bacterial Mutagenicity	4 *Salmonella* strains & 1 *E. coli* strain with and without exogenous metabolic activation system	Fermentation-derived Reb A	≥ 95%	Up to 5,000 µg 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 µg/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 µg/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	≥ 95%	Up to 5,000 µg/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 µg/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 (comet assay)	Male BDF1 mouse stomach, colon, liver	Stevia extract	Stevioside, 52%;	250 – 2,000 mg/kg bw	Negative[a]	Sekihashi et al. (2002)

End-Point	Test System	Material	Purity (%)	Concentration / Dose	Result	Reference
			Reb A, 22%			
Chromosomal aberration	CHL/IU Chinese hamster lung fibroblasts	Reb A	NS	1.2 - 55 mg/mL	Negative[b]	Nakajima (2000a)
Micronucleus formation	BDF1 mouse bone marrow	Reb A	NS	500-2,000 mg/kg bw/ day for 2 days	Negative[c]	Nakajima (2000b)
Forward mutation	*S. typhimurium* TM677	Reb A	NS	10 mg/plate	Negative[b]	Pezzuto et al. (1985)

NS = Not specified.

[a] Sacrificed at 3 hours and 24 hours.

[b] With or without metabolic activation (source not specified in original monograph).

[c] 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_0 or F_1 generations. The survival and general condition of the F_1 and F_2 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; b) further support the above described findings from published studies. In a two-generation dietary reproduction study, four groups of male and female Crl: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_0 and F_1 generations, rebaudioside A doses were 0, 500, 1,000, and 2,000 mg per kg per day. At initiation of study, F_0 animals were approximately 7 weeks of age. The test diet was offered to the offspring selected to become the F_1 generation following weaning [beginning on postnatal day (PND) 21]. The F_0 and F_1 males continued to receive rebaudioside A throughout mating, continuing through the day of euthanasia. The F_0 and F_1 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 ≥ 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 ≥ 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 ~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 ± 0.06%, mean ± standard error) and placebo (0.09 ± 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 ± 3.7 mg per dL and 11.2 ± 4.5 mg per dL), insulin (1.0 ± 0.64 µU per mL and 3.3 ± 1.5 µU per mL), and Cpeptide (0.13 ± 0.09 ng per mL and 0.42 ± 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 13 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_{50} 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_{50} 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 13-1.

Table 13-1. Mutagenicity & Genotoxicity Studies on Steviol

	In Vivo/In Vitro	System	Test Sample Purity	Author Conclusion	Results and Remarks
Sekihashi et al. (2002)[a]	*In Vivo/In Vitro*	Comet Assay	Not reported	Negative	In *in vitro* 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 *in vivo* 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 *in vivo* experiment with stevia extract performed, which also gave negative results.
Oh et al. (1999)[b]	*In Vivo?*	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)[c]	*In Vivo?*	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)[a]	*In Vitro*	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)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Positive	Mutagenic effects of steviol and/or metabolites found in *S.typhimurium* TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene. Magnitude of increase of these mutations over the control not reported.
Klongpanichpak et al. (1997)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	Steviol and stevioside inactive in TA strains of *S. typhimurium, E. coli WP2, uvrA/PKM101* and rec assay using *B. subtilis* even when microsomal activated fraction present. Magnitude of increase of these mutations over the control not reported.
Matsui et al. (1996)[a]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	Testing of Southern Blot technique with probe for gpt gene DNA of *E. coli*. The chromosomal DNA of TM677 and steviol-induced TM677 mutants

	In Vivo/In Vitro	System	Test Sample Purity	Author Conclusion	Results and Remarks
					digested by restriction enzymes and probed. No significant differences found in fragment length between wild-type and mutant DNA.
Matsui et al. (1996)[a]	*In Vitro*	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.
Procinska et al. (1991)[c]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Negative	The direct mutagenic activity of 15-oxo-steviol was refuted.
Compadre et al. (1988)[a]	*In Vitro*	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)[d]	*In Vitro*	Bacterial Mutagenicity	Not Reported	Positive	Using *S. typhimurium* 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)[c]	*In Vivo*	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)[c]	*In Vivo*	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)[a]	*In Vivo*	Micronucleus (mouse)	Not Reported	Negative	Steviol did not increase number of micronuclei observed in this study.
Temcharoen et al. (2000)[c]	*In Vivo*	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.

[a] Abstract only. [b] As reported in WHO (2006). [c] As reviewed by Geuns (2003). [d] 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* may not be a safe alternative to sugar or synthetic sweetners, 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.

END