PureCircle

745

Natural Extracted Ingredients

November 6, 2017

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Notice for Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, I am submitting one hard copy and one electronic copy (on CD), as the notifier [PureCircle Ltd., 915 Harger Road, Suite 250, Oak Brook, Illinois, 60523], a Notice of the GRAS status on the basis of scientific procedures, that steviol glycosides with a high rebaudioside M content produced by enzymatic conversion of rebaudioside A, produced by PureCircle Ltd., as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, and therefore, is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act*. Information setting forth the basis for the GRAS status, which includes detailed information on the notified substance and a summary of the basis for the GRAS status, as well as a consensus opinion of an independent panel of experts in support of the safety of steviol glycosides with a high rebaudioside M content produced by enzymatic conversion of rebaudioside A under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Notice for Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Sidd Purkayastha, Ph.D.
VP, Head of Global Scientific and Regulatory Affairs
PureCircle USA

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GRAS NOTICE FOR STEVIOL GLYCOSIDES WITH A HIGH REBAUDIOSIDE M CONTENT PRODUCED BY ENZYMATIC CONVERSION OF REBAUDIOSIDE A FROM STEVIA LEAF EXTRACT

PREPARED FOR:
Office of Food Additive Safety (FHS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Drive
College Park, MD
20740

DATE:
03 November 2017

GRAS Notice for Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

GRAS Notice for Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, PureCircle Ltd. hereby informs the United States (U.S.) Food and Drug Administration (FDA) of the view that its steviol glycosides with a high rebaudioside M content produced *via* enzymatic conversion of rebaudioside A from stevia leaf extract is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on its conclusion that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Part 1.3 below. In addition, as a responsible official of PureCircle Ltd., the undersigned hereby certifies that all data and information presented in this notice constitutes a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to PureCircle Ltd. and pertinent to the evaluation of the safety and GRAS status of steviol glycosides with a high rebaudioside M content produced *via* enzymatic conversion of rebaudioside A from stevia leaf extract as a general purpose sweetener, as described herein.

Signed,

(b) (6)

03/Nov/2017

Sidd Purkayastha, Ph.D.
VP, Head of Global Scientific & Regulatory Affairs
PureCircle Limited
sidd.purkayastha@purecircle.com

Date

1.1 Name and Address of Notifier

PureCircle Limited
915 Harger Road, Suite 250
Oak Brook, Illinois
60523

1.2 Common Name of Notified Substance

Steviol glycosides with a high rebaudioside M content produced by enzymatic conversion of rebaudioside A from stevia leaf extract.

Steviol glycosides; Rebaudioside M; Reb M; Steviol glycosides (modified Stevia leaf extract); Reb M (modified Stevia leaf extract); Modified Stevia leaf extract; Modified Stevia extract

1.3 Conditions of Use

PureCircle intends to market steviol glycosides with a high rebaudioside M (reb M) content produced by enzymatic conversion of rebaudioside A (reb A) as a general purpose sweetening agent in the U.S., in accordance with current Good Manufacturing Practice (cGMP), excluding infant formulas and meat and poultry products.

Most other high-intensity sweeteners have been approved by the FDA as general purpose sweeteners without their uses being restricted to specific foods or use-levels. Hence, the foods to which high-intensity sweeteners are added and the use-levels are controlled by their technological properties (*e.g.,* sweetness potency). Considering that steviol glycosides, including PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, are characterized by a sweetness intensity that is, for the most part, comparable to that of other high-intensity sweeteners (*e.g.,* aspartame and steviol glycosides produced through enzymatic conversion of reb A are approximately 200 times as sweet as sucrose), the uses and use-levels of steviol glycosides with a high reb M content are likely to be similar to those currently permitted for other high-intensity sweeteners in the U.S.

1.4 Basis for GRAS

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR), steviol glycosides with a high reb M content produced by enzymatic conversion of reb A has been determined by PureCircle to be GRAS on the basis of scientific procedures. The GRAS status of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is based on data generally available in the public domain pertaining to the safety of steviol glycosides and the production strains as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A as a component of food [see Appendix A, entitled, "**Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract for Use as a General Purpose Sweetener**"].

1.5 Availability of information

The data and information that serve as the basis for this GRAS Notification will be made available to the FDA for review and copying upon request during business hours at the offices of:

PureCircle Limited
915 Harger Road, Suite 250
Oak Brook, Illinois
60523

In addition, should the FDA have any questions or additional information requests regarding this notification during or after the Agency's review of the notice, PureCircle will supply these data and information.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is PureCircle's view that all data and information presented in Parts 2 through 7 of this notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552.

Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2.1.1 Common or Usual Name

Steviol glycosides; Rebaudioside M; Reb M; Steviol glycosides (modified Stevia leaf extract); Reb M (modified Stevia leaf extract); Modified Stevia leaf extract; Modified Stevia extract

2.1.2 Chemical and Physical Characteristics

The food ingredient identified as steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is a white to off-white powder that has a clean taste with no abnormal or off odor and is freely soluble in water. Steviol glycosides with a high reb M content produced by enzymatic conversion is approximately 200 times sweeter than sucrose and is consistent with the sweetness intensity of steviol glycosides in general (FAO, 2016).

PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is composed of >30% reb M and also contains other steviol glycosides, including those listed in Table 2.2-1. The final purified product contains ≥95% total steviol glycosides, which is consistent with the purity criteria for steviol glycosides as established by the Joint FAO/WHO Expert Committee on Food Additives [JECFA] (JECFA, 2016a). All steviol glycosides are glycosylated derivatives of the aglycone steviol and as such, all share the same backbone structure (Figure 2.1.2-1) and differ only with respect to the type and number of glycoside units at positions R_1 and R_2. The chemical structures of the different steviol glycosides that may be present in steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are presented in Table 2.1.2-1.

Figure 2.1.2-1 Backbone Structure for Steviol Glycosides

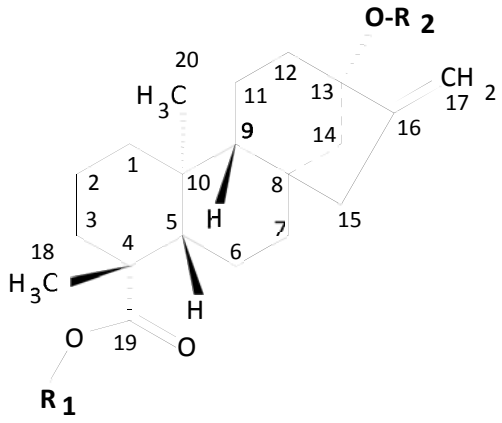


Table 2.1.2-1 Individual Steviol Glycosides That Have Been Identified and May be Present in Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A (see Figure 2.1.2-1 for backbone structure)

Common name	Trivial formula	Mol. Wt.	R_1	R_2
Rebaudioside A	SvG4	967	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside D	SvG5	1,129	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside I	SvG5	1,129	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M	SvG6	1,291	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M2	SvG6	1,291	Glcβ(1-2)[Glcβ (1-6)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-

2.2 Method of Manufacturing

PureCircle's steviol glycosides with a high reb M content food ingredient produced by enzymatic conversion of reb A is manufactured using enzymes (UDP-glucosyltransferases and a sucrose synthase) derived from genetically modified *E. coli* K-12 that convert reb A extracted from the leaves of *S. rebaudiana* to reb M. In the first stage of the manufacturing process reb A is extracted from the leaves of *S. rebaudiana* and purified to greater than 95% reb A, consistent with the methods and specifications outlined by JECFA for steviol glycosides from S. *rebaudiana* Bertoni (FAO, 2016; JECFA, 2016a). In the second stage, genetically modified strains of *E. coli* K-12 are fermented to produce UDP-glucosyltransferase and sucrose synthase enzymes that are then isolated for use in the enzymatic conversion process. The purified reb A powder is reacted with the enzymes in the third stage of the manufacturing process to generate a mixture of steviol glycosides containing >30% reb M. In the fourth and final stage, the steviol glycoside mixture is purified in accordance with the methodologies outlined in the Chemical and Technical Assessment (CTA) published by FAO/JECFA for steviol glycosides (FAO, 2016), yielding a final product that contains ≥95% total steviol glycosides, specifically comprised of reb M and other steviol glycosides, including those listed in Table 2.1.2-1.

2.2.1 Raw Materials and Processing Aids

All raw materials, processing aids, and purification equipment used to manufacture steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are food-grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses (Table 2.2.1-1).

[1] Compliant with the specifications set forth in the Food Chemicals or equivalent international food or pharmacopeia standard [*e.g.,* JECFA, Food Chemical Codex (FCC), United States Pharmacopeia (USP), European Pharmacopeia (EP)].

Table 2.2.1-1 Raw Materials, Processing Aids, and Equipment Used to Manufacture Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Raw Material/Processing Aid	Technological Function	Regulatory Status
Stevia rebaudiana Bertoni leaves	Source of rebaudioside A	NA
Sucrose	Reactant	GRAS
UDP disodium salt (5'-UDP-Na_2)	Processing aid	NA
UDP-glucosyltransferases and sucrose synthase	Processing aids	NA
High-purity calcium hydroxide	Flocculant	Permitted for use in food as a direct food additive with no limitations apart from cGMP, 21 CFR §184.1205 (U.S. FDA, 2017a)
Ethanol, food-grade	Crystallization and desorption solvent	GRAS when used in accordance with cGMP, 21 CFR §184.1293 (U.S. FDA, 2017a)
Activated carbon, food-grade	Decolorizing agent	GRAS
Adsorption and ion-exchange resin	Purification	Used in accordance with 21 CFR §173.25 (U.S. FDA, 2017a)

GRAS = Generally Recognized as Safe; NA = not applicable

2.2.2 Enzymes

The enzymes used in the manufacturing process to convert reb A to reb M include UDP-glucosyltransferases (UGT-Sr, UGT-Sl) and sucrose synthase (SuSy-At). These enzymes are manufactured in accordance with cGMP for food and the principles of Hazard Analysis of Critical Control Points (HACCP) (see stage 2 of the manufacturing process in Section 2.2.3). These enzymes are produced by microbial fermentation of the *E. coli* production strain LE1B109 carrying the expression vector for the corresponding enzyme gene (see Section 2.2.4 for further details on the genetic modification). The *E. coli* LE1B109 production organism is of a biosafety category level 1. Separate specifications for the 3 enzymes have been established and the analytical results from 3 non-consecutive lots of each product are presented in Tables 2.2.2-1 to 2.2.2-3 below. These data demonstrate product consistency and the absence of the production microorganism in the final enzyme preparations. Furthermore, the enzymes are free of antibiotics as no antibiotics were used in the manufacturing process. The enzymes are food-grade quality and conform to the recommended purity criteria established by the Food Chemicals Codex (FCC, 2016) and JECFA (2006).

Table 2.2.2-1 Product Specifications for Sucrose Synthase SuSy-At

Specification Parameter	Specification	Manufacturing Lot		
		PM2-34-001	PM-39-001	
Activity	≥400 U/mL	413	547	512
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.11	0.14	0.11
TOS (%)	NS	9.48	10.49	9.62

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

Table 2.2.2-2 Product Specifications for UDP-Glucosyltransferase UGT-Sr

Specification Parameter	Specification	Manufacturing Lot		
		FAH-a-U3D1	FAH-a-U4D1	FAH3-002
Activity	≥1 U/mL	1.22	1.66	2.00
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.08	0.07	0.08
TOS (%)	NS	10.53	13.61	14.17

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

Table 2.2.2-3 Product Specifications for UDP-Glucosyltransferase UGT-Sl

Specification Parameter	Specification	Manufacturing Lot		
		SK4-14-001	SK4-18-001	SK4-19-001
Activity	≥7 U/mL	9.6	12.0	9.2
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.12	0.06	0.09
TOS (%)	NS	10.47	13.47	11.41

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

2.2.3 Manufacturing Process

A schematic overview of the manufacturing process for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is illustrated below in Figure 2.2.3-1. The steviol glycoside purification processes utilized prior to and following the enzymatic conversion are consistent with the methodologies for the manufacture of steviol glycosides as described in the CTA published by FAO/JECFA (FAO, 2016). Steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is manufactured in a facility certified under Food Safety System Certification 22000:2010.

In stage 1, *S. rebaudiana* leaves are placed in hot water at 50 to 60°C for 1 to 2 hours in continuous countercurrent extractors. The filtrate is separated using mesh screens, collected in a holding tank, and treated with flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and coloring agents. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionized by ion-exchange resins in (H^+) and (OH^-) form. The deionized filtrate is fed to a column system packed with macroporous adsorption resin that retains the glycosides. The column is washed with deionized water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. The obtained glycoside solution is treated with activated carbon and the carbon is separated from the solution by a plate-and-frame filter press. A standard

evaporator is used to remove the ethanol, and the resulting aqueous solution is deionized again by ion-exchange resins in (H^+) and (OH^-) forms. The refined solution is concentrated using a nanofiltration membrane and the concentrated solution is spray dried to yield stevia extract powder containing >50% reb A (RA50). The RA50 powder is further purified by dissolving in aqueous ethanol and incubating at low temperature for several hours to allow for reb A to crystallize. The reb A crystals containing >95% reb A are separated by conventional centrifugation and dried in a rotary drum vacuum dryer at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminum foil bags.

In stage 2 of the manufacturing process, *E. coli* production strain LE1B109 carrying the expression vector for the corresponding enzyme is inoculated in sterilized culture medium composed of the ingredients listed in Table 2.2.3-1 and fermented.

Table 2.2.3-1 Raw Materials Used for Fermentation of *E. coli* Enzyme Production Strain LE1B109

Raw Material		
Glucose	Fermentation Nutrient	Permitted for use in food as ingredient with no limitations apart from cGMP, 21 CFR §184.1857
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Inducer for enzyme expression	
Defined mineral components	Fermentation Nutrient	Permitted for use in food as food additive, food substance, ingredient, flavor enhancer, flavoring agent, processing aid or nutrient supplement, with no limitations apart from cGMP, each being selected from 21 CFR Parts §184, §172, §573, §182, §582.
Suitable antifoam agent	Processing aid	Listed in the U.S. FDA September 11, 2003 letter to ETA as acceptable for use in enzyme manufacturing
Nuclease	Processing aid	

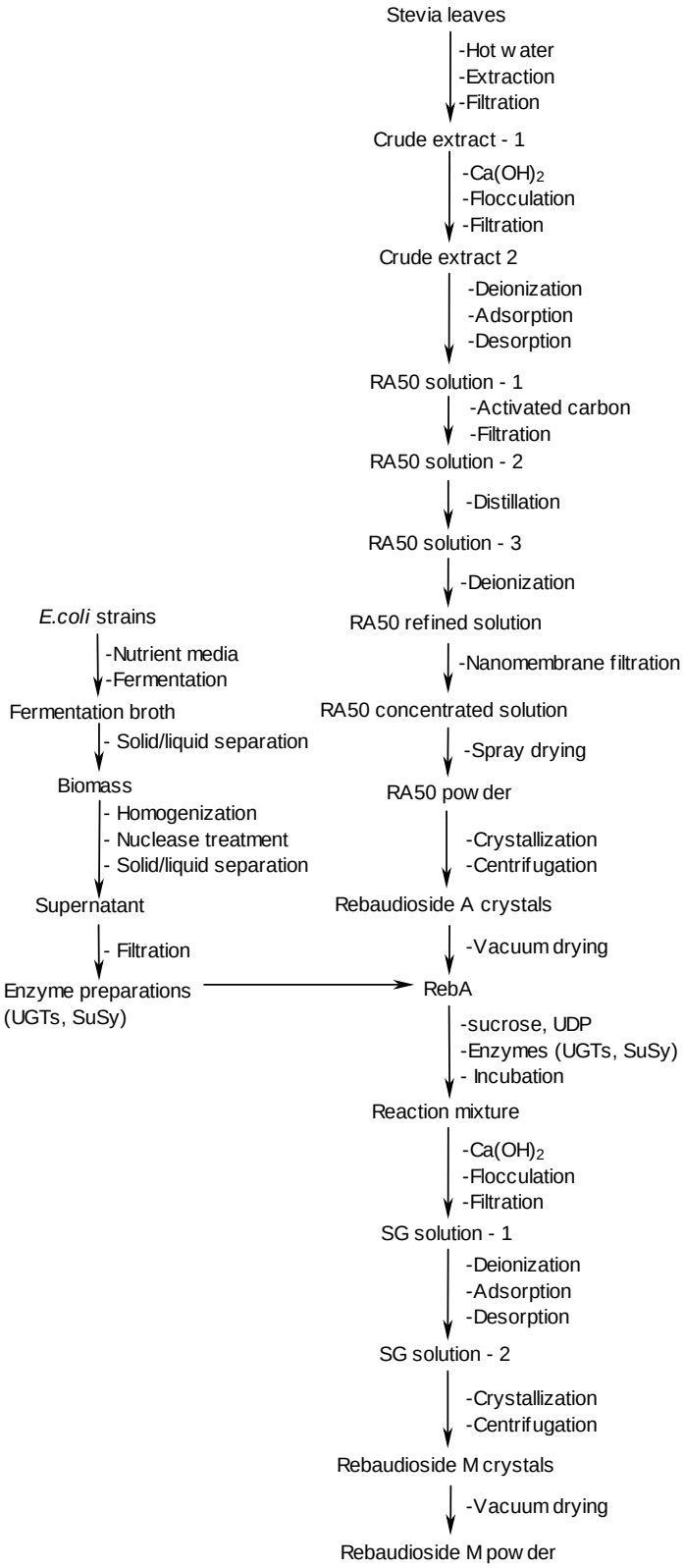
The fermentation conditions are a pH of between 6 to 8 and a temperature of between 25 to 37°C. The fermentation process is continued until laboratory test data shows the desired enzyme production yield. Usually, after at least 15 hours, the fermentation is stopped. In a subsequent recovery process, the enzyme is isolated from the biomass. In a first solid/liquid separation, the biomass is separated from the culture broth by standard techniques (*e.g.,* is centrifuged and/or filtered). The biomass is homogenized to disrupt the bacterial cells and treated with a nuclease to degrade the DNA/RNA nucleic acids released upon cell disruption. This is followed by solid/liquid separation steps to further remove cell debris and other insoluble matter. The cell-free supernatant is filtered to obtain the purified enzyme preparation. All raw materials used for fermentation and recovery are of food-grade quality or have been assessed to be fit for their intended use.

In stage 3, the products of stage 1 (reb A, >95%) and stage 2 (UGT-Sr, UGT-Sl, and SuSy-At enzymes) are mixed to initiate the enzymatic conversion process. First, the reb A (>95%) powder and sucrose are dissolved in reverse-osmosis water. Next, 5'-UDP-Na_2 and UGT-Sr, UGT-Sl, and SuSy-At enzymes are added to formulate the reaction mixture. The reaction mixture is incubated at 40 to 50°C for 10 to 48 hours. The use of different reaction times yields steviol glycoside mixtures with different ratios of starting glycoside reb A, intermediate glycosides such as reb D, and the primary final glycoside product reb M. The resulting reaction mixture containing a mixture of steviol glycosides, including those listed in Table 2.1.2-1, is heated to 80 to 100°C for 10 minutes to inactivate the enzymes.

In the last stage of manufacturing, the reaction mixture is treated with a flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and other impurities. A plate-and-frame filter

press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionized by ion-exchange resins in (H^+) and (OH^-) form. The deionized filtrate is fed to a column system packed with macroporous adsorption resin that retains the reb M and other steviol glycosides. The column is washed with deionized water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. Next, the filtrate is maintained at low temperatures for several hours to allow reb M to crystallize. The reb M crystals containing >30% reb M are separated by conventional centrifugation and dried in a rotary drum vacuum at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminum foil bags. The bags are placed in high-density polyethylene drums sealed with tamper evident seals.

Figure 2.2.3-1 Schematic Overview of the Manufacturing Process for Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A



2.2.4 Construction of the Enzyme Production Microorganisms

The production strain LE1B109 is a genetically modified derivative strain of the laboratory strain *E. coli* K-12 W3110. The parental strain *E. coli* K-12 W3110 has been modified by site-directed recombination at different chromosomal loci to suit production purposes in terms of genetic stability, especially plasmid stability, and efficiency of expression and biotransformation. The expression of a number of proteases has been eliminated by deletion of the corresponding genes. Antibiotic-free selection of target clones has been enabled through deletion of one gene. One further gene has been deleted to prevent unwanted recombination effects. The gene coding for the T7 RNA polymerase from *E. coli* T7 phage and another gene copy of lacI, a repressor naturally present in *E. coli* K-12 W3110, have been inserted into the genome of W3110 to achieve a strong and regulated enzyme expression. Furthermore, the strain might carry certain deletions of endogenous enzyme genes connected to the degradation of biotransformation reactants in order to avoid side reactions. Insertions and deletions of chromosomal DNA are in general performed by integration of plasmid-based fragments carrying antibiotic resistance genes. After selection of the correct chromosomal mutants, resistance genes are excised and all plasmids are removed. No residual vector sequences or antibiotic resistance genes are left in the final cell.

The final production strain used for manufacturing each enzyme is created from the LE1B109 recipient strain by introducing an expression vector carrying the specific gene for one of the enzymes listed in Table 2.2.4-1. The plasmids used to transform the *E. coli* recipient strain are based on the well-known vector pRSF-1b. The plasmids have been fully sequenced and do not carry antibiotic resistance genes or any other sequences of concern. The production strain LE1B109 has been sequenced to confirm absence of antibiotic resistance genes or any other sequences of concern.

Table 2.2.4-1 Summary of Enzymes and their Respective Functions in the Production Strains

Enzyme	Function	
Sucrose synthase	Catalyzes the formation of UDP-glucose	*Arabidopsis thaliana*
UDP-glucosyltransferase UGT-Sr	Catalyzes the addition of glucose to steviol glycosides	*Stevia rebaudiana*
UDP-glucosyltransferase UGT-Sl	Catalyzes the addition of glucose to steviol glycosides	*Solanum lycopersicum*

2.3 Product Specifications and Batch Analysis

2.3.1 Physical and Chemical Specifications

The product specifications for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are presented in Table 2.3.1-1.

Table 2.3.1-1 Physical and Chemical Specifications for Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Specification Parameter	Steviol glycosides with a high reb M content	Current JECFA specifications for steviol glycosides (JECFA, 2016a)	Method of analysis
Appearance	White to off-white powder	White to light yellow powder	Sensory Evaluation
Total steviol glycosides (anhydrous basis)	≥95%	≥95% total steviol glycosides[a]	HPLC (JECFA, 2016a)
Loss on drying	≤6.0%	≤6% (105°, 2h)	FAO/JECFA Vol 4[b] (p. 61)
pH (1% solution)	4.5 to 7.0	4.5 to 7.0	FAO/JECFA Vol 4 (p. 36-38)
Residual ethanol	<0.30%	≤0.5%	USP[c] Method 467
Residual methanol	<0.02%	≤0.02%	USP Method 467
Total ash	<1.0%	≤1%	AOAC[d] Method 945.46
Lead (as Pb)	<1.0 ppm	≤1 ppm	AOAC Method 993.14
Arsenic (as As)	<1.0 ppm	≤1 ppm	AOAC Method 993.14
Cadmium (as Cd)	<1.0 ppm	NS	AOAC Method 993.14
Mercury (as Hg)	<1.0 ppm	NS	AOAC Method 993.14
Residual protein	Not detected	NA	SDS-PAGE[e]
Residual DNA	Not detected	NA	PCR[e]

FCC = Food Chemicals Codex; HPLC = high performance liquid chromatography; NA = not applicable; NS = not specified; PCR = polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; USP = United States Pharmacopeia

[a] Where steviol glycosides "consists of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, deoxyglucose, galactose, and arabinose". (JECFA, 2016a, 2017).

[b] FAO/JECFA (2006). *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 65th JECFA Meetings, 1956–2005. (FAO JECFA Monographs 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf [Last updated (Web version): August 2011].

[c] USP (2012). United States Pharmacopeia, 35th edition & National Formulary, 30th edition [Online]. Rockville (MD): U.S. Pharmacopeia (USP) Convention Inc. Available at: http://www.uspnf.com/ [Subscription Only].

[d] AOAC (2005). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition* (Current through Revision 1, 2006). Arlington (VA): Association of Official Analytical Chemists (AOAC).

[e] Method described in Section 2.3.5.

2.3.2 Microbiological Specifications

The microbiological specifications for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are presented in Table 2.3.2-1.

Table 2.3.2-1 Microbiological Specifications for Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Specification Parameter	Specification	Method of Analysis
Total plate count	<1,000 CFU/g	AOAC[a] Method 966.23
Yeast and mold (CFU/g)	Not detected	Standards Australia[b] Method 1766.2.2
Total coliforms (MPN/g)	Not detected	ISO 4831[c]
Escherichia coli count (MPN/g)	Not detected	ISO 7251[d]
Salmonella sp.	Absent in 25 g	ISO 6579[e]

CFU = colony forming units; MPN = most probable number

[a] AOAC (2005). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition* (Current through Revision 1, 2006). Arlington (VA): Association of Official Analytical Chemists (AOAC).

[b] Standards Australia (1997). *Food microbiology. Method 2.2: Examination for specific organisms—Colony count of yeasts and moulds*. (Australian/New Zealand Standard AS 1766.2.2). Sydney, Australia: Standards Association of Australia/SAI Global.

[c] BSi (1991). *Methods for Microbiological examination of food and animal feeding stuffs — Part 3: Enumeration of coliforms — Most probable number technique*. (British Standard (BS) / International Organization for Standardization (ISO), BS 5763-3:1991 ISO 4831:1991). London, Engl.: British Standards Institution (BSi).

[d] BSi (1993). *Methods for Microbiological examination of food and animal feeding stuffs — Part 8: Enumeration of presumptive Escherichia coli. Most probable number technique*. (British Standard (BS) / International Organization for Standardization (ISO), BS 5763-8:1994 ISO 7251:1993). London, Engl.: British Standards Institution (BSi).

[e] BSi (2012). Microbiology of Food and Animal Feed. Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella. Enumeration by a miniaturized most probable number technique. (PD CEN ISO/TS 6579-2:2012). London, Engl.: British Standards Institution (BSi). Information available at: http://shop.bsigroup.com/en/ProductDetail/?pid=000000000030255346.

2.3.3 Batch Analyses

2.3.3.1 Physical and Chemical Analysis

Data from the analysis of 3 non-consecutive lots of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A demonstrating the consistency of the manufacturing process and compliance with the physical and chemical specifications are presented in Table 2.3.3.1-1.

Table 2.3.3.1-1 Physical and Chemical Product Analysis for 3 Non-Consecutive Lots of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Specification Parameter	Limit	Manufacturing Lot		
		BM050517	SK-B-U2D1	SK-B-U3D1
Appearance	White to off-white powder	Conforms	Conforms	Conforms
Total steviol glycosides (anhydrous basis)	≥95%	98.88%	97.91%	97.20%
Loss on drying	≤6.0%	1.64%	1.64%	3.85%
pH (1% solution)	4.5 to 7.0	6.32	5.99	5.89
Residual ethanol	<0.30%	0.041%	0.134%	0.133%
Residual methanol	<0.02%	ND	0.001%	0.001%
Total ash	<1.0%	0.05%	<0.005%	0.02
Lead (as Pb)	<1.0 ppm	0.021 ppm	0.035 ppm	0.038 ppm
Arsenic (as As)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm

Table 2.3.3.1-1 Physical and Chemical Product Analysis for 3 Non-Consecutive Lots of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Specification Parameter	Limit	Manufacturing Lot		
		BM050517	SK-B-U2D1	SK-B-U3D1
Cadmium (as Cd)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm
Mercury (as Hg)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm
Residual protein	Not detected	ND	ND	ND
Residual DNA	Not detected	ND	ND	ND

ND = not detected; ppm = parts-per-million

2.3.3.2 Microbiological Analysis

Data from the analysis of 3 non-consecutive lots of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A demonstrating the consistency of the manufacturing process and compliance with the microbiological specifications are presented in Table 2.3.3.2-1.

Table 2.3.3.2-1 Microbiological Product Analysis for 3 Non-Consecutive Lots of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Specification Parameter	Limit	Manufacturing Lot		
		BM050517	SK-B-U2D1	SK-B-U3D1
Total plate count	<1,000 CFU/g	ND	ND	ND
Yeast and mold (CFU/g)	Not detected	ND	ND	ND
Total coliforms (MPN/g)	Not detected	ND	ND	ND
Escherichia coli count (MPN/g)	Not detected	ND	ND	ND
Salmonella sp.	Absent in 25 g	Absent	Absent	Absent

CFU = colony forming units; MPN = most probable number; ND = not detected

2.3.4 Steviol Glycoside Distribution Analysis

As described in Section 2.2.3, the distribution of steviol glycosides in the final product is dependent upon the length of reaction time of the enzymes with the starting material reb A extracted from the leaves of *S. rebaudiana*. Example data from 2 production lots (SK-BU2D1, SK-BU3D1) presented in Table 2.3.4-1 demonstrates that as the enzyme reaction time proceeds from 10 to 40 hours the steviol glycoside distribution changes, with increasing amounts of reb M being produced as the reaction proceeds. Example intermediate glycosides include rebaudiosides D and I.

Table 2.3.4-1 Changes in the Steviol Glycoside Distribution with Different Enzyme Reaction Times

Steviol Glycoside (%)	Time (hours)					
	0	**14**	**16**	**18**	**21**	**40**
Lot SK-BU2D1						
Rebaudioside A	100	30.4	25.6	NM	14.2	2.1
Rebaudioside D	ND	69.2	74.1	NM	43.6	1.7
Rebaudioside I	ND	0	0.1	NM	3.4	6.6
Rebaudioside M2	ND	0.38	0.12	NM	0.14	0.19
Rebaudioside M	ND	ND	ND	NM	38.6	89.4
Total Steviol Glycosides (%)	**100**	**99.98**	**99.92**	**NA**	**99.94**	**99.99**
Lot SK-BU3D1						
Rebaudioside A	100	NM	28.6	21.1	9.4	1.2
Rebaudioside D	ND	NM	71.1	77.3	60.0	1.8
Rebaudioside I	ND	NM	ND	0.3	3.1	4.2
Rebaudioside M2	ND	NM	0.28	0.35	0.34	0.37
Rebaudioside M	ND	NM	ND	0.9	27.1	92.5
Total Steviol Glycosides (%)	**100**	**NA**	**99.98**	**99.95**	**99.94**	**100.1**

NA = not applicable; ND = not detected; NM = not measured

As per the defined product specifications in Table 2.3.1-1 for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the final product contains ≥95% steviol glycosides, comprised of >30% reb M and other steviol glycosides such as those listed in Table 2.1.2-1. The steviol glycoside distribution, measured by high performance liquid chromatography (HPLC), is provided for 3 non-consecutive lots of final product manufactured with a 40-hour enzyme reaction time (Table 2.3.4-2) and demonstrates that the manufacturing process produces a product with a consistent steviol glycoside distribution and that the total steviol glycosides measured is consistently ≥95%.

Table 2.3.4-2 Steviol Glycoside Distribution for 3 Lots of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Steviol Glycoside (%)	Manufacturing Lot			Average
	BM050517	**SK-BU2D1**	**SK-BU3D1**	
Rebaudioside D	1.78[a]	0.23	0.41	0.81
Rebaudioside M	95.98	95.71	95.43	95.71
Rebaudioside I	0.91	1.54	0.93	1.13
Rebaudioside A	0.09	0.28	0.12	0.16
Total Steviol Glycosides (%)	**98.76**	**97.76**	**96.89**	**97.80**

[a] Average of 3 duplicates is reported

2.3.5 Residual Protein and DNA

To confirm the success of the purification techniques and confirm the absence of protein in steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the final product is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of steviol glycosides with a high reb M content are dissolved to a concentration of 1,000 ppm, and about 10 µL from each dissolved sample is stained with 3X protein loading dye and loaded onto a precast polyacrylamide gel. Electrophoresis is conducted at 60 minutes at 130 V and the gel is stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% water for 1 hour. Gels are destained by soaking for 4 hours in a

mixture of 10% acetic acid, 50% methanol, and 40% water. If protein is present in the sample, it will be visually detected on the gel (limit of detection = 0.1 µg protein). No visible protein bands have been detected in any batches of final product.

To confirm the absence of residual DNA in steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, a polymerase chain reaction (PCR) method was developed and primers were designed to amplify the gene of interest. Genomic DNA is extracted using a DNA extraction kit according to the manufacturer's protocol. The genomic DNA is quantified using a spectrophotometer and the extracted genomic DNA is evaluated for the presence of the gene of interest. The thermal profile used is 2 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C. Results of the PCR analysis have not detected any PCR products in any of the batches of final product (limit of detection = 0.00002 ng DNA).

2.4 Stability Data

The stability of steviol glycosides has been previously reviewed by a number of the scientific advisory bodies involved in the evaluation of steviol glycoside safety (JECFA, the European Food Safety Authority [EFSA], and the Food Standards Australia/New Zealand [FSANZ]) and is also discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999). Specifically, JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in foods at their 68th meeting (JECFA, 2007). The Committee noted that steviol glycosides do not undergo browning or caramelization when heated, and are reasonably stable under elevated temperatures used in food processing. Under acidic conditions (pH 2 to 4), steviol glycosides (approximately 90 to 94% purity), are stable for at least 180 days when stored at temperatures up to 24°C. When exposed to elevated temperatures (80°C, in water, 8 hours), however, 4 and 8% decomposition was observed in solutions of steviol glycosides at pH 4.0 and 3.0, respectively, indicating that the stability of steviol glycosides is pH and temperature dependent. When the temperature was increased to 100°C, expectedly higher rates of steviol glycoside decomposition (10 and 40% at pH 4.0 and 3.0, respectively) were observed. Based on the above findings, as well as additional publicly available stability studies, JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions.

In a recent publication, the structural and compositional stability of 3 commercial batches each of the dried stevia leaves, the first aqueous infusion of the ground stevia, and a high-purity stevia leaf extract (≥95% steviol glycosides, was evaluated to determine whether the manufacturing process adversely impacts steviol glycoside composition (Oehme *et al.,* 2017). Changes in steviol glycoside composition were analyzed by HPLC-UV and HPLC-ESI-MS/MS. The authors noted that all 9 steviol glycosides defined by JECFA were detected in all samples. The results also demonstrated that stevia extract processing does not chemically alter or modify the individual steviol glycoside content.

Although the stability of all steviol glycosides were not specifically addressed during JECFA's evaluation, it is expected that the stability of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A would be similar to individual steviol glycosides given the similarities in structure. Additional stability studies of steviol glycosides with a high reb M content produced by enzymatic conversion as powders under normal and/or accelerated storage conditions as well as in solution at various pH levels and temperatures were conducted for confirmation. These studies are summarized in Sections 2.4.1 and 2.4.2 and demonstrate that the stability of steviol glycosides with a high reb M content produced by enzymatic conversion is similar to individual steviol glycosides, as previously concluded by JECFA.

2.4.1 Storage Stability

The storage stability of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A (Lot LB110117) was assessed. Steviol glycosides with a high reb M content powder samples were stored in glass containers for up to 12 weeks at 1) 25°C, 60% relative humidity and 2) 40°C, 75% relative humidity. To assess storage stability, samples were tested by HPLC at baseline and at various time points thereafter, based upon measured values of individual steviol glycosides as well as total steviol glycosides. As reported in Table 2.4.1-1, steviol glycosides with a high reb M content powder stored under both conditions for 12 weeks was stable in its individual steviol glycoside content as well as total steviol glycosides (<1% degradation).

Table 2.4.1-1 Storage Stability of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A (Lot LB110117), as percent (%) dry basis

Week	0	4	8	12
25°C, 60% relative humidity				
Reb D	0.67	0.69	0.72	0.72
Reb M	96.72	96.59	96.47	96.06
Reb A	ND	ND	ND	ND
Total steviol glycosides	**97.39**	**97.28**	**97.19**	**96.78**
40°C, 75% relative humidity				
Reb D	0.67	0.70	0.73	0.75
Reb M	96.72	96.72	96.15	96.08
Reb A	ND	ND	ND	ND
Total steviol glycosides	**97.39**	**97.42**	**96.88**	**96.83**

ND = not detected; Reb = rebaudioside

2.4.2 pH Stability

The general stability of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A (Lot LB110117) was assessed over a pH range of 2.0 to 8.0 for a total of 12 weeks at 4 different temperatures, 4, 25, 37, and 56°C. Samples were prepared at concentrations of approximately 1,000 mg/L in 500 mL of buffer solution and stored in amber glass vials. Buffer was prepared by mixing different ratios of 0.1 M phosphate buffer, 0.1 M phosphorous acid, or 0.1 M di-sodium hydrogen phosphate buffer to obtain the target pH. Total steviol glycosides present in the stability samples were measured by HPLC at baseline as well as various time points over the study period, determined by the sum of the measured concentrations of rebaudiosides A, D, and M. Table 2.4.2-1 summarizes the results of the stability for solutions of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A.

The extent and rate of degradation of steviol glycosides with a high reb M content, based on measured total steviol glycosides, was shown to be dependent on pH, temperature, and time. In general, steviol glycosides with a high reb M content at all pH levels tested (2.0 to 8.0) was most stable when stored at 4°C and the least stable at 56°C. Over the 12-week study period, samples tested at pH 4.0 to 8.0 at 5, 25, and 37°C remained stable within at least 7% of the starting material percentage value. A significant loss in stability was noted when samples were stored at 56°C at the majority of pH levels, with the pH 4.0 and 5.0 samples remaining the most stable over the 12 weeks. Overall, at pH values ranging from 4.0 to 8.0, no significant degradation was observed over 12 weeks at 5, 25, and 37°C.

Similar to individual steviol glycosides, the stability of the steviol glycosides with a high reb M content followed the same degradation pathway and was pH-, temperature-, and time-dependent. Therefore, the conclusions regarding the stability of steviol glycosides made by JECFA and other scientific bodies (that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions) can be extended to steviol glycosides with a high reb M content produced by enzymatic conversion of reb A that are the subject of this safety assessment.

Table 2.4.2-1 Stability of Steviol glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A (Lot LB110117) in Solution at Varying Temperature and pH

Week	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
4°C	**Total steviol glycosides (%)**						
0 (baseline)	97.44	97.24	97.18	97.29	97.31	97.21	97.59
2	97.04	97.50	96.87	96.88	97.06	96.99	96.85
4	95.11	97.72	97.11	97.32	97.48	97.01	97.36
6	94.81	97.08	96.97	97.08	97.50	97.08	96.85
8	94.27	96.28	96.60	96.72	97.06	96.32	96.62
10	93.60	96.25	95.91	96.72	96.74	95.75	96.08
12	93.78	96.87	96.37	96.16	96.45	96.28	94.70
25°C	**Total steviol glycosides (%)**						
0 (baseline)	97.44	97.24	97.18	97.29	97.31	97.21	97.59
2	92.86	96.78	96.82	96.78	96.75	96.92	96.67
4	83.37	96.16	97.08	97.10	97.65	96.78	96.62
6	79.54	95.82	97.02	97.18	97.25	97.66	96.03
8	70.25	94.01	96.22	96.86	96.34	96.08	95.13
10	66.85	93.16	95.75	96.38	95.52	94.76	94.07
12	64.64	93.42	96.43	96.25	95.66	95.59	94.60
37°C	**Total steviol glycosides (%)**						
0 (baseline)	97.44	97.24	97.18	97.29	97.31	97.21	97.59
2	64.01	93.46	96.34	97.29	96.73	96.26	95.71
4	32.01	87.34	95.97	95.83	95.27	95.22	96.01
6	24.28	85.21	95.90	95.50	94.33	95.34	95.85
8	16.89	75.69	95.37	95.30	94.13	92.79	94.50
10	11.64	75.10	93.46	94.61	91.52	91.15	93.07
12	9.16	75.18	93.99	94.92	91.76	90.49	93.19
56°C	**Total steviol glycosides (%)**						
0 (baseline)	97.44	97.24	97.18	97.29	97.31	97.21	97.59
2	4.88	69.94	92.42	94.66	95.62	94.48	94.22
4	0.79	36.96	84.74	89.33	77.82	70.94	85.77
6	1.01	30.40	80.78	86.56	70.14	65.35	83.73
8	0.11	23.91	77.01	83.92	65.39	62.51	80.05
10	0.03	14.75	72.92	76.89	56.32	54.64	71.90
12	0.03	11.62	70.67	73.89	52.36	51.57	68.94

[a] Sum of the following individual steviol glycosides: rebaudiosides A, D, and M

Part 3. §170.235 Dietary Exposure

3.1 Intended Use of Steviol Glycosides with a High Reb M Content and Levels of Use in Foods

Steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is approximately 200 times sweeter than sucrose and is intended for use as a general purpose sweetening agent, in accordance with cGMP. Most other high-intensity sweeteners have been approved by the FDA as general purpose sweeteners without their uses being restricted to specific foods or use-levels. Hence, the foods to which high-intensity sweeteners are added and the use-level are controlled by their technological properties (*e.g.,* sweetness potency). Considering that steviol glycosides, including PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, are characterized by a sweetness intensity that is, for the most part, comparable to that of other high-intensity sweeteners (*e.g.,* aspartame and steviol glycosides produced through enzymatic conversion of reb A are approximately 200 times as sweet as sucrose), the uses and use-levels of steviol glycosides with a high reb M content are likely to primarily reflect those currently permitted for other high-intensity sweeteners in the U.S.

3.2 Estimated Dietary Consumption of Steviol Glycosides with a High Reb M Content Based Upon Intended Food Uses

3.2.1 History of Consumption of Steviol Glycosides

Since it was first discovered in the West in 1887 by Antonio Bertoni (a South American natural scientist), *S. rebaudiana* and its isolated steviol glycosides (most commonly stevioside) have been consumed by humans in various countries as sweeteners in foods and beverages (Geuns, 2003). In Brazil and Paraguay, *S. rebaudiana* has been used by its native people for hundreds of years as both a food ingredient and as a tea (Blumenthal, 1995; Geuns, 2003). Use of *S. rebaudiana* leaves as a sweetener by the native Indians of the Guarani Tribe since pre-Columbian times has been documented (Ferlow, 2005). In the 1980s, *S. rebaudiana* became a popular herbal tea ingredient in the U.S. (Blumenthal, 1995; Ferlow, 2005). Stevioside has been used as a sweetener in Japan for more than 30 years (Geuns, 2003; Ferlow, 2005). In 1995, the use of stevioside in Asia was reported to be approximately 160,000 metric tons sucrose equivalents, increasing to approximately 200,000 metric tons sucrose equivalents in 1999 (International Sugar Organization, 2001).

3.2.2 Estimated Consumption of Steviol Glycosides with a High Reb M Content from Proposed Food Uses

Numerous surveys have been completed in various global jurisdictions (U.S., Canada, Brazil, Australia/New Zealand, and countries in the European Union) to assess daily consumption estimates of other well-established high-intensity sweeteners in the marketplace (*e.g.,* aspartame, cyclamate, saccharin, and sucralose). Renwick (2008) used the available post-market surveillance data for other high-intensity sweeteners as the basis for the assessment of dietary exposure for reb A by assuming full replacement of the currently approved intense sweeteners with the new sweetener. This intake assessment methodology yields intake estimates that while conservative, as it is unlikely that the novel sweetener would entirely replace all other sweeteners in the marketplace, are realistic in that they reflect actual post-market intakes of high-intensity sweeteners. Specifically, in order to estimate reb A intakes, Renwick (2008) first expressed the post-market surveillance intake estimates for intense sweeteners presently used in the global

marketplace as sucrose equivalents in various population groups (for average and high-end non-diabetic and diabetic adult and child consumers) (see Table 3.2.2-1). The data used in these analyses were primarily derived from studies that used specifically designed food diaries combined with actual use-levels or approved levels in different foods and beverages (Renwick, 2008). In order to predict dietary exposure to reb A, the intake estimates for the high-intensity sweeteners (expressed as sucrose equivalents) were adjusted for the sweetness intensity of reb A relative to sucrose (approximately 200).

In the case of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the same methodology as applied by Renwick (2008) was used to estimate intake values. Based on a sweetness potency test, steviol glycosides with a high reb M content produced by enzymatic conversion of reb A was determined to be approximately 200 times sweeter than sucrose. The intake values for intense sweeteners presented in Table 3.2.2-1 below were adjusted accordingly to derive an estimated intake range for steviol glycosides with a high reb M content. The estimated intake ranges were then converted to steviol equivalents based upon the molecular weight for reb M of 1,291.3 g/mol.

Table 3.2.2-1 Estimated Consumption of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A Using Renwick's (Renwick, 2008) Methodology of Intense Sweetener Intake Assessment

Population Group	Intakes of intense sweeteners (expressed as sucrose equivalents) (mg/kg bw/day)		Consumption estimates for:			
			Steviol glycosides with a high reb M content[a] (mg/kg bw/day)		Steviol glycosides with a high reb M content as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	1.28	3.38	0.32	0.85
Diabetic Adults	280	897	1.40	4.49	0.35	1.12
Non-diabetic Children	425	990	2.13	4.95	0.53	1.24
Diabetic Children	672	908	3.36	4.54	0.84	1.14

bw = body weight, reb = rebaudioside

[a] Approximately 200 times as sweet as sucrose.

[b] Calculated based on the molecular weights of steviol (318.45 g/mol) and reb M (1,291.3 g/mol) [steviol conversion factor of 0.25]

For non-diabetic adults, average and high-end intakes of steviol glycosides with a high reb M content of up to 0.32 and 0.85 mg/kg body weight/day expressed as steviol equivalents, respectively, were calculated. For diabetic adults, average and high-end intakes were slightly higher at up to 0.35 and 1.12 mg/kg body weight/day. Average and high-end exposures to steviol glycosides with a high reb M content, expressed as steviol equivalents, in non-diabetic children were calculated to be up to 0.53 and 1.24 mg/kg body weight/day, respectively. Although average intakes of steviol glycosides with a high reb M content, expressed as steviol equivalents, were estimated to be higher at up to 0.84 mg/kg body weight/day in diabetic children compared to values for non-diabetic children, high-end values in diabetic children (1.14 mg/kg body weight/day) were lower than high-end values in non-diabetic children. The predicted intakes of steviol glycosides with a high reb M content, expressed as steviol equivalents, are all below the current acceptable daily intake (ADI) defined by the JECFA for steviol glycosides (FAO, 2016) of 0 to 4 mg/kg body weight/day as steviol.

As part of their evaluation of the safety of steviol glycosides in 2008, JECFA considered various intake models for the estimation of dietary exposure to steviol glycosides, including the intake analysis conducted by Renwick (2008). Although higher intake estimates than those presented by Renwick (2008) were

identified using other methodologies, including ones considering replacement of all sweeteners used in or as food (up to approximately 6 mg/kg body weight/day, expressed as steviol equivalents), it was noted by JECFA that such replacement estimates were highly conservative and that actual exposures to steviol glycosides (expressed as steviol equivalents) would be 20 to 30% of these values (1 to 2 mg/kg body weight/day, expressed as steviol equivalents). Furthermore, JECFA noted that the intake estimates based on post-market surveillance further confirmed the lower range.

Part 4. §170.240 Self-Limiting Levels of Use

The use of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is largely limited by the desired sweetness intended for a particular food or beverage product. Therefore, the use of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A as a general purpose sweetener in foods is self-limiting based on its organoleptic properties.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable as steviol glycosides with a high reb M content produced by enzymatic conversion of reb A was not used in food before 1958.

Part 6. §170.250 Narrative and Safety Information

Over the last few decades, the safety of steviol glycosides has been considered by several scientific bodies and regulatory agencies, including the FDA, JECFA, the European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada. Interest in the use of steviol glycosides as sweeteners encouraged extensive testing of the compounds and as such a large safety database exists. This database includes a thorough examination of the comparative metabolism and pharmacokinetics of steviol glycosides in experimental animals and humans, acute toxicity studies, short- and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies. Although many earlier studies examining the safety of steviol glycosides were conducted with stevioside due to the predominance of stevioside in *S. rebaudiana* leaves (Aze *et al.*, 1991; Toyoda *et al.*, 1997), the database pertaining to the safety of steviol glycosides was expanded following the completion of additional short-term toxicity, reproductive toxicity, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies on reb A (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). Although the majority of toxicity studies have been conducted with either purified stevioside or reb A, the extensive database on the common metabolic fate of steviol glycosides has permitted the scientific bodies and regulatory agencies to extend their safety opinions to all steviol glycosides from the *S. rebaudiana* leaf, rather than just individual glycosides (JECFA, 2016a).

Given the metabolic fate of steviol glycosides, the safety of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A can be established based on the conclusions of the steviol glycoside safety reviews conducted by numerous scientific bodies and regulatory agencies, as well as the publicly available scientific literature related to the safety of steviol glycosides. In the sections that follow, a detailed summary of: i) the metabolic fate of steviol glycosides; ii) the data deemed pivotal in establishing the safety of steviol glycosides and conclusions by the scientific bodies and regulatory agencies (*i.e.,* JECFA, EFSA, FSANZ, Health Canada); and iii) the studies available in the scientific literature published since the FDA

review of the related GRAS notice GRN 667 for reb M produced from stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes derived from genetically modified strains of *P. pastoris*.

6.1 Absorption, Distribution, Metabolism, and Elimination of Steviol Glycosides

In vitro and *ex vivo* studies have demonstrated that steviol glycosides are not hydrolyzed by digestive enzymes of the upper gastrointestinal tract due to the presence of β-glycosidic bonds and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea *et al*., 1997; Koyama *et al*., 2003a; Geuns *et al*., 2003, 2007). Therefore, steviol glycosides enter the colon intact, where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Gardana *et al*., 2003; Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon, reviewed extensively by Renwick and Tarka (2008), have confirmed the ability of gut microflora from mice, rats, hamsters, and humans to hydrolyze steviol glycosides completely to steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Nikiforov *et al.*, 2013; Purkayastha *et al.,* 2016).

Steviol glycosides are hydrolyzed sequentially, removing one sugar moiety at a time, with differences in the degradation rates depending on the structural complexities of each steviol glycoside (Wingard *et al*., 1980; Koyama *et al*., 2003b). Stevioside, for example, is degraded to steviolbioside, steviolmonoside, and finally to steviol, with glucose released with each sequential hydrolysis, whereas reb A is first converted to either stevioside (major pathway) or reb B (minor pathway) prior to being ultimately degraded to steviol (Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b). Despite these structural differences, several parallel *in vitro* comparisons between reb A and various individual steviol glycosides have demonstrated a remarkable similarity with respect to the rate of hydrolysis of different steviol glycosides to steviol in the presence of human fecal homogenates, particularly during the first 24 hours of incubation (Purkayastha *et al.,* 2014, 2015, 2016). For example, reb M and reb A (0.2 mg/mL) were incubated with human fecal homogenates samples at 37°C for up to 24 hours under anaerobic conditions, and by 16 hours both compounds were reported to be completely metabolized to steviol (Purkayastha *et al.,* 2016). These experiments demonstrate that steviol glycosides are metabolized by human fecal homogenates to steviol at generally similar hydrolysis rates, indicating that the number and location of sugar units attached to the steviol backbone does not significantly affect the rate of hydrolysis.

Steviol is absorbed systemically into the portal vein and distributed to a number of organs and tissues, including the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and Renwick, 2008). In the liver, steviol is conjugated to glucuronic acid to form steviol glucuronide. In rats, free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) were identified in the plasma 8 hours after oral administration with either reb A or stevioside (Roberts and Renwick, 2008). Similarly, in humans steviol glucuronide was detected in the plasma following ingestion of stevioside or reb A, with maximal concentrations detected 8 and 12 hours after administration, respectively (Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The toxicokinetic/ pharmacokinetic differences of steviol and steviol glucuronide were recently examined in rats and humans by Roberts *et al.* (2016) following administration of stevioside (40 mg/kg body weight). Peak plasma concentrations (C_{max}) of steviol were similar in both rats and humans but were slightly delayed in humans compared to rats. Similarly, C_{max} values for steviol glucuronide were also delayed in humans but were approximately 25-fold higher in humans than rats. Systemic exposure to steviol and steviol glucuronide based on the area under the curve ($AUC_{0\text{-}72h}$) was reported to be 2.8-fold and 57-fold greater in humans,

when compared to rats, respectively. These data show that the extent of conjugation of steviol to glucuronic acid is higher in humans than in rats. More detail is presented in Section 6.3.4.

In rats, free and conjugated steviol, as well as any un-hydrolyzed fraction of the administered glycosides, are excreted primarily in the feces *via* the bile (generally within 48 hours), with smaller amounts appearing in the urine (less than 3%) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Roberts and Renwick, 2008). In contrast, steviol glycosides are excreted in humans primarily as steviol glucuronide *via* the urine, along with very small amounts of the unchanged glycoside or steviol. Relative to amounts recovered in urine, larger amounts of steviol (unabsorbed steviol released from steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile) were also eliminated in the feces in humans (Kraemer and Maurer, 1994; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2007). The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water-soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are considered applicable to the assessment of the safety of steviol glycosides in humans given the similarities in metabolic fate.

In summary, with the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol. This common metabolite steviol is absorbed systemically, conjugated to glucuronic acid, and eliminated primarily *via* the urine in humans. Numerous *in vitro* studies have demonstrated that steviol glycosides have very similar rates of microbial hydrolysis in the gastrointestinal tract, despite differences in the number of sugar units attached to the steviol backbone. Therefore, the safety database that has been established for individual steviol glycosides (*e.g.,* stevioside, reb A, reb D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless of the steviol glycoside distribution of the preparation, including steviol glycosides with a high reb M content produced by enzymatic conversion of reb A.

6.2 Summary of Steviol Glycoside Safety Opinions by Scientific and Regulatory Authorities

6.2.1 United States

In the U.S., the FDA has raised no objections to 45 GRAS notices (GRN 252, 253, 275, 278, 282, 287, 303, 304, 318, 323, 329, 337, 348, 349, 354, 365, 367, 369, 375, 380, 388, 389, 393, 395, 418, 448, 452, 456, 461, 467, 473, 493, 512, 516, 536, 548, 555, 607, 619, 626, 632, 638, 656, 662, 667) submitted since 2008 for major individual steviol glycosides (stevioside, rebaudiosides A, C, D, and X/M), mixtures of steviol glycosides, and glucosylated/enzyme-modified steviol glycosides for use as general purpose sweeteners in food and beverages products. Of particular relevance, GRAS notice GRN No. 667 received no questions from the FDA regarding the GRAS status of reb M produced *via* an enzymatic bioconversion process for use as a sweetener in foods (U.S. FDA, 2017b,c). Similar to PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the reb M in GRN No. 667 is produced from stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes, which are derived from microorganisms (strains of *Pichia pastoris*) that have been genetically modified to produce these enzymes.

6.2.2 The Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The safety of steviol glycosides was reviewed by JECFA at 5 separate meetings (51st, 63rd, 68th, 69thand 82nd) in 1998, 2004, 2007, 2008, 2016. At the first meeting in 1998, JECFA was asked to specifically review the safety of stevioside. Following review of the available information, the Committee concluded that the data on stevioside were limited and highlighted the need for specifications for commercial materials. An ADI could not be established.

Subsequently in 2004, the Committee determined that the material of commerce for which tentative specifications were developed should be known as "steviol glycosides". New data as per the requests made at the earlier meeting were provided to the Committee for review. The Committee reviewed the newly available data which demonstrated that stevioside and reb A were not genotoxic and that the positive *in vitro* results for steviol and its oxidative derivatives were not confirmed *in vivo*. Although the Committee reviewed the results of a developmental study showing adverse effects on fertility following treatment of male rats with a crude aqueous extract of *S. rebaudiana*, the Committee referred back to the studies reviewed at the preceding meeting noting that in studies conducted with higher purity material, no reproductive or developmental effects were observed, and thus, the reproductive effects noted following administration of the crude extract were unlikely to be related to steviol glycosides. Although the Committee did not raise any further questions regarding the potential toxicity of steviol glycosides at this review, the Committee noted that pharmacological effects in patients with hypertension or type 2 diabetes were observed at doses of 12.5 to 25 mg/kg body weight/day of steviol glycosides (5 to 10 mg/kg body weight/day as steviol equivalents). Consequently, further information regarding the potential effects of steviol glycosides in subjects with diabetes and in normotensive and hypotensive populations was requested. At this time, a temporary ADI of 2 mg/kg body weight/day (expressed as steviol) for steviol glycosides was allocated, based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and a safety factor of 200 (JECFA, 2006).

In 2007, the Committee received additional data pertaining to the potential pharmacological effects of steviol glycosides in humans; yet, none of these studies were conducted with a material that met the specifications for steviol glycosides. However, the Committee was made aware of an ongoing human study that was designed to specifically address the Committee's previous concerns (Maki *et al*., 2008a,b) and thus the temporary ADI was extended until 2008. The specifications were revised and the tentative designation was removed.

In 2008, the Committee was presented with new data pertaining to the metabolic fate of steviol glycosides in rats and humans (Roberts and Renwick, 2008; Wheeler *et al*., 2008), subchronic and reproductive/ developmental toxicity of reb A specifically (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008), and the potential pharmacological effects of steviol glycosides in diabetic populations and individuals with normal or low-normal blood pressure (Maki *et al*., 2008a,b). The Committee concluded that the results of the human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose were sufficient to remove the additional safety factor of 2 and establish a full ADI of 4 mg/kg body weight (expressed as steviol) for steviol glycosides. The specifications for steviol glycosides were revised further, requiring not less than 95% of the 7 named steviol glycosides (stevioside, rebaudiosides A, B, C, dulcoside A, rubusoside, and steviolbioside).

During the Committee's 73rd meeting in 2010, JECFA revised the specifications for steviol glycosides to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria (JECFA, 2010). Although no specific studies have been conducted with these steviol glycosides individually, their

inclusion within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all steviol glycosides are degraded to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general.

At the 82nd meeting, the Committee reviewed data related to the safety of steviol glycosides that had become available since the 69th meeting and confirmed the acceptable daily intake of 0 to 4 mg/kg body weight, expressed as steviol (FAO, 2016). A new specifications monograph was prepared for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (the Committee also confirmed its inclusion in the ADI) based on details of a new manufacturing process that utilizes a strain of genetically modified *Yarrowia lipolytica* overexpressing the steviol glycoside biosynthetic pathway to produce reb A (JECFA, 2016b). New 'tentative' specifications were established for "Steviol Glycosides from *Stevia rebaudiana* Bertoni", showing a separation of the specifications based on source material used in the manufacturing process, and recognizing commercial products that contain not less than 95% of total steviol glycosides (on a dried basis), where steviol glycosides are defined as "a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, and deoxyglucose" (JECFA, 2016a). At the 84th meeting the tentative designation was removed, and 2 additional sugar moieties arabinose and galactose are to be included in the definition (JECFA, 2017).

6.2.3 Food Standards Australia/New Zealand (FSANZ)

Immediately prior to JECFA's 69th meeting, FSANZ conducted their own evaluation of the safety of steviol glycosides (FSANZ, 2008). In its assessment, FSANZ considered the data previously reviewed by JECFA, as well as supplementary data consisting of published and unpublished studies. FSANZ considered the toxicological database for stevioside to cover a range of toxicological endpoints, and concluded that the supplementary data were sufficient to revise JECFA's temporary ADI to a full ADI of 4 mg/kg body weight/day by removing the additional uncertainty factor of 2. Similar to JECFA, FSANZ has recently published specifications for steviol glycosides from *S. rebaudiana* that broaden the definition to include all individual steviol glycosides present in the *S. rebaudiana* Bertoni leaf, so long as the total steviol glycoside content is not less than 95% on a dried basis (FSANZ, 2017).

6.2.4 European Food Safety Authority (EFSA)

In 1985, the European Commission's SCF evaluated stevioside as a sweetener and concluded that its use was "not toxicologically acceptable" due to limited data on metabolism, mutagenicity, long-term, and reproductive and developmental toxicity (SCF, 1985). In a subsequent evaluation, the SCF examined newly available data on metabolism, genotoxicity, and long-term toxicity, but maintained that these data were inadequate to sufficiently assess the safety of stevioside (SCF, 1999). Specifically, the SCF continued to raise concerns related to the potential reproductive effects of steviol glycosides and recommended that a study in a rat strain other than the F344 rat be conducted (rat strain used in the 2 carcinogenicity studies on stevioside [Yamada *et al*., 1985; Toyoda *et al*., 1997]), since it is not possible to evaluate any potential effects on the testicular system in this strain of rats as it normally seems to develop testicular changes. The SCF (1999) also questioned the relevance of numerous other studies because the composition of the test material was not clearly defined. The potential mutagenic effects of steviol also continued to be a concern (SCF, 1999). Based on the SCF's review of stevioside, the European Commission rejected *Stevia* and stevioside for use as a sweetener (Geuns, 2003). However, in an independent review of the safety data previously reviewed by JECFA at its 69th meeting, EFSA corroborated JECFA's conclusion regarding the safety and concurred with the ADI previously established by JECFA of 4 mg/kg body weight/day for steviol glycosides, expressed as steviol equivalents (EFSA, 2010). Moreover, in a subsequent examination of steviol

glycoside safety, in response to a request to amend the specifications for steviol glycosides, EFSA recently concluded that safety studies conducted with reb A and stevioside (*i.e.,* individual steviol glycosides) can extend to other steviol glycosides due to the shared metabolic fate (EFSA, 2015). The EFSA Panel concluded that "extending the current specifications to include [two additional steviol glycosides], rebaudiosides D and M, as alternatives to reb A in the predominant components of steviol glycosides would not be of safety concern" and further to that, "considered that the ADI of 4 mg/kg body weight/day can also be applied where total steviol glycosides comprise more than 95% of the material".

6.2.5 Health Canada

Health Canada has conducted its own independent review of the available safety data for steviol glycosides (Health Canada, 2012). Further corroborating the conclusions by JECFA, FSANZ, and EFSA, Health Canada established an ADI of 4 mg/kg body weight/day for steviol glycosides, expressed as steviol glycosides, based on the NOAEL from the 2-year carcinogenicity study conducted by Toyoda *et al.* (1997) and an uncertainty factor of 100. In addition, based on their latest review, Health Canada expanded the definition of steviol glycosides to include all steviol glycosides in the *S. rebaudiana* Bertoni plant and no safety concerns were raised in their assessment (Health Canada, 2017).

6.3 New Data Related to the Safety of Steviol Glycosides

The safety of steviol glycosides was evaluated in the related GRAS notice GRN 667 for reb M produced using UDP-glucosyltransferase and sucrose synthase enzymes derived from genetically modified strains of *Pichia pastoris*, which included a search of the scientific literature to capture relevant publications, and therefore the safety information presented in GRN 667 is incorporated by reference. To identify new data related to the safety of steviol glycosides since the FDA review in 2017 of GRN 667, a comprehensive search of the scientific literature was conducted. The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The studies identified included genotoxicity studies and several studies in animals evaluating the safety, antidiabetic, and immune effects of steviol glycosides. In general, the results of these recent studies provide further support for the safety of steviol glycosides.

6.3.1 Genotoxicity

The results of a bacterial reverse mutation assay, conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Test Guideline 471, was recently published in which the genotoxic potential of rebaudioside A (>95% purity) produced by fermentation (by genetically modified yeast, *Yarrowia lipolytica*) was evaluated (Rumelhard *et al.*, 2016). In the study, rebaudioside A was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2 uvrA at concentrations of up to 5,000 µg/plate in the presence or absence of exogenous metabolic activation. The results indicate that rebaudioside A produced by fermentation is not genotoxic. The same preparation was tested in an *in vitro* micronucleus assay in cultured peripheral human lymphocytes conducted in accordance with OECD Test Guideline 487 (Rumelhard *et al.*, 2016). Consistent with the results of the preceding study, rebaudioside A was determined to lack genotoxic potential following incubation with lymphocytes in the presence and absence of exogenous metabolic activation at concentrations of up to 5,000 µg/mL. In studies using a crude ethanolic extract obtained from *S. rebaudiana* leaves, negative results were reported in a reverse mutation assay in *S. typhimurium*, an *in vivo* mouse micronucleus test, and an *in vivo* mouse sperm malformation assay; these findings support the safety of products derived from *S. rebaudiana* Bertoni

leaves (Zhang *et al*., 2017). These findings corroborate the previous conclusions by JECFA (2010) that steviol glycosides and steviol are not genotoxic.

To investigate the anticancer potential of stevioside, the cytotoxicity and genotoxicity of stevioside (purity not reported) was evaluated using CCD18Co myofibroblast cells (non-targeted cell) and human colon derived cancer cells HCT 116 (targeted cell) (Sharif *et al*., 2017). The MTT assay, an indicator of toxicity, was used to assess cell viability in the presences of stevioside at concentrations of 0, 12.5, 25, 50, 100, and 200 µM. An alkaline comet assay, an indicator of genotoxicity, was employed to measure the presence of DNA strand breaks when cells were treated with 200 µM stevioside. A CometScore software program was used to quantify DNA tail intensity and tail moment. Stevioside was not cytotoxic to either cell line at up to 100 µM, and although both cell lines reported significant decreases in cell viability when exposed to 200 µM stevioside, the relative decrease between the 2 cells lines was not significantly different. With respect to genotoxicity, no differences in DNA tail intensity were measured in either cell line compared to control, and no change in tail moment was measured in the CCD18Co cells when exposed to 200 µM stevioside. A significant increase in tail moment was reported in HCT 116 cells compared to control, and slight DNA fragmentation was observed in these cells using fluorescence microscopy. The authors concluded that stevioside did not elicit cytotoxic or genotoxic effects in the non-targeted CCD18Co myofibroblast cells, and although some evidence of DNA damage was reported in the targeted HCT 116 cancer cells, the results do not suggest that stevioside has potent anticancer potential in HCT 116 cells.

6.3.2 Repeat-Dose Studies

Rebaudioside A (>95% purity) produced by fermentation (by genetically modified yeast, *Y. lipolytica*) was administered to Sprague-Dawley rats for a total of 90 days and was mixed in the diet at dose levels of 0, 500, 1,000, or 2,000 mg/kg body weight/day (N=20 per sex per group) (Rumelhard *et al.*, 2016). No test article-related systemic or local toxicity was reported based on daily clinical observations and weekly physical examinations, and no deaths occurred in any group throughout the study. Males in the highest dose group experienced significantly lower changes in body weight, body weight gain, and cumulative body weight gain, resulting in mean body weights that were 5.9% lower than the control group at the end of the study. Females in the highest dose group also experienced some statistically significant decreases in body weight during the study, but at the end of the study, body weights between the synthesized rebaudioside A and control groups were equivalent. Consumption of rebaudioside A was not reported to influence food consumption. The study authors associated the changes in body weight with the decreased caloric value of the diet containing rebaudioside A and therefore did not consider these changes to be adverse. Neurological evaluations conducted during the final week of the study reported no differences between the control and test-article treated groups, and no ophthalmological findings were considered test-article related. Following 90 days of exposure, rebaudioside A was not reported to induce any changes in the hematology profile, serum chemistry, or urinalysis parameters, and had no effect upon gross pathological findings, organ weights, or histopathology. Based on these results, the authors concluded that the NOAEL for rebaudioside A (described as 'fermentative') was at least the highest dose tested (2,000 mg/kg body weight/day) and that the safety profile of rebaudioside A is similar to plant derived rebaudioside A (Rumelhard *et al*., 2016).

In another 90-day repeat-dose oral toxicity study, groups of male and female Sprague-Dawley rats (10/sex/group) were provided diets containing an ethanolic extract of *S. rebaudiana* Bertoni leaves at doses of 570, 1,163, and 1,700 mg/kg body weight for females and 724, 1,464, and 2,238 mg/kg body weight for males (*i.e.,* up to 270 times the manufacturer-recommended daily intake) (Zhang *et al*., 2017). There were no mortalities and no treatment-related adverse clinical effects throughout the study. Clinical chemistry and hematological findings revealed no consistent dose-dependent trends. Organ weights, macroscopic

evaluations, and microscopic evaluations reported no treatment-related effects. It is noted that this study did not evaluate the complete set of organs recommended by the OECD (OECD, 1998). The study also evaluated a test article that does not meet the purity specifications established by JECFA, which contained approximately 47.78% polyphenols (mostly isochlorogenic acids) with the remainder consisting of soluble fibers and glucose. Regardless of these limitations, the results of this study support the safety of stevia leaf-derived products.

6.3.3 Antidiabetic Effects

In vitro and *in vivo* studies were conducted to examine the effects of steviol and steviol glycosides on pancreatic β-cell function and taste preferences of mice (Philippaert *et al*., 2017). In particular, the authors were interested in the relationship between steviol glycosides and TRPM5, an ion channel present in pancreatic β-cells and type II taste receptors that is associated with sweet, bitter, and umami taste perception. The *in vitro* and *in vivo* studies conducted using *Trpm5-/-* mice were able to demonstrate that: a) stevioside, reb A, and steviol potentiate the activity of TRPM5; b) TRPM5 facilitates insulin release from the islet cells; c) potentiation of TRPM5 activity by steviol glycosides modulates and intensifies bitter, sweet, and umami taste responses, and d) the glucose lowering effect of stevioside is dependent on TRPM5 expression in pancreatic islets. In addition, the effect of chronic stevioside treatment (25 mg/kg, 0.1% solution in drinking water) on the development of diabetes induced by a high-fat diet (HFD) on male mice (C57BI6/J wildtype or *Trpm5-/-*, n=8 per group) was examined. Mice either served as the control group (HFD) or were treated with stevioside (HFD plus stevioside). Following consumption of the HFD for 20 weeks, a time-dependent development of glucose intolerance was observed in the wildtype control group using an intraperitoneal glucose tolerance test, whereas wildtype mice treated with stevioside (HFD plus stevioside) had normal glycemic profiles after 20 weeks. *Trpm5-/-* mice showed no differences in control (HFD) and treatment (HFD plus stevioside) groups. The authors also considered reversal of glucose homeostasis by stevioside withdrawal in male mice (C57BI6/J, n=8 to 10 per group). The mice were divided into the following groups: a 15-week HFD with stevioside treatment (124 µM stevioside in drinking water; mg/kg dose not stated), a 10-week HFD with stevioside followed by a 5-week HFD without stevioside, and a control group on a 15-week HFD. Results demonstrated an improved glucose tolerance when mice on a HFD were administered stevioside. However, deteriorated glucose tolerance was observed in mice on a HFD treated with stevioside for 10 weeks, followed by removal of stevioside for 5 weeks, with levels similar to that of untreated mice. The authors concluded that targeting TRPM5 may have the potential to prevent and treat type 2 diabetes. It was also suggested that other modulators of TRPM5 including, stevioside, reb A, and steviol may play a role in the future development of TRPM5-targetted antidiabetic drugs.

Chronic reb A exposure in circadian rhythms, insulin action *in vivo*, and susceptibility to diet-induced obesity was evaluated in male C57BL6/J mice (10/group) (Reynolds *et al*., 2017). Groups were administered reb A at a concentration of 0.1% (116 to 207 mg/kg body weight/day) in drinking water or were provided with normal drinking water over a period of about 7 months. During the 32-day treatment period, mice were placed in cages with running wheels, and wheel running activity was monitored over a 12-hour light-dark cycle and in complete darkness. Following a 3-month recovery period, mice were tested for glucose, pyruvate, and insulin tolerance (*i.e., in vivo* insulin action) with additional 7- to 10-day recovery periods between each test. The authors also assessed the mice in their susceptibility to obesity by providing a high fat diet for 2 months. Glucose, insulin, and pyruvate tolerance tests were conducted again and showed similar results among treatment and control groups. In the same manner, exposure to reb A had no effect on the susceptibility to diet-induced obesity.

6.3.4 Other Physiological Effects

The effects of stevioside (>95% purity) were studied in *in vivo* and *in vitro* studies using rat plasma levels of tumor necrosis factor-*alpha* (TNF-α) and IL-1β, and their release from isolated rat peripheral blood mononuclear cells (PBMCs) (Noosud *et al*., 2017). Stevioside was administered *via* oral gavage to male Wistar rats (170 to 220 g in weight; n=6/group) at doses of 0, 500, and 1,000 mg/kg body weight/day over a period of 6 weeks. Plasma and PBMCs were isolated from the rats' blood after the exposure period. PBMCs were stimulated with and without lipopolysaccharide (LPS) *in vitro* for 24 hours to induce cytokine production. Supernatant fluids were collected and the release and concentrations of TNF-α and IL-1β were measured using rat enzyme-linked immunosorbent assay (ELISA) kits. Cell viability between stevioside-treated and control groups were comparable, indicative of the non-toxic nature of stevioside following oral intake. Concentrations of TNF-α and IL-1β were not detected in the plasma of control or treatment groups. When PBMCs were stimulated with LPS *in vitro*, stevioside exposed cells (both doses) released TNF-α and IL-1β. However, the levels of cytokines were significantly decreased when compared to the control group, indicating the inhibitory effect of stevioside on cytokine release. The authors concluded that stevioside may have the ability to inhibit release TNF-α and IL-1β (pro-inflammatory cytokines) *in vivo,* however, further studies should be conducted. It is noted that the doses utilized in this study greatly exceed the current ADI for steviol glycosides.

A study by Potočnjak *et al.* (2017) investigated the impact of stevioside exposure in mice with cisplatin-induced nephrotoxicity. Groups of male BALB/cN mice received either water (n=4), water combined with a single intraperitoneal injection of cisplatin (13 mg/kg, n=5), or stevioside (98% purity) combined with a single intraperitoneal injection of cisplatin (n=5). Cisplatin was administered 48 hours prior to 2 daily doses of oral stevioside (50 mg/kg). Treatment with stevioside was reported to: a) normalize relative kidney weight, blood urea nitrogen, and serum creatinine levels to control levels; b) attenuate the morphological changes, inflammation, and oxidative stress in the kidney induced by cisplatin; and c) reduce apoptosis and cell-cycle arrest induced by cisplatin in kidney cells. The authors concluded that stevioside exhibited renoprotective effects in this mouse-model of cisplatin-induced acute kidney injury, and that further studies are needed to confirm these protective effects in patients.

6.3.5 Revision of the Acceptable Daily Intake for Steviol Glycosides

The ADI for steviol glycosides of 4 mg/kg body weight/day (expressed as steviol) is calculated based on a NOAEL of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from the 2-year carcinogenicity study in rats conducted by Toyoda *et al.* (1997) and application of a safety factor of 100 (FSANZ, 2008; JECFA, 2009; EFSA, 2010; Health Canada, 2012). As defined by the World Health Organization, the standard safety factor value of 100 to account for inter- and intra-species differences (a 10-fold factor for each) may be adjusted using chemical-specific adjustment factors (CSAFs). For example, using appropriate toxicokinetic/toxicodynamic data the safety factor of 10 that is applied to account for inter-species differences can be modified based on the chemical-specific data, and can be broken down into its 2 components that account for toxicokinetic (4-fold factor) and toxicodynamic (2.5-fold factor) differences.

In a recent study published by Roberts *et al.* (2016), the toxicokinetic differences of steviol and steviol glucuronide were compared in rats and humans following a single oral dose of 40 mg stevioside/kg body weight. Blood samples were collected pre-dose and through 72 hours post-dose and were assayed for steviol and steviol glucuronide. Peak plasma concentrations (C_{max}) of steviol were similar in both rats and humans (see below) but were slightly delayed in humans compared to rats. Similarly, C_{max} values for steviol glucuronide were also delayed in humans but were approximately 25-fold higher in humans than rats

(approximately 4,440 ng/mL *vs.* 180 ng/mL). Systemic exposure to steviol and steviol glucuronide assessed using the area under the curve ($AUC_{0\text{-}72h}$) was 2.8-fold (~1,650 ng·h /mL *vs*. ~590 ng·h /mL) and 57-fold (~136,000 ng·h /mL *vs*. ~2,400 ng·h /mL) greater in humans than rats, respectively. As such, the AUC and C_{max} data were used to calculate the CSAF as follows:

a) the $AUC_{0\text{-}72}$ for free steviol in humans (1,631 ng·h/mL) is higher than the AUC_{last} in male and female rats (581 and 605 ng·h/mL, respectively), and therefore the ratio of AUC between humans and rats is 2.8;

b) the C_{max} values for free steviol in humans (77.21 ng/mL) are approximately equivalent to those in male and female rats (76.0 and 87.1 ng/mL, respectively), and therefore the ratio of C_{max} values is approximately one;

c) the standard safety factor of 4 for toxicokinetic interspecies differences can therefore be revised to range from 1 to 2.8;

Applying the CSAF of 1 to 2.8 for toxicokinetic differences between rats and humans when calculating the ADI for steviol glycosides revises the standard safety factor of 10 for interspecies differences to range from 2.5 [$1_{(toxicokinetic)}$ x $2.5_{(toxicodynamic)}$] to 7 [$2.8_{(toxicokinetic)}$ x $2.5_{(toxicodynamic)}$], and decreases the overall safety factor of 100 to range from 25 to 70 (human variability), providing an ADI between 6 and 16 mg/kg body weight, as steviol equivalents (Roberts *et al.,* 2016). Currently, the ADI assigned by JECFA is 0 to 4 mg/kg body weight, as steviol equivalents for stevia leaf extracts.

6.4 Safety of the Enzyme Production Microorganisms

The enzyme production strain *E. coli* LE1B109 is a derivative of the parental strain *E. coli* K-12 W3110. Its genome has been analyzed and absence of antibiotic resistance genes or any other sequence of concern has been confirmed. The enzyme production strain was evaluated using the decision tree developed by Pariza and Johnson (2001), and was accepted based on the conclusion that the final product (steviol glycosides with a high reb M content) meets JECFA specifications, as discussed in Section 2.3. The absence of the production microorganism in the final enzyme preparations is demonstrated for each enzyme batch, according to the product specifications.

The parental strain *E. coli* K-12 W3110 belongs to the well-defined taxonomic family of the *Enterobacteriaceae*. The primary habitat of *E. coli* is the lower intestinal tract of warm-blooded animals, where it represents the predominant aerobic microorganism. Non-pathogenic strains of *E. coli* are considered as commensal, although the host also derives some beneficial effects, mainly by preventing colonization by pathogens (Tenaillon *et al.*, 2010).

6.4.1 History of Use of the Parental Strain

The K-12 strain, and in particular the W3110 substrain, has been safely used as a laboratory organism for more than 50 years and is one of the most extensively characterized bacteria (Bachmann, 1972; Jensen, 1993).

E. coli K-12 has a long history of safe use in the industrial production of specialty chemicals and human drugs (U.S. EPA, 1997). For example, a food enzyme preparation (chymosin) obtained from a genetically modified *E. coli* K-12 strain was affirmed as GRAS by the FDA in 1990 (Flamm, 1991; Olempska-Beer *et al.*, 2006) and has been used safely for cheese production worldwide. In the European Union there are

currently 3 food enzyme preparations derived from *E. coli* K-12 being assessed by EFSA as part of the requirements for authorization in accordance with Regulation (EC) 1331/2008 (European Commission, 2016). One of them, D-allulose 3-epimerase, has recently been the subject of a GRAS notification, receiving no questions from the FDA (U.S. FDA, 2016). The other 2 food enzyme preparations derived from *E. coli* K-12, two different cyclomaltodextrin glucotransferases, have been safely used for years in the production of the novel food ingredients alpha- and gamma-cyclodextrin, authorized by the European Commission in 2008 and 2012, respectively.

6.4.2 Pathogenicity and Toxicogenicity of the Parental Strain

E. coli K-12 is not considered a human or animal pathogen and has accordingly been classified as belonging to Risk Group 1 in the NIH Guidelines (NIH, 2016). Moreover, it is often used as a non-pathogenic reference when studying the virulence factors of pathogenic *E. coli* strains (Blanc-Potard *et al.*, 2002; Kaper *et al.*, 2004). *E. coli* K-12 and its derivatives are essentially unable to colonize the mammalian gastrointestinal tract, do not produce toxins that cause illness upon ingestion, including Shiga toxin, and are unable to persist in either water or soil (Bogosian *et al*., 1996; U.S. EPA, 1997). The parental laboratory strain W3110 does not carry any introduced antimicrobial resistance genes. The complete genomes of *E. coli* K-12 and specifically of the sub-strain W3110 have been sequenced, confirming the absence of toxigenic potential (Blattner *et al.*, 1997; Hayashi *et al.*, 2006).

6.5 Allergenicity

As discussed in Section 2.3.5, the final product does not contain residual protein and DNA as per the defined product specifications, and as demonstrated in 3 non-consecutive batches of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A. However, in order to confirm the lack of potential for cross-reactivity among the inserted heterologous gene sequences in the production strain, a sequence homology search was conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001) and the Codex Alimentarius (2009) using the AllergenOnline Database version 17 (available at http://www.allergenonline.org; updated January 18, 2017) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2017). This was done to determine whether the genes encoding for UDP-glucosyltransferase and sucrose synthase enzymes used in the manufacturing process of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A contains amino acid sequences similar to other known allergens that might produce an allergenic response. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety.

E-values ranging between 0.07 to 0.77 were identified for the sequences of interest in comparison to known allergens from Bermuda grass, snails, oysters, wheat, or cypress trees. E-scores larger than $1x10^{-7}$ are unlikely to identify proteins that may share immunologic or allergic cross-reactivity to known allergens (Hileman *et al.*, 2002). Additionally, none of the sequences encoding UDP-glucosyltransferase and sucrose synthase enzymes shared greater than 50% identity with the identified allergens, indicating the unlikely potential for cross-reactivity. Furthermore, in addition to the full-length FASTA search, and in accordance with FAO/WHO guideline, the database was also searched using a sliding window of 80-amino acid sequences derived from the full-length UGTSr1, UGTSl2, and SuSy amino acid sequences. The 80-amino acid alignment search was conducted using default settings (*E* value cut-off = 1 and maximum alignments of 20). According to the approach adopted by the Codex Alimentarius Commission, significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen must be considered a possibility. Using this search strategy, no identity matches of greater than 35% were identified.

6.6 Expert Panel Evaluation

PureCircle has concluded that steviol glycosides with a high reb M content produced by enzymatic conversion of reb A meeting appropriate food-grade specifications and manufactured consistent with cGMP is GRAS for use as an ingredient in various food products, as described in Part 1.3, on the basis of scientific procedures. Steviol glycosides manufactured by PureCircle *via* enzymatic conversion of reb A from stevia leaf extract are substantially equivalent to steviol glycoside products currently in the market, including those extracted from the leaves of *S. rebaudiana.*

The GRAS status of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is based on conclusions of scientific bodies and regulatory authorities regarding steviol glycoside safety, data generally available in the public domain pertaining to the safety of steviol glycosides, and a unanimous opinion among a panel of experts ("Expert Panel"), who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of the following qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley M. Tarka Jr., Ph.D. (The Tarka Group Inc., and The Pennsylvania State University, College of Medicine).

The Expert Panel, convened by PureCircle, independently and critically evaluated all data and information presented herein, and concluded that steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is GRAS for use as a general purpose sweetener, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the Expert Panel and evaluation of such data as it pertains to the proposed GRAS uses of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, are presented in Appendix A.

6.7 Conclusions

Based on the data and information presented herein, PureCircle has concluded that steviol glycosides with a high reb M content produced by enzymatic conversion of reb A from stevia leaf extract, meeting appropriate food-grade specifications and manufactured according to cGMP, is safe for use as a general purpose sweetener as presented in Section 1.3. PureCircle also has further concluded that pivotal data and information relevant to the safety of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are publicly available and therefore the intended uses of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A can be concluded to be GRAS on the basis of scientific procedures.

Part 7. §170.255 List of Supporting Data and Information

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Part	Section §	Section Title
Subchapter B—Food for Human Consumption		
172—Food Additives Permitted for Direct Addition to Food for Human Consumption		[all sections of Part 172]
173—Secondary Direct Food Additives Permitted in Food for Human Consumption	173.25	Ion-exchange resins
182—Substances Generally Recognized as Safe		[all sections of Part 182]
184—Direct Food Substances Affirmed as Generally Recognized as Safe		[all sections of Part 184]
	184.1205	Calcium hydroxide
	184.1293	Ethyl alcohol
	184.1857	Corn sugar
Subchapter E—Animal Drugs, Feeds, and Related Products		
573—Food Additives Permitted in Feed And Drinking Water of Animals		[all sections of Part 573]

Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
582—Substances Generally Recognized as Safe		[all sections of Part 582]

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Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of Steviol Glycosides with a High Rebaudioside M Content Produced by Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract for Use as a General Purpose Sweetener

October 20th, 2017

INTRODUCTION

PureCircle Ltd. (herein "PureCircle") intends to market steviol glycosides with a high rebaudioside M (reb M) content, produced *via* a manufacturing process that utilizes enzymes derived from *Escherichia coli* K-12 (*E. coli* K-12) to convert rebaudioside A (reb A) from stevia leaf extract to a high reb M containing steviol glycoside mixture, as a general purpose sweetener in the United States (U.S.). Steviol glycosides are natural constituents of the *Stevia rebaudiana* Bertoni (*S. rebaudiana*) plant and are typically extracted from the dried leaves *via* a hot water extraction process. PureCircle has developed an alternative manufacturing process for producing a blend of steviol glycosides consisting of >30% reb M (hereinafter referred to as "steviol glycosides with a high reb M content") that utilizes enzymes (UDP-glucosyltransferases and sucrose synthase) derived from genetically modified *E. coli* K-12 that convert reb A extracted from the leaves of *S. rebaudiana* to reb M. Following the conversion process, steviol glycosides with a high reb M content is purified to meet or exceed the ≥95% steviol glycoside purity definition established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

At the request of PureCircle, an Expert Panel of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether, under the conditions of intended use as a sweetening agent, steviol glycosides with a high reb M content produced by enzymatic conversion of reb A would be "Generally Recognized as Safe" (GRAS), based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley M. Tarka Jr., Ph.D. (The Tarka Group Inc., and The Pennsylvania State University, College of Medicine). For purposes of the Expert Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined by the U.S. Food and Drug Administration (FDA) in 21 CFR 170.3(i) (U.S. FDA, 2016).

The Expert Panel independently and collectively evaluated a dossier [Documentation Supporting the Enzymatic Conversion of Rebaudioside A from Stevia Leaf Extract to a High Rebaudioside M Steviol Glycoside Mixture as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener] that included a comprehensive summary of scientific information on steviol glycosides with a high reb M content produced by enzymatic conversion of reb A. This dossier was prepared from information available within the public domain and also included details pertaining to the method of manufacture, product specifications, supporting analytical data, intended use-levels in food and beverages, consumption estimates for all intended uses, and a summary of the comprehensive safety literature for steviol glycosides. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent, critical evaluation of such data and information, the Expert Panel convened on October 20th, 2017 *via* teleconference and unanimously concluded that the intended use described herein for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, meeting appropriate food-grade specifications as described in the supporting dossier and manufactured according to current Good Manufacturing Practice (cGMP), is safe, suitable, and GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

CHEMISTRY AND MANUFACTURING

The ingredient that is the subject of this GRAS evaluation is a mixture of steviol glycosides, consisting of >30% reb M along with other individual steviol glycosides (*e.g.,* rebaudiosides A, D, I, M, M2), which is produced by enzymatic conversion of reb A extracted from the leaves of the *S. rebaudiana* plant. The final purified product contains ≥95% total steviol glycosides, consistent with the purity criteria for steviol glycosides as established by JECFA (2016a). The molecular structures of all steviol glycosides are similar, consisting of a common steviol backbone linked to differing sugar moieties (*e.g.,* glucose, xylose, rhamnose, fructose, deoxyglucose, arabinose, and/or galactose) *via* 1,2-; 1,3-; 1-4- or 1,6- α or β-glycosidic linkages. Despite these small differences in structure, all steviol glycosides share a common metabolic pathway in which they are hydrolyzed in the gastrointestinal tract to steviol, the metabolite that is absorbed systemically, conjugated with glucuronic acid, and excreted primarily *via* the urine in humans.

Steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is manufactured in a facility certified under Food Safety System Certification (FSSC) 22000:2010 and all raw materials, processing aids, and purification equipment used are food-grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses, and/or are considered safe and suitable for use in the production of probiotic ingredients or microbial-derived enzyme preparations. In the first stage of the manufacturing process, reb A is extracted from the leaves of *S. rebaudiana* and purified to ≥95% reb A consistent with the methods and specifications outlined by JECFA for steviol glycosides from S. *rebaudiana* Bertoni (FAO, 2016; JECFA, 2016a). In the second stage of the manufacturing process, genetically modified strains of *E. coli* K-12 are fermented in sterilized culture medium to produce UDP-glucosyltransferase and sucrose synthase enzymes. The Expert Panel reviewed information pertinent to the construction of the enzyme production microorganisms and noted that the incorporated DNA was sourced from natural sources (*i.e.,* plants) and is not associated with any known allergens or toxins, and that the parental strain *E. coli* K-12 is not toxigenic or pathogenic. The enzymes are isolated from the fermentation biomass by standard techniques and the final purified enzyme preparations are free of antibiotics, are food-grade, and conform to the recommended purity criteria for enzyme preparations established by the Food Chemicals Codex. In the third stage of the manufacturing process, the purified reb A powder (≥95%) from stevia leaf extract is reacted with the purified UDP-glucosyltransferase and sucrose synthase enzymes to generate a mixture of steviol glycosides. The use of different reaction times yields steviol glycoside mixtures with different ratios of starting glycoside reb A, intermediate glycosides such as reb D, and the primary final glycoside product reb M. In the fourth and final stage of the manufacturing process, the steviol glycoside mixture is purified in accordance with the methodologies outlined in the Chemical and Technical Assessment (CTA) published by FAO/JECFA for steviol glycosides (FAO, 2016), yielding a final product that contains ≥95% total steviol glycosides specifically comprised of reb M and other steviol glycosides (*e.g.,* rebaudiosides A, D, I, M, M2).

[1] Compliant with the specifications set forth in the Food Chemicals or equivalent international food or pharmacopeia standard (*e.g.,* JECFA, CODEX, USP, EP).

Physical and chemical specifications for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A from stevia leaf extract were established based on the specifications set by JECFA for steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2016a). Microbiological specification parameters have been established to ensure safe use in food, and parameters for residual protein and DNA are included to ensure that the *E. coli* production strain and the enzymes used to convert reb A to reb M are not present in the final product. Total steviol glycoside content is measured using the high-performance liquid chromatography (HPLC) method described in the most recent JECFA specification monograph for steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2016a). Batch samples of steviol glycoside extract preparations are routinely tested to verify compliance with the established chemical and microbiological parameters, and the Expert Panel reviewed data provided for 3 non-consecutive lots of the final product. The Expert Panel also reviewed data demonstrating that the individual steviol glycoside distribution in the final product may vary depending on the length of the enzyme reaction time with reb A, yet, the final product consistently contains no less than 95% total steviol glycosides.

Although JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions (JECFA, 2007), PureCircle undertook a series of studies to confirm the storage stability and pH/temperature stability of powder and in solution samples of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, respectively. Similar to the conclusions made by JECFA for other steviol glycosides, steviol glycosides with a high reb M content were shown to be stable at pH values ranging from 4.0 to 8.0 for 12 weeks at 5, 25, and 37°C.

INTENDED FOOD USES AND ESTIMATED INTAKE

The Expert Panel understands that the proposed use of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A will be as a general purpose sweetener that will be added to a variety of food products, consistent with the current uses of other related high-intensity sweeteners that are already in the market. Based on post-market surveillance data for other high-intensity sweeteners and adjusting for relative sweetness intensity of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A (approximately 200 times sweeter than sucrose), the estimated intakes were calculated for adults and children (Table 1). The mean intake of steviol glycosides with a high reb M content produced by enzymatic conversion was predicted to range across all groups from 1.28 mg/kg body weight/day for non-diabetic adults to 3.36 mg/kg body weight/day for diabetic children, equivalent to 0.32 and 0.84 mg steviol equivalents/kg body weight/day for non-diabetic adults and diabetic children, respectively. Predicted intakes for heavy consumers ranged across all groups from 3.38 mg/kg body weight/day for non-diabetic adults to 4.95 mg/kg body weight/day for non-diabetic children, equivalent to 0.85 and 1.24 mg steviol equivalents/kg body weight/day for non-diabetic adults and non-diabetic children, respectively. Accordingly, the highest intake estimate for steviol glycosides with a high reb M content of 1.24 mg/kg body weight/day, as steviol equivalents, derived for non-diabetic children under the proposed conditions of use is below the current Acceptable Daily Intake (ADI) for steviol glycosides of 0 to 4 mg/kg body weight, expressed as steviol, as established by JECFA (2010).

Table 1 **Estimated Consumption of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A Using Renwick's (Renwick, 2008) Methodology of Intense Sweetener Intake Assessment**

Population Group	Intakes of intense sweeteners (expressed as sucrose equivalents) (mg/kg bw/day)		Consumption estimates for:			
			Steviol glycosides with a high reb M content[a] (mg/kg bw/day)		Steviol glycosides with a high reb M content as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	1.28	3.38	0.32	0.85
Diabetic Adults	280	897	1.40	4.49	0.35	1.12
Non-diabetic Children	425	990	2.13	4.95	0.53	1.24
Diabetic Children	672	908	3.36	4.54	0.84	1.14

bw = body weight, reb = rebaudioside

[a] Approximately 200 times as sweet as sucrose.

[b] Calculated based on the molecular weights of steviol (318.45 g/mol) and reb M (1,291.3 g/mol) [steviol conversion factor of 0.25]

INFORMATION TO ESTABLISH SAFETY

The Expert Panel reviewed the available data to support the safety of steviol glycosides in general, and utilized these data to establish the safety of PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A. This information included a detailed discussion of the metabolic fate of steviol glycosides, a summary of the conclusions made by global scientific and regulatory authorities regarding the safety of steviol glycosides and the data deemed pivotal in determining safety, and a review of any new studies published in the scientific literature. Furthermore, information related to the safety of the *E. coli* parental and production strains for the enzymes was considered by the Expert Panel, including assessment of the potential allergenicity of the inserted heterologous gene sequences in the production strains.

In vitro and *ex vivo* studies have demonstrated that steviol glycosides are not hydrolyzed by digestive enzymes of the upper gastrointestinal tract due to the presence of β-glycosidic bonds and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea *et al*., 1997; Geuns *et al*., 2003, 2007; Koyama *et al*., 2003a). Therefore, steviol glycosides enter the colon intact, where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Gardana *et al*., 2003; Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon, reviewed extensively by Renwick and Tarka (2008), have confirmed the ability of gut microflora from rodents and humans to hydrolyze steviol glycosides completely to steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Nikiforov *et al.*, 2013; Purkayastha *et al.,* 2016). Steviol glycosides are hydrolyzed sequentially, removing one sugar moiety at a time, with differences in the degradation rates depending on the structural complexities of each steviol glycoside (Wingard *et al*., 1980; Koyama *et al*., 2003b). Despite these structural differences, several parallel *in vitro* comparisons between rebaudioside A and individual steviol glycosides have demonstrated a remarkable similarity with respect to the rate of hydrolysis of different steviol glycosides to steviol in the presence of human fecal homogenates, indicating that the number and location of sugar units attached to the steviol backbone does not significantly affect the rate of microbial hydrolysis (Purkayastha *et al.,* 2014, 2015, 2016). Steviol is absorbed systemically into the portal vein and distributed to a number of organs and tissues, including the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and

Renwick, 2008). In the liver, steviol is conjugated with glucuronic acid to form steviol glucuronide (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). In humans, steviol glycosides are eliminated as steviol glucuronide with very small amounts of the unchanged glycoside or steviol *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). Based on this shared metabolic fate of steviol glycosides, the safety database that has been established for individual steviol glycosides (*e.g.,* stevioside, rebaudioside A, rebaudioside D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless of the steviol glycoside distribution of the preparation, including steviol glycosides with a high reb M content produced by enzymatic conversion of reb A.

Stevia plant extracts have a long history of human consumption due to the characteristically sweet taste of steviol glycosides. JECFA has reviewed the safety of steviol glycosides at 5 separate meetings (51st, 63rd, 68th, 69th and 82nd) and established an ADI of 0 to 4 mg/kg body weight, expressed as steviol equivalents, based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year carcinogenicity study in rats (Toyoda *et al.*, 1997). At the 82nd meeting, a new specifications monograph was prepared for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (the Committee also confirmed its inclusion in the ADI) based on details of a new manufacturing process that utilizes a strain of genetically modified *Yarrowia lipolytica* overexpressing the steviol glycoside biosynthetic pathway to produce rebaudioside A (JECFA, 2016b). Also, new 'tentative' specifications were established for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" recognizing commercial products that contain not less than 95% of total steviol glycosides[2] (on a dried basis). The safety of steviol glycosides has been extensively reviewed by JECFA and numerous other scientific bodies and regulatory agencies, including the U.S. FDA, the European Commission's Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA), Food Standards Australia/New Zealand (FSANZ), and Health Canada, who have all concluded that preparations containing no less than 95% steviol glycosides are safe when used in accordance with cGMP and have confirmed the JECFA ADI (SCF, 1985, 1999; FSANZ, 2008, 2017; EFSA, 2010, 2015; Health Canada, 2012, 2017). Numerous other jurisdictions throughout the world have also approved the use of steviol glycosides in food and beverage products. The FDA has reviewed over 40 GRAS notifications for a variety of steviol glycoside preparations and to date has raised no objections regarding the GRAS status of steviol glycoside products for use as general purpose sweeteners in food and beverage products. Of particular relevance, this includes GRAS notice GRN No. 667 for reb M produced by enzymatic conversion of stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes derived from genetically modified strains of *Pichia pastoris* (U.S. FDA, 2017), which describes a similar manufacturing process to that employed by PureCircle to produce steviol glycosides with a high reb M content *via* enzymatic conversion of reb A.

E. coli K-12 is a well-characterized bacterium that has an extensive history of safe-use in the industrial production of specialty chemicals and drugs for human use (U.S. EPA, 1997). Several food enzyme preparations derived from strains of *E. coli* K-12 have GRAS status for use in foods in the U.S. (*i.e.,* chymosin, D-allulose 3-epimerase) and/or are authorized for use in the European Union, and have a history of safe use in the production of foods and food ingredients. *E. coli* K-12 is not considered a human or animal pathogen and has accordingly been classified as belonging to Risk Group 1 in the NIH Guidelines (NIH, 2016). The parental *E. coli* K-12 genome does not carry any introduced antimicrobial resistance genes and is absent of toxigenic potential. The potential for cross-reactivity among the inserted heterologous gene sequences in the modified *E. coli* K-12

[2] Steviol glycosides are defined as "a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, and deoxyglucose" (JECFA, 2016a). At the 84th meeting the tentative designation was removed, and 2 additional sugar moieties arabinose and galactose are to be included in the definition (JECFA, 2017).

enzyme production strain was investigated in accordance with the FAO/WHO protocol for bioinformatic allergenicity assessment (FAO/WHO, 2001) and Codex Alimentarius (2009). A search of the amino acid sequences of the inserted heterologous gene sequences in the production strain for matches to known putative allergens using the web-based database AllergenOnline (FARRP, 2017). Full-length FASTA alignment did not identify any similarity greater than 50% to known allergen sequences, indicating the unlikely potential for cross-reactivity to any known allergens. Furthermore, given that no protein or DNA is present in the final steviol glycoside product as per the defined product specifications, the Expert Panel concluded that the potential allergenicity of the heterologous gene sequences inserted in the enzyme production strains should not be a health concern.

The scientific evidence examined by the Expert Panel demonstrates that under the conditions of intended use, steviol glycosides with a high reb M content produced by enzymatic conversion of reb A would not produce any adverse health effects.

CONCLUSION

We, the Expert Panel, have independently and collectively, critically evaluated the data and information summarized above as well as other information that we deemed pertinent to the safety of the proposed use of PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A as a general purpose sweetener. We unanimously conclude that under the conditions of intended use in foods specified herein, PureCircle's steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, meeting appropriate food-grade specifications and produced in accordance with current Good Manufacturing Practice (cGMP), is safe and Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts, critically evaluating the same information, would concur with our conclusion.

(b) (6)

25 October 2017

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From: Sidd Purkayastha
To: Perrier, Judith
Subject: GRN 745
Date: Wednesday, February 21, 2018 11:59:30 AM

Dear Dr Parrier
The terminal date for lit review was Sept 7 2017, same as for other notification.

Best regards
Sidd

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