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August 31, 2018

Via FedEx & CD-ROM

Dr. Susan Carlson
Director, Division of Biotechnology and GRAS
Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Amyris Inc. Steviol Glycosides Rebaudioside M

Dear Dr. Carlson:

We respectfully submit the attached GRAS Notification on behalf of our client, Amyris Inc. for Steviol Glycosides Rebaudioside ("Reb") M produced by fermentation to be used as a general-purpose sweetening agent, excluding infant formulas and meat and poultry products. The uses and use-levels of Amyris's Steviol Glycosides Reb M produced by fermentation are to reflect those currently permitted for other high-intensity sweeteners in the United States. More detailed information regarding product identification, intended use levels, and the manufacturing and safety of the ingredient is set forth in the attached GRAS Notification.

Amyris Inc. has determined that their Steviol Glycosides Reb M is GRAS based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and in conformance with the guidance issued by the Food and Drug Administration (FDA) under 21 C.F.R. § 170.36, 81 Fed. Reg. 54960 (Aug. 17, 2016). Therefore, the use of the Steviol Glycosides Reb M as described in this GRAS Notification is exempt from the requirement of premarket approval as set forth in the Federal Food, Drug, and Cosmetic Act.

The analytical data, published studies, and information that are the basis for this GRAS Notification are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001, or will be sent to FDA upon request.

We look forward to the Agency's review of this submission and would be happy to provide Agency officials with any information they may need to complete their assessment. Thank you for your attention to this matter.

Sincerely,

(b) (6)

Evangelia C. Pelonis

GRAS Notice for Steviol Glycosides Rebaudioside M

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

Submitted by:

Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

On behalf of our client

Amyris Inc.
5885 Hollis Street, Suite 100
Emeryville, CA 94608
United States

August 31, 2018

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Part 1 – Signed statements and certification

(1) Applicability of 21 C.F.R. part 170, subpart E

We submit this generally recognized as safe (GRAS) notice in accordance with proposed 21 C.F.R. part 170, subpart E.

(2) Name and address of the notifier

Company: Amyris Inc.
Name: Fernando Garcia
Address: 5885 Hollis Street, Suite 100, Emeryville, CA 94608
Phone: (510) 597-4835
Email: garcia@amyris.com

All communications on this matter are to be sent to Counsel for Amyris Inc.

Evangelia C. Pelonis
Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington DC 20005
Tel: 202-434-4106
Fax: 202-434-4646
Email: pelonis@khlaw.com

(3) Name of the notified substance

Steviol glycosides rebaudioside ("reb") M.

(4) Applicable conditions of use of the notified substance

Amyris Inc. intends to market steviol glycosides reb M produced by fermentation as a general-purpose sweetening agent in the United States, 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 technological properties (*e.g.,* sweetness potency). Considering that steviol glycosides, including Amyris's

steviol glycosides reb M produced by fermentation, are characterized by a sweetness intensity that is comparable to that of other high-intensity sweeteners (*e.g.*, aspartame is approximately 200 times as sweet as sucrose, steviol glycosides reb M produced by fermentation is approximately 200-300 times sweeter than sucrose), the uses and use-levels of steviol glycosides reb M produced by fermentation primarily reflect those currently permitted for other high-intensity sweeteners in the U.S.

(5) Basis for the GRAS determination

Keller and Heckman LLP, on behalf of Amyris Inc., hereby notifies the Agency of its determination that the steviol glycosides reb M produced by fermentation is Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This GRAS conclusion is based on scientific procedures in accordance with 21 C.F.R. §170.30(a) and (b) and conforms to the guidance issued by the Food and Drug Administration (FDA) under 21 C.F.R. §170.36, 81 Fed. Reg. 54960 (Aug. 17, 2016). The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with proposed 21 C.F.R. § 170.36. The GRAS status of steviol glycosides reb M produced by fermentation is based on data generally available in the public domain and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of steviol glycosides reb M produced by fermentation as a component of food [see **Appendix I GRAS Expert Panel Report**].

(6) Exclusion from premarket approval

The notified substance is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (FD&C Act) based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

(7) Availability of data and information

The information for this GRAS conclusion including analytical data, published studies, and information that are the basis for this GRAS determination are available to FDA upon request as

required by 21 C.F.R. § 170.225(c)(7)(ii)(A) or (B) by contacting Keller and Heckman LLP at the below address.

Evangelia C. Pelonis
Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington DC 20005
Tel: 202-434-4106
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(8) Applicability of FOIA exemptions

Amyris Inc. is not claiming any information in Parts 2 through 7 of this document as trade secret, confidential or financial information that is privileged or confidential. Thus, all information and data in this submission are not exempt from the Freedom of Information Act (FOIA), 5 U.S.C. Section 552.

(9) Certification

We certify on behalf of our client, Amyris Inc., that this GRAS conclusion is based on representative data from Amyris Inc. required for the safety and GRAS status for steviol glycosides reb M produced by fermentation. To the best of our knowledge it is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(10) Signature and name and title of the person signing this GRAS notice:

(b) (6)

8/31/18

Evangelia C. Pelonis
Partner
Keller and Heckman LLP

Date: August 31, 2018

Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

(1) Scientific data and information that identifies the notified substance

(a) Common or usual name

The name of the notified substance is steviol glycosides rebaudioside ("reb") M.

(b) Chemical, physical, and microbiological characteristic properties

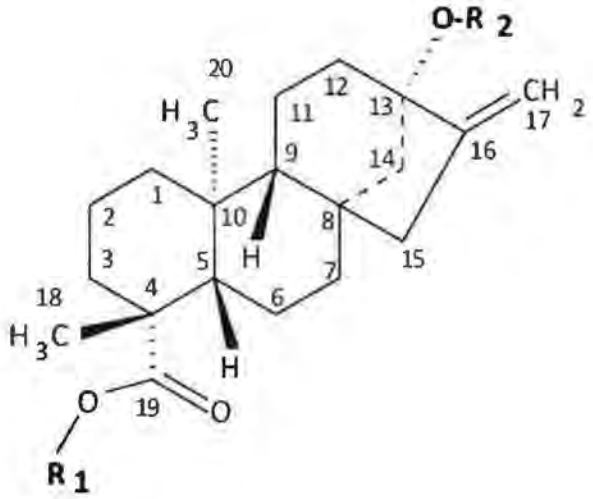
The substance is a white to off-white powder that has a clean taste with no abnormal or off odor and is freely soluble in water. Steviol glycosides reb M is produced by fermentation and is approximately 200-300 times sweeter than sucrose and is consistent with the sweetness intensity of steviol glycosides in general (FAO, 2016). Amyris's steviol glycosides reb M produced by fermentation is composed of ≥95% reb M and contains other steviol glycosides, including those listed in **Table 1**. The final 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 therefore, all share the same backbone structure (**Figure 1**) and differ only with respect to the type and number of glycoside units at positions R_1 and R_2. **Table 1** below provides a list of the other steviol glycosides that may be present in Amyris's steviol glycosides reb M produced by fermentation.

Table 1. Steviol glycosides present in Amyris's steviol glycosides reb M produced by fermentation

Common name	Trivial formula	Mol. Wt.	R_1	R_2
Steviolmonoside	SvG1	481	H	Glcβ1-
Steviol-19-O-B-D-glucoside	SvG1	481	Glcβ1-	H
Rubusoside	SvG2	643	Glcβ1-	Glcβ1-
Steviolbioside	SvG2	643	H	Glcβ(1-2)Glcβ1-
Stevioside	SvG3	805	Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside B	SvG3	805	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside E	SvG4	967	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-

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	1129	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1
Rebaudioside M	SvG6	1291	Glcβ(1-2)[Glcβ(1-3)]Glcβ1	Glcβ(1-2)[Glcβ(1-3)]Glcβ1

Figure 1. Backbone structure for steviol glycosides



(2) Material specifications

(a) Parental strain

The parental microorganism, hereinafter referred to as the parental strain, used to construct the steviol glycoside-producing yeast is *Saccharomyces cerevisiae* ("*S. cerevisiae*") strain CEN.PK113-7D. The parental strain is auxotrophic for histidine, leucine, tryptophan, uracil, and adenine through base-pair deletions or changes of *HIS3, LEU2, TRP1, URA3,* and *ADE1*, respectively. Antibiotic resistance markers kanMX, hphA, and natA were used at specific points of strain construction and are not present in the final production strain. The parental strain is restored to full prototrophy by insertion of copies of *HIS3, LEU2, TRP1, URA3,* and *ADE1* from wild-type *S. cerevisiae*.

(b) Production Strain

The parental strain *S. cerevisiae* CEN.PK113-7D was genetically engineered to increase flux through the endogenous yeast mevalonate pathway to increase carbon flux to the farnesyl pyrophosphate (FPP) precursor as described by Westfall *et al.* (2012) and Meadows *et al.* (2016). The genetically-engineered parental strain with high flux to FPP precursor was converted into a steviol glycoside-producing yeast, herein referred to as the *S. cerevisiae* production strain, by a series of site-specific genomic integrations of DNA constructs in stable, non-essential regions of the genome via homologous recombination. These regions include, but are not limited to, *PDC6, NDT80,* and *HO*. The genes used to generate the production strain encode for enzymes required for steviol glycoside synthesis and improve the overall production efficiency of steviol glycosides. All promoters and terminators used to express the genes are native to *S. cerevisiae*, and include but are not limited to, promotors of *GAL1* and *GAL10* proteins, and terminators of *PGK1* and *TDH3*. **Table 2** provides a summary of the representative enzymes and their technological functions. The incorporated DNA to produce Reb M from FPP is all sourced from biosafety level 1 organisms that are not associated with any known allergens or toxins. In addition, the production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens. Antibiotic resistance markers are not present in the final production strain.

Table 2. Summary of enzymes and their respective functions in Amyris's production strain

Enzyme	Function
Geranylgeranyl pyrophosphate (GGPP) synthase	Converts FPP to GGPP
Copalyl diphosphate (CDP) synthase	Converts GGPP to CDP
Kaurene synthase	Converts CDP to kaurene
Kaurene oxidase	Converts kaurene to kaurenoic acid
Kaurenoic acid hydroxylase (KAH)	Converts kaurenoic acid to steviol
Cytochrome P450 reductase	Works in conjunction with P450 enzymes in pathway
UDP-glucosyl transferases	Adds a glucose to steviol or steviol glycosides

(c) Construction of Production Strain

DNA constructs consisting of genomic DNA homologous to the upstream and downstream DNA sequence of the desired integration site are inserted into the yeast genome via standard methods as described in Rothstein (1991). A single DNA construct may contain one to four open reading frames, which consist of a native yeast promotor and terminator and a gene of interest (*i.e.*, a gene required for steviol glycoside production). DNA constructs with more than one open reading frame may contain spacer DNA obtained from amplified genomic DNA of *E. coli* K-12 to prevent interference during transcription. Spacer DNA constructs are used as structural DNA elements inside of the engineered integrations as they do not have sequence homology to yeast chromosomes. In addition, spacer DNA does not express heterologous proteins as they do not encode functional protein sequences and/or do not include promotors expected to allow expression in yeast.

The parental strain is a stable haploid yeast and therefore does not undergo mating-type switching or mating events (Jensen *et al.*, 1985). The production strain is rendered haploid negative (HO^-) by deletion of the *HO* gene and replaced with a DNA construct containing a kaurene synthase gene and a copalyl-diphosphate synthase gene. Replacement with a DNA construct ensures that the production strain remains haploid negative and will not undergo mating events/unwanted genetic rearrangement.

The identity of the production strain is confirmed through PCR analysis of the inserted DNA construct. In addition, whole genome sequencing of the production strain can be used to confirm that the DNA construct was correctly inserted, and no unexpected genetic elements were inserted into the genome. As the DNA construct was inserted by homologous recombination, the introduced genetic elements are stable, and the production strain does not contain any plasmid or other exogenous mobile genetic elements. The cell line stability is demonstrated by using primary and secondary cell banks and comparing productivities. Extended seed trains are routinely tested to ensure retention of phenotype over generations of the production strain. Furthermore, the production strain is consistently tested for contaminating bacteria and strain performance according to internal standard operation procedures.

(3) Raw Materials and Processing Aids

All raw materials, processing aids, and purification equipment used to manufacture Amyris's steviol glycosides reb M produced by fermentation are food-grade and have an appropriate regulatory status in the United States. **Table 3** below lists the raw materials, processing aids, equipment, and their respective technological function and regulatory status. The production process also utilizes food grade antifoaming agents that have an appropriate regulatory status for this use.

Table 3. Raw materials, processing aids, and equipment used in the manufacture of Amyris's steviol glycosides reb M produced by fermentation

Raw Material/Processing Aid	Technological Function	Regulatory Status
Indirect Additives - Fermentation Medium Ingredients		
Magnesium sulfate heptahydrate	Fermentation nutrient	No limitation other than cGMP as flavor enhancer, nutrient supplement, and processing aid, 21 CFR § 582.5443, 21 CFR § 184.1443
Ammonium sulfate	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR § 582.1143, 21 CFR § 184.1143
Baker's Yeast extract	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR § 184.1983
Monopotassium phosphate (KH_2PO_4)	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §160.110
Succinic acid	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §582.1091, 21 CFR §184.1091
L-(+)-Lysine monohydrochloride	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §582.5411, 21 CFR §172.320
Sodium hydroxide (NaOH)	Fermentation nutrient	pH control agent and processing aid with no limitation other than cGMP, 21 CFR §582.1763, 21 CFR §184.1763
Ammonium Hydroxide (NH_4OH)	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR § 582.1139, 21 CFR § 184.1139
Potassium hydroxide (KOH)	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §582.1631, 21 CFR §184.1631
Ethylenediaminetetraacetic acid (EDTA)	Fermentation nutrient	Permitted in a number of foods as a food additive at specified levels, 21 CFR §172.135
Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5997, 21 CFR §182.8997
Copper sulfate ($CuSO_4$) anhydrous	Fermentation nutrient	Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1261

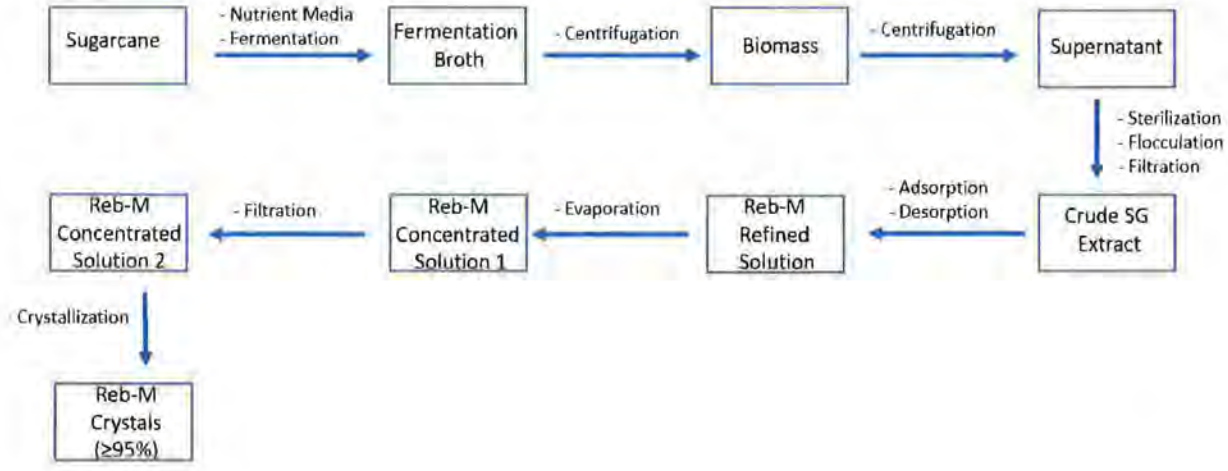
Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5446, 21 CFR §184.1446
Cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	Fermentation nutrient	As an animal feed trace mineral (21 CFR §582.80) and agricultural chemical additive
Sodium molybdate dihydrate ($NaMoO_4 \cdot 2H_2O$)	Fermentation nutrient	As an agricultural chemical additive, chemical additive, processing aid; considered a plant nutrient under 40 CFR §180.920 and exempt from a tolerance in food
Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Fermentation nutrient	Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1315
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Fermentation nutrient	Used as an anticaking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, processing aid, stabilizer and thickener, surface-active agent, synergist, texturizer in accordance with cGMP, 21 CFR §582.1193, 21 CFR §582.6193, 21 CFR §184.1193
Biotin	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §582.5159, 21 CFR §182.8159
para-amino-benzoic acid	Fermentation nutrient	EAFUS listed
Calcium pantothenate	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5212, 21 CFR §184.1212
Nicotinic acid	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1530
Myo-inositol	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5370, 21 CFR §184.1370
Thiamine.HCl	Fermentation nutrient	Used as a flavoring agent and nutrient supplement with no limitation other than cGMP, 21 CFR §582.5875, 21 CFR §184.1875
Pyridoxine.HCl	Fermentation nutrient	Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5676, 21 CFR §184.1676
Ammonium phosphate monobasic ($NH_4H_2PO_4$)	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR §184.1141a, 21 CFR §582.1141
Sulfuric Acid (H_2SO_4)	Fermentation nutrient	GRAS when used in accordance with cGMP, 21 CFR § 184.1095
Cane syrup / Brazilian maltose syrup	Raw material	GRAS
Ethanol, food-grade	Crystallization and desorption solvent	GRAS when used in accordance with cGMP, 21 CFR §184.1293
Adsorption resin	Purification	Used in accordance with 21 CFR §177.2710

(4) Description of the method of manufacture

Amyris's steviol glycosides reb M produced by fermentation is manufactured using a strain of *S. cerevisiae* that has been modified through genetic engineering to express the steviol glycoside biosynthetic pathway. In the first stage of the manufacturing process food-grade sugarcane is mixed with the *S. cerevisiae* production strain Y47220 and fermented to produce the Reb M and other steviol glycosides. The fermentation broth goes through centrifugation to separate the biomass from the aqueous phase, followed again by centrifugation. The supernatant product is then sterilized, which then goes through flocculation and filtration to obtain the crude steviol glycosides extract. That extract enters an adsorption and desorption process to become the Reb M refined solution, which is evaporated into a Reb M concentrated solution. That solution is filtered and crystallized, which results in a final product that contains ≥95% Reb M powder.

The purification processes used after fermentation are consistent with the methodologies for the manufacture of steviol glycosides as described in the CTA published by FAO/JECFA (FAO, 2016). Steviol glycosides reb M produced by fermentation is manufactured in a facility certified under Food Safety System Certification (FSSC) 22000:2010. The flow chart for the manufacturing process is shown below in **Figure 2.**

Figure 2. Manufacturing process of Amyris's steviol glycosides reb M produced by fermentation



(5) Product Specifications and Batch Analyses

(a) Physical, Chemical, and Microbiological Specifications

The product specifications for steviol glycosides reb M produced by fermentation are presented in **Table 4**.

Table 4. Physical and microbiological characteristics of Amyris's steviol glycosides reb M produced by fermentation

Component	Limits	Unit of Measure
Physical Analysis		
Appearance (powder)	White to off-white	N/A
Total steviol glycosides (anhydrous)		
Rebaudioside M Content (anhydrous)	≥95	(wt/wt) %
Ash	≤1.0	(wt/wt) %
Moisture (loss on drying)	≤5.0	(wt/wt) %
pH (measured at 1% dilution)	4.5 – 7.0	
Residual Ethanol	< 0.30	%
Residual Methanol	< 0.02	%
Heavy Metals		
Lead (Pb)	< 1.0	ppm
Arsenic (As)	< 1.0	ppm
Cadmium (Cd)	< 1.0	ppm
Mercury (Hg)	< 1.0	ppm
Cobalt	< 1.0	ppm
Microbiological Analysis		
Total Plate Count (TPC)	< 1000	CFU/g
Yeast	< 10	CFU/g
Mold	< 10	CFU/g
Total Coliforms	< 3	MPN/g
E. coli	< 10	CFU/g
Staphylococcus aureus	Non-detect	CFU/g
Salmonella	Negative / 25g	
Listeria	Negative / 25g	
Protein	Non-detect	ng / ml
DNA	Non-detect	pg / ul

wt% = weight percent

ppm = parts per million

CFU = colony-forming unit

MPN = most probable number

(b) Batch Analyses

Data from the analysis of three non-consecutive lots of steviol glycosides reb M produced by fermentation, which demonstrate the consistency of manufacturing process and compliance with the physical and chemical specifications, are presented in **Table 5**.

Table 5. Physical, chemical, and microbiological product analysis for 3 non-consecutive lots of Amyris's steviol glycosides reb M produced by fermentation

Specification Parameter	Limit	Manufacturing Lot		
		18RGT0506RM001	18RGT0511RM002	18RGT0606RM003
Appearance (powder)	White to off-white powder	White powder	White powder	White powder
Rebaudioside M content (anhydrous) by HPLC-UV	≥95 wt%	98%	98%	99 %
Ash	≤1.0 wt%	0.01%	0.02%	0.02 %
Moisture (loss on drying)	≤5.0 wt%	1.02%	1.31%	0.1 %
pH (measured at 1% dilution)	4.5 – 7.0	5.5	5.7	5.4
Arsenic (As)	< 1.0 ppm	0.001 ppm	0.003 ppm	0.003 ppm
Cadmium (Cd)	< 1.0 ppm	0.003 ppm	0.003 ppm	< 0.002 ppm
Lead (Pb)	< 1.0 ppm	0.042 ppm	0.025 ppm	0.017 ppm
Mercury (Hg)	< 1.0 ppm	0.001 ppm	0.004 ppm	< 0.002 ppm
Cobalt	< 1.0 ppm	< 0.10 ppm	< 0.10 ppm	< 0.10 ppm
Residual Ethanol	< 0.30%	0.04%	< 0.02%	0.16%
Residual Methanol	< 0.02%	< 0.01%	< 0.01%	< 0.01 %
Total Plate Count (TPC)/Aerobic Plate Count (APC)	< 1000 CFU/g	10 CFU/g	< 10 CFU/g	< 10 CFU/g
Yeast	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Mold	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Coliforms	< 3 MPN/g	< 3 MPN/g	< 3 MPN/g	< 3 MPN/g
Escherichia coli	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Staphylococcus aureus	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Salmonella	Negative / 25g	Not detected / 25g	Not detected / 25g	Not detected/25g

Listeria	Negative 25/g	Not detected / 25g	Not detected / 25g	Not detected/25g
Protein	Non-detect (ng/ml)	Not detected	Not detected	Not detected
DNA	Non-detect (pg/µl)	Not detected	Not detected	Not detected

wt% = weight percent

ppm = parts per million

CFU = colony-forming unit

MPN = most probable number

Data for three production lots of Steviol Glycosides Reb M Produced by Fermentation in **Table 6** shows the difference in the distribution of steviol glycosides present in the mother liquor following the fermentation and in the final purified product following crystallization. Steviol Glycosides Reb M Produced by Fermentation produces a final product ≥95% reb M and other steviol glycosides such as those listed in **Table 6**. The manufacturing process purification steps are effective and produces a product with a consistent steviol glycoside distribution. This was measured by high performance liquid chromatography (HPLC) and is provided for three non-consecutive lots of final product illustrated in **Table 6**.

Table 6. Similarity of the stability of Amyris's steviol glycosides reb M produced by fermentation as compared to individual steviol glycosides as measured by HPLC

	Lot 18RGT0506RM001		**Lot 18RGT0506RM002**		**Lot 18RGT0606RM003**	
Steviol Glycoside (wt%)	**Mother liquor**	**Crystal**	**Mother liquor**	**Crystal**	**Mother liquor**	**Crystal**
Rebaudioside D	0.082	2.564	0.086	2.761	0.596	1.512
Rebaudioside M	0.139	98.353	0.141	98.34	1.281	98.59
Rebaudioside A	0.005	ND	0.006	ND	0.045	ND
Rebaudioside E	0.025	ND	0.026	ND	0.230	ND
Stevioside	0.012	ND	0.014	ND	0.105	ND
Rubusoside	0.008	ND	0.009	ND	0.092	ND
Rebaudioside B	0.001	ND	0.002	ND	0.007	0.417
Steviolbioside	0.002	ND	0.002	ND	0.009	ND
i-Steviolmonoside	0.011	ND	0.012	ND	0.136	ND
Steviolmonoside	0.019	ND	0.018	ND	0.102	ND

(c) Residual Protein and DNA

To confirm the success of the purification and confirm the absence of protein in steviol glycosides reb M produced by fermentation, the final product is analyzed by sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of steviol glycosides reb M produced by fermentation 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 50 minutes at 200 V and the gel is stained with Coomassie Blue for 1 hour. Gels are destained by soaking in milli-q 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 reb M produced by fermentation, 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 manufacturer's protocol. The thermal profile used is 2 minutes at 98°C followed by 35 cycles of 25 seconds at 98°C, 30 seconds at 55°C, and 60 seconds at 72°C (or longer than 60 seconds at 72°C if >1Kb) followed by 1 cycle for 2 minutes at 72°C. The genomic DNA is quantified by loading the sample with an agarose gel loading dye diluted to 1x and ran onto a 1% agarose gel for 20 to 30 minutes. The gel is visualized under UV light to image the DNA bands. Results of the PCR analysis have not detected any PCR products in any of the batches of final product (limit of detection for a single heterologous gene = 0.1 pg/μL DNA).

(6) Stability Data

The stability data of steviol glycosides have been reviewed by scientific advisory bodies involved in the evaluation of steviol glycosides 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, 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 reported 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 reported. Based on the above, and in addition to 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 three commercial batches were evaluated to determine whether the manufacturing process adversely impacts steviol glycoside composition, with each batch containing a sample of untreated stevia leaves, the first water extract and high-purity end product (≥95% steviol glycosides) (Oehme et al., 2017). Changes in steviol glycoside composition were analyzed by HPLC-UV and HPLC-ESI-MS/MS. The authors reported that all nine JECFA-defined steviol glycosides were detected in all samples. The results also demonstrated that stevia extract processing does not chemically alter or modify the individual steviol glycoside content.

(a) pH Stability

The general stability of steviol glycosides with a high reb M content (Lot 18RGT0511RM002) was assessed at pH 2, 5 and 8 for a total of 8 weeks at 4 different temperatures, 4, 22, 40, and 50°C. pH 2, pH 5 and pH 8 solutions were prepared using phosphoric acid and/or di-sodium hydrogen phosphate. Steviol glycosides with a high reb M content was suspended in 250 mL solution to obtain 1g/L concentration at each pH solution. 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 the following specific steviol glycosides: rebaudiosides A, B, D, E, M, Steviol-19-O-B-D-glucoside, rubusoside, steviolbioside, Steviolmonoside, and stevioside. Steviol Glycosides with a high Reb-M content tested at pH level 2 was most stable when stored at 4°C and least stable at 50°C. However, at pH 2 when stored at 22°C, 40°C and 50°C, Reb-M degrades at a comparable level as reported in the reference GRN 744. Overall, no significant degradation is observed over 8 weeks for content tested at pH 2 stored at 4°C. Steviol Glycosides with a high Reb-M content tested at pH level 5 and 8 was stable when stored at 4°C, 22°C, 40 °C and 50°C for 4 weeks. No significant degradation is observed over 8 weeks for content tested at pH 5 stored at 4°C, 22°C, 40°C and 50°C. No significant degradation is observed over 8 weeks for content tested at pH 8 stored at 4°C, 22°C and 40°C. However, Reb-M shows slight degradation (0.74 g/Kg to 0.58 g/Kg) stored at 50°C for 8 weeks. **Table 7** summarizes the results of the stability for solutions of steviol glycosides with a high reb M content. Stability results are comparable to those reported in the reference GRN 744.

Table 7. Stability data results (pH solution 2, 5, 8, and powder) Amyris's steviol glycosides reb M produced by fermentation

	Time Point (wk)	Temperature (°C)	Steviol-19-O-B-D-glucoside (g/kg)	RebA (g/kg)	RebB (g/kg)	RebD (g/kg)	RebE (g/kg)	RebM (g/kg)	Rubus-oside (g/kg)	Steviol bioside (g/kg)	Steviol monoside (g/kg)	Stevioside (g/kg)
pH 2	0	4	0.00	0.00	0.00	0.02	0.00	0.91	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.03	0.00	0.99	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.01	0.03	0.00	1.00	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.00	0.00	0.99	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.98	0.00	0.00	0.00	0.00
	0	22	0.00	0.00	0.00	0.02	0.00	0.91	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.02	0.02	0.00	0.84	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.00	0.02	0.00	0.90	0.00	0.00	0.00	0.00

	6		0.00	0.00	0.02	0.00	0.00	0.68	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.02	0.00	0.00	0.66	0.00	0.00	0.00	0.00
	0	40	0.00	0.00	0.00	0.02	0.00	0.91	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.05	0.01	0.00	0.24	0.00	0.07	0.00	0.00
	4		0.00	0.00	0.04	0.00	0.00	0.12	0.00	0.13	0.00	0.00
	6		0.00	0.00	0.20	0.00	0.00	0.03	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.01	0.00	0.00	0.03	0.00	0.16	0.00	0.00
	0	50	0.00	0.00	0.00	0.02	0.00	0.91	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.16	0.00	0.00
	4		0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.16	0.00	0.00
	6		0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00
	Time Point (wk)	**Temperature (°C)**	**Steviol-19-O-B-D-glucoside (g/kg)**	**RebA (g/kg)**	**RebB (g/kg)**	**RebD (g/kg)**	**RebE (g/kg)**	**RebM (g/kg)**	**Rubusoside (g/kg)**	**Steviol bioside (g/kg)**	**Steviol monoside (g/kg)**	**Stevioside (g/kg)**
pH 5	0	4	0.00	0.00	0.00	0.02	0.00	0.81	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.89	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.00	0.02	0.00	0.89	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.01	0.00	0.90	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.90	0.00	0.00	0.00	0.00
	0	22	0.00	0.00	0.00	0.02	0.00	0.81	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.89	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.03	0.02	0.00	0.78	0.00	0.01	0.00	0.00
	6		0.00	0.00	0.00	0.01	0.00	0.89	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.90	0.00	0.00	0.00	0.00
	0	40	0.00	0.00	0.00	0.02	0.00	0.81	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.87	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.01	0.02	0.00	0.89	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.01	0.00	0.91	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.90	0.00	0.00	0.00	0.00
	0	50	0.00	0.00	0.00	0.02	0.00	0.81	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.01	0.02	0.00	0.88	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.01	0.02	0.00	0.88	0.00	0.00	0.00	0.00
	6.7		0.00	0.00	0.00	0.01	0.00	0.82	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.01	0.01	0.00	0.79	0.00	0.00	0.00	0.00
	Time Point (wk)	**Temperature (°C)**	**Steviol-19-O-B-D-glucoside (g/kg)**	**RebA (g/kg)**	**RebB (g/kg)**	**RebD (g/kg)**	**RebE (g/kg)**	**RebM (g/kg)**	**Rubusoside (g/kg)**	**Steviol bioside (g/kg)**	**Steviol monoside (g/kg)**	**Stevioside (g/kg)**
pH 8	0	4	0.00	0.00	0.00	0.02	0.00	0.74	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.84	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.00	0.02	0.00	0.86	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.01	0.00	0.84	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.86	0.00	0.00	0.00	0.00
	0	22	0.00	0.00	0.00	0.02	0.00	0.74	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.83	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.00	0.03	0.00	0.85	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.00	0.00	0.86	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.01	0.00	0.84	0.00	0.00	0.00	0.00
	0	40	0.00	0.00	0.00	0.02	0.00	0.74	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.00	0.02	0.00	0.82	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.01	0.03	0.00	0.82	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.03	0.00	0.76	0.00	0.00	0.00	0.00

	8		0.00	0.00	0.00	0.03	0.00	0.75	0.00	0.00	0.00	0.00
	0	50	0.00	0.00	0.00	0.02	0.00	0.74	0.00	0.00	0.00	0.00
	2		0.00	0.00	0.01	0.03	0.00	0.75	0.00	0.00	0.00	0.00
	4		0.00	0.00	0.01	0.04	0.00	0.68	0.00	0.00	0.00	0.00
	6		0.00	0.00	0.00	0.05	0.00	0.59	0.00	0.00	0.00	0.00
	8		0.00	0.00	0.00	0.05	0.00	0.58	0.00	0.00	0.00	0.00

(b) Storage Stability

The storage stability of steviol glycosides reb M produced by fermentation (Lot 18RGT0511RM002) was assessed. Powder samples were stored in aluminum food grade bags for up to 8 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. Reb M to total steviol glycosides content stored at 25°C, 60% relative humidity and 40°C, 75% relative humidity storage conditions was stable and no significant degradation was observed at 4 weeks. As reported in **Table 8**, steviol glycosides with reb M powder stored under both conditions for 8 weeks was stable in total steviol glycosides (TSG). Stability results are comparable to those reported in the referenced GRN 744.

Table 8. Storage stability of Amyris's steviol glycosides reb M produced by fermentation (Lot 18RGT0511RM002), (g/kg)

Time Point (wk)	Temperature (C°/%RH)	Steviol-19-O-B-D-glucoside (g/kg)	RebA (g/kg)	RebB (g/kg)	RebD (g/kg)	RebE (g/kg)	RebM (g/kg)	Rubus-oside (g/kg)	Steviol bioside (g/kg)	Steviol monoside (g/kg)	Stevioside (g/kg)	Reb-M/TSG
0	25C/60% RH	0.00	0.67	4.16	19.74	0.31	952.67	0.00	0.32	0.00	0.30	97.4%
4		0.00	0.68	5.49	19.16	0.25	861.21	0.00	0.24	0.00	0.00	97.1%
8		0.00	0.33	4.46	14.23	0.00	691.26	0.00	0.00	0.00	0.16	97.3%
0	40C/75% RH	0.00	0.67	4.16	19.74	0.31	952.67	0.00	0.32	0.00	0.30	97.4%
4		0.00	0.79	6.62	21.06	0.28	962.67	0.00	0.39	0.00	0.37	97.0%
8		0.00	0.42	5.33	15.52	0.00	724.34	0.00	0.00	0.00	0.16	97.1%

Part 3 – Dietary exposure

(1) Estimate of Dietary Exposure

Steviol glycosides reb M produced by fermentation is approximately 200-300 times sweeter than sucrose and is intended for use as a general-purpose sweetening agent, in accordance with cGMP. The majority of 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. The foods to which high-intensity sweeteners are added and their use-levels are controlled by technological properties (e.g., sweetness potency). Steviol glycosides have a sweetness intensity that is comparable to that of other high-intensity sweeteners (e.g., aspartame is approximately 200 times as sweet as sucrose, steviol glycosides reb M produced by fermentation is approximately 200-300 times sweeter than sucrose), and therefore the uses and use-levels of steviol glycosides reb M produced by fermentation are likely to reflect those currently permitted for other high-intensity sweeteners in the U.S.

S. rebaudiana and its isolated steviol glycosides (most commonly stevioside) have been consumed by humans in various countries as sweeteners in foods and beverages since it was first discovered in the West by Antonio Bertoni in 1887 (Geuns, 2003). In Brazil and Paraguay, *S. rebaudiana* has been used as both a food ingredient and as a tea for hundreds of years (Blumenthal, 1995; Geuns, 2003). There is also documentation of the use of *S. rebaudiana* leaves as a sweetener by the native Indians of the Guarani Tribe, dating back to pre-Colombian times (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) and 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).

Numerous surveys have been conducted in various 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, are realistic in that they reflect actual post-market intakes of high-intensity sweeteners. Specifically, 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 9** below). 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 250).

In regards to steviol glycosides reb M produced by fermentation, the same methodology Renwick (2008) applied was used to estimate intake values. Based on a sweetness potency test, steviol glycosides reb M produced by fermentation was determined to be approximately 200-300 times sweeter than sucrose. The intake values for intense sweeteners presented in **Table 9** below were adjusted accordingly to derive an estimated intake range for steviol glycosides reb M produced by fermentation. 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 9. Estimated consumption of Amyris's steviol glycosides reb M produced by fermentation using Renwick's (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 reb M[a] (mg/kg bw/day)		Steviol glycosides with reb M 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	1.02	2.70	0.26	0.68
Diabetic Adults	280	897	1.12	3.59	0.28	0.90
Non-diabetic Children	425	990	1.70	3.96	0.43	0.99
Diabetic Children	672	908	2.69	3.63	0.68	0.91

bw = body weight; reb = rebaudioside

[a] Approximately 200-300 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 reb M produced by fermentation of up to 0.26 and 0.68 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 0.28 and 0.90 mg/kg body weight/day. Average and high-end exposures to steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, in non-diabetic children were calculated to be up to 0.43 and 0.99 mg/kg body weight/day, respectively. Although average intakes of steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, were estimated to be higher at up to 0.68 mg/kg body weight/day in diabetic children compared to values for non-diabetic children, high-end values in diabetic children (0.91 mg/kg body weight/day) were lower than high-end values in non-diabetic children. The predicted intakes of steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, are all below the current ADI defined by the JECFA for steviol glycosides (FAO, 2016) of 0 to 4 mg/kg body weight/day as steviol.

For non-diabetic adults, average and high-end intakes of steviol glycosides reb M produced by fermentation of up to 0.26 and 0.68 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 0.28 and 0.90 mg/kg body weight/day. Average and high-end exposures to steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, in non-diabetic children were calculated to be up to 0.43 and 0.99 mg/kg body weight/day, respectively. Although average intakes of steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, were estimated to be higher at up to 0.68 mg/kg body weight/day in diabetic children compared to values for non-diabetic children, high-end values in diabetic children (0.91

mg/kg body weight/day) were lower than high-end values in non-diabetic children. The predicted intakes of steviol glycosides reb M produced by fermentation, expressed as steviol equivalents, are all below the current ADI defined by 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 – Self-limiting levels of use

Steviol glycosides reb M produced by fermentation has self-limiting levels caused by the desired sweetness intended for a particular food or beverage product. Therefore, the use of steviol glycosides reb M produced by fermentation as a general-purpose sweetener in foods is self-limiting based on its organoleptic properties.

Part 5 – Experience based on common use in food before 1958

The statutory basis for the conclusion of GRAS status of steviol glycosides reb M produced by fermentation is not based on common use in foods before 1958. The GRAS determination is based on scientific procedures.

Part 6 – Narrative

(1) Reference to GRAS No. 744

Amyris's steviol glycosides reb M produced by fermentation bears similarities to the substance at the focus of GRAS No. 744, to which FDA had no questions. The similarities between the steviol glycosides reb M produced by fermentation in this GRAS notification and that of GRAS No. 744 include the type of raw ingredients and the production strain, in addition to certain other features. Amyris incorporates by reference the dietary exposure and safety information provided in GRAS No. 744, noting that FDA had no questions in response.

Due to the similarities between GRAS No. 744 and Amyris's steviol glycosides reb M produced by fermentation, certain aspects of the dietary exposure and safety analysis have been repeated as they appear in GRAS No. 744.

Amyris's steviol glycosides reb M produced by fermentation is differentiated from GRAS No. 744 because Amyris's contains reb M at an amount greater than 95%, whereas GRAS No. 744 contains a total amount of steviol glycosides at 95% with reb M content of approximately 90%

Additionally, the production strain that is the focus of GRAS No. 744 contains 18 inserted genes, whereas the production strain for Amyris's steviol glycosides reb M produced by fermentation contains a total of 16 genes. Additionally, Y47220 contains two versions of the KAH gene, one version is the wild-type gene and a second version containing two altered amino acids resulting in higher enzyme activity.

Amyris's manufacturing process of steviol glycosides reb M produced by fermentation differs from that of GRAS No. 744. Notably, Amyris simplifies the manufacturing process by processing through two steviol glycoside solutions, instead of three solutions, and removing the activated carbon step between the 1st steviol glycoside solution and a Reb-M refined solution. Additionally, Amyris's process does not include nanofiltration and spray drying to produce Reb-M powder (< 95%), and instead uses only crystallization to produce the Reb-M powder (≥95%).

(2) Overview of Safety of Steviol Glycosides

Over the last few decades, several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, the European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada, have reviewed the safety of steviol glycosides. Interest in the use of steviol glycosides as sweeteners initiated extensive testing of the compounds and, in turn, generated a large safety database. 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 information available on the common metabolic fate of steviol glycosides has permitted 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 reb M produced by fermentation can be established based on the conclusions of the steviol glycoside safety reviews, and on the publicly available scientific literature related to the safety of steviol glycosides. Furthermore, although the production strain is not present in the final product, information related to the safety of the *S. cerevisiae* parental and production strains was compiled, including assessment of the potential allergenicity of the heterologous gene sequences inserted in the production strain.

(3) Existing clearances for steviol glycosides with reb M

Effective GRAS notices pertaining to steviol glycosides with reb M are described in **Table 10** below.

Table 10. GRAS notices for steviol glycosides with reb M

Year	Clearance
2014	GRN 512, High purity Rebaudioside M; FDA has no questions
2016	GRN 667, Rebaudioside M; FDA has no questions
2018	GRN 744, Steviol glycosides consisting primarily of rebaudioside M; FDA has no questions
2018	GRN 745, Steviol glycosides consisting primarily of rebaudioside M; FDA has no questions
2018	GRN 759, Steviol glycosides consisting primarily of rebaudioside M produced in *Yarrowia lipolytica*; FDA has no questions

In the U.S., the FDA has raised no objections to 51 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, 702, 715, 733, 744, 745, 759) 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 Nos. 744 and 745 received no questions from the U.S. FDA regarding the GRAS status of steviol glycosides consisting primarily of rebaudioside M for use as a general-purpose sweetener in foods, excluding meat and poultry products and infant formula, at levels in accordance with current good manufacturing practices. (U.S. FDA, 2018a, U.S. FDA, 2018b). Similar to Amyris's steviol glycosides reb M produced by fermentation, the final products in GRAS Nos. 744 and 745 contain ≥95% steviol glycosides, and consists of rebaudiosides A, B, C, D, E, F, M, stevioside, steviolbioside, rubusoside and dulcoside A in varying percentages.

(4) 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; 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 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 rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside 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 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, particularly during the first 24 hours of incubation (Purkayastha et al., 2014, 2015, 2016). For example, reb M and rebaudioside 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 rebaudioside A or stevioside (Roberts and Renwick, 2008). Similarly, in humans steviol glucuronide was detected in the plasma following ingestion of stevioside or rebaudioside 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 (AUC0-72h) 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.

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 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 (via the bile in rats and in the urine in humans) 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 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, 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 reb M produced by fermentation.

(5) Safety data

The safety of steviol glycosides was evaluated in the related GRAS Nos. 744 and 745 for a mixture of steviol glycosides produced using genetically modified *S. cerevisiae*, which included a search of the scientific literature to capture relevant publications, and therefore the safety information presented in GRAS Nos. 744 and 745 is incorporated by reference. To identify new data related to the safety of steviol glycosides since the U.S. FDA review in 2018 of GRAS Nos. 744 and 745, we conducted a comprehensive search of the scientific literature through August 2018. 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 and humans evaluating the safety, antidiabetic, and immune effects of steviol glycosides. The results of these recent studies provide further support for the safety of steviol glycosides.

(a) 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 the 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 cells) (Sharif et al., 2017). The MTT assay, an indicator of toxicity, was used to assess cell viability in the presence 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 reported in either cell line compared to control, and no change in tail moment was reported 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 reported 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 (Sharif et al., 2017).

(b) Repeat-Dose Studies

Rebaudioside A (> 95% purity) produced by fermentation (by genetically modified yeast, Y. lipolytica) was administered to Sprague-Dawley rats as a dietary mixture at concentrations of 0, 500, 1,000, or 2,000 mg/kg body weight/day (N=20 per sex per group) for a total of 90 days (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 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 0, 1.04, 2.08, and 3.12% of the diet which correspond to targeted doses of 0, 830, 1670, and 2500 mg/kg bw/day. (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, 1998b). 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.

(c) Glycemic Effects

To evaluate the antihyperglycemic effects of steviol glycosides, groups of male normoglycemic (6/group, with the exception of glibenclamide treatment, where n = 12) and streptozotocin-induced diabetic (4/group) Wistar rats were given one of the following for 28 days in food: rebaudioside B, rebaudioside C, rebaudioside D, dulcoside A or steviolbioside at a dose of 20 mg/kg body weight/day (Aranda-Gonzalez et al., 2016). Distilled water and glibenclamide (5 mg/kg body weight per day) served as controls and food was available ad libitum once the initial treatment pellet was consumed each day. Prior to the 28-day oral treatment, an intraperitoneal glucose tolerance test (IPGTT) was performed with 1 g/kg body weight glucose and the same doses and groups listed previously. Prior to the test, and after 6 hours of fasting, blood was collected from the tip of the tail to measure glucose levels. After the 28-day oral treatment with steviol glycosides,

IPGTT was repeated, except only glucose (1 g/kg body weight) was administered. Acute administration of rebaudioside B, rebaudioside D, dulcoside A or steviobioside had no effect on IPGTT in normoglycemic rats. At 15 minutes, there was a significant decrease in glucose in the rebaudioside C group compared with the control group; however, at 120 minutes, only glibenclamide induced an antihyperglycemic effect that was statistically significant from the control group. The authors concluded that acute intraperitoneal or oral administration of minor steviol glycosides at doses of 20 mg/kg body weight/day for 28 days had no antihyperglycemic effect in normoglycemic or induced-diabetic rats.

The hypoglycemic and hypolipidemic effects of stevia leaf powder were studied in 20 human volunteers with type 2 diabetes mellitus (Ritu and Nandini, 2016). Commercially produced stevia leaf powder was utilized in the study, containing stevioside and rebaudioside A, however, the overall glycoside purity of the product was not reported. Prior to the onset of the study, the subjects were given thorough medical examinations, and 10 were assigned to the 'intervention group' to receive 1 g of stevia leaf powder (no mg/kg body weight dose reported), and 10 served as controls. It was unclear if stevia was administered daily, and how it was delivered. Prior to the 'intervention' and at 30 and 60 days following, biochemical parameters of blood glucose (fasting and post-prandial), triglycerides, cholesterol (total, low-density lipoprotein [LDL] and very low-density lipoprotein [VLDL], high-density lipoprotein [HDL] and LDL/HDL ratio), and atherogenic index were measured. After 60 days, a statistically significant decrease in fasting and post-prandial blood glucose levels compared to baseline was reported in the stevia group. No differences were reported at 30 days post-intervention. It was reported by the authors that stevia exposure led to a significant reduction in serum cholesterol, triglycerides and very low-density lipoprotein-cholesterol (VLDL-C). Additionally, a 3-day dietary evaluation was conducted on each subject during the study to analyze intake of energy, carbohydrates, proteins, fats and fibers. Mean caloric intake was lower in the stevia group than the control (statistical significance not reported), and on average, the stevia group consumed more protein and fewer carbohydrates.

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, specifically 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 (Philippaert et al., 2017). The in vitro and in vivo studies conducted using Trpm5-/- mice demonstrated that stevioside, rebaudioside A, and steviol: a) potentiate the activity of TRPM5; b) TRPM5 facilitates insulin release from the islet cells; c) potentiate TRPM5 activity and intensify bitter, sweet, and umami taste responses, and d) the glucose lowering effect of stevioside is dependent on TRPM5 expression in pancreatic islets. The effect of 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 (C57BL6/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 reported 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 (C57BL6/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, equivalent to 0.01% 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. Deteriorated glucose tolerance was reported 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.

Chronic rebaudioside 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 rebaudioside 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 approximately 4 months. During the first 32 days of treatment, mice were placed in cages with running wheels. Following a one-week acclimatization period (days 1-7) wheel running activity was monitored over a 12-hour light-dark cycle for 14 days (days 8-22) and in complete darkness for 10 days (days 23-32). 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 susceptibility to obesity by providing a high fat diet to the mice for 2 months. Glucose, insulin, and pyruvate tolerance tests were conducted and showed similar results among treatment and control groups. In the same manner, exposure to rebaudioside A had no effect on the susceptibility to diet-induced obesity.

(d) 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 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. The levels of cytokines were significantly decreased when compared to the control group, indicating the inhibitory effect of stevioside on cytokine release.

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), drinking 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) Revision of the Acceptable Daily Intake for Steviol Glycosides

The ADI for steviol glycosides of 4 mg/kg body weight/day (expressed as steviol) was 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 be Toyoda et al. (1997) and application of a safety factor of 100 (FSANZ, 2008; JECFA, 2009; EFSA, 2010; Health Canada, 2012a). 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.

Roberts et al. (2016) reported on the toxicokinetic differences of steviol and steviol glucuronide 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 (Cmax) of steviol were similar in both rats and humans (see below) but were slightly delayed in humans compared to rats. Cmax 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 (AUC0-72h) 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. The AUC and Cmax data were used to calculate the CSAF as follows:

a) the AUC0-72 for free steviol in humans (1,631 ng·h/mL) was higher than the AUC 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 Cmax values for free steviol in humans (77.21 ng/mL) were approximately equivalent to those in male and female rats (76.0 and 87.1 ng/mL, respectively), and therefore the ratio of Cmax 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.

(7) Safety of the Parental Strain

Saccharomyces cerevisiae, also known as brewer's yeast or baker's yeast, has an extensive history of safe-use in the food industry. In the U.S., according to 21 CFR §172.896 dried yeast, including *S. cerevisiae*, is permitted for use in food so long as the total folic acid content is no greater than 0.04 mg/g of yeast (U.S. FDA, 2017a). Protein isolated from *S. cerevisiae* (baker's yeast protein) and the dried cell walls of S. cerevisiae (baker's yeast glycan) are food additives permitted for the direct addition to food for human consumption (21 CFR §172.325 and 172.898, respectively) (U.S. FDA, 2017a). Baker's yeast extract, the concentrated or dried soluble component of mechanically ruptured cells of *S. cerevisiae*, is GRAS for use as a flavoring agent and adjuvant at a level not to exceed 5% in food (21 CFR §184.1983 - U.S. FDA, 2017a). Vitamin D2 baker's yeast, which is generated by exposing *S. cerevisiae* to UV light, resulting in the conversion of endogenous ergosterol to vitamin D2, is also a food additive permitted for direct addition to food for human consumption (21 CFR §172.381 - U.S. FDA, 2017a). Food enzymes produced by *S. cerevisiae* (e.g., invertase, GRN No. 88) (U.S. FDA, 2002) as well as several *S. cerevisiae* strains genetically-modified to alter the expression of specific endogenous enzymes or pathways (GRN No. 120, 175,

350, 422, 604) (U.S. FDA, 2002, 2003, 2006, 2011b, 2012, 2016c) have GRAS status with no objection from the U.S. FDA.

S. cerevisiae has been granted Qualified Presumption of Safety (QPS) status in the European Union by EFSA and therefore is considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes, as long as the following qualification is met in the safety assessment: "Absence of resistance to antimycotics used for medical treatment of yeast infections in cases where viable cells are added to the food or feed chain *S. cerevisiae* this qualification applies for yeast strains able to grow above 37°C" (EFSA, 2017).

Despite the extensive history of safe use of *S. cerevisiae* in the food industry, rare reports of *S. cerevisiae* infections in humans indicate that *S. cerevisiae* is also regarded as an opportunistic pathogen. A comprehensive review conducted by Enache-Angoulvant and Hennequin (2007) reported 92 cases of Saccharomyces invasive infection, with the most common predisposing factors being antibiotic therapy and intravascular catheter. *S. cerevisiae* strain YJM789, for example, was isolated from the lung of an AIDS patient with polymicrobial pneumonia (Tawfik et al., 1989; Wei et al., 2007) and de Llanos et al. (2006) reported 4 clinical cases of *S. cerevisiae* detection in the blood. Amyris's steviol glycosides reb M produced by fermentation does not contain any viable production organisms, as evidenced by the absence of protein and residual DNA in the final product, and therefore the aforementioned reports are of no safety concern.

(8) Safety of Production Strain

As discussed in Part 2(2)(b), the production strain contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. The genes used to create the production strain are naturally-occurring or from biosafety level 1 organisms, listed in **Table 11**. The fermentation broth is subjected to a heat treatment step to kill the yeast cells prior to the purification/concentration steps wherein the production strain is removed. As evidenced by the absence of protein and residual DNA in the final product and the high purity content of the steviol glycosides reb M produced by fermentation, the inserted DNA from these source organisms is of no safety concern.

Table 11. Source organisms for genes inserted in Amyris's production strain

Organism from which gene was derived	Description
E. coli K-12	A non-pathogenic / non-toxic strain of E. coli
Dickeya zeae	Bacterium; harmless to humans
Saccharomyces kluyveri	Yeast similar to *S. cerevisiae*; laboratory model organism; harmless to humans
Zymomonas mobilis	Bacterium; makes ethanol; originally isolated from alcoholic beverages like African palm wine
Blakeslea trispora	Fungus that infects soy; used commercially to produce beta-carotene
Arabidopsis thaliana	Mouse-ear cress; a weed in the brassicaceae family (*i.e.*, broccoli and cauliflower) commonly used for molecular plant research
Pisum sativum	Garden pea
Oryza sativa	Rice
Picea glauca	White spruce
Stevia rebaudiana	Leaf extracts from this plantine are consumed and are classified as GRAS (Generally Recognized as Safe)
Setaria italic	Foxtail millet; a variety of cultivated millet

(9) Allergenicity

As demonstrated in 3 non-consecutive batches of steviol glycosides reb M produced by fermentation, the final product does not contain residual protein and DNA as per the defined product specifications. The potential for cross-reactivity among the inserted heterologous gene sequences in the production strain was investigated in accordance with the FAO/WHO protocol for bioinformatic allergenicity assessment (FAO/WHO, 2001). In the assessment, potential linear IgE epitopes were identified by searching for any match of 6 consecutive amino acids from each inserted gene sequence to an allergen database. Potential conformational IgE epitopes were identified by searching for greater than 35% sequence identity over a sliding 80-mer amino acid window. Amyris's steviol glycosides reb M produced by fermentation contains a total of 16 genes including two different copies of the KAH gene; therefore16 gene sequences were searched against the AllergenOnline Database Version 18 (available at http://www.allergenonline.org; updated March 23, 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2017). The database contains a comprehensive list of putative allergenic proteins developed via a peer reviewed process for the purpose of evaluating food safety.

Part one of the bioinformatics assessment searched for 6-mer matches between the engineered Reb M constructs, and the AllergenOnline database. This search returned 324 hits. Part two of the bioinformatics assessment, requiring >35% sequence similarity of any 80-mer amino acid window, returned 429 hits. In addition, total protein sequences queried for >35% similarity against the entire allergen database returned zero hits.

Based on the search of 6 consecutive amino acids, all inserted gene sequences had 100% identity to known allergens, however, it should be noted that the use of a 6-mer amino acid identity search can generate false positives (Goodman, 2006; EFSA, 2010). The FARRP indicates that a single identity match of 6 to 8 contiguous amino acids does not imply similar IgE binding in the absence of more extensive identity alignments (Goodman et al., 2008). Evaluation of sequence identity over a sliding 80-mer amino acid window indicated that several gene sequences had greater than 35% similarity to known allergen sequences. However, none of the sequences shared greater than 35% identity with any identified allergens over their full sequence length, indicating the unlikely potential for cross-reactivity to any known allergens. Therefore, based on the assessment conducted, the inserted heterologous gene sequences in the production strain to produce steviol glycosides reb M produced by fermentation have low potential for allergenicity. Neither protein nor DNA is present in the final product of steviol glycosides reb M produced by fermentation, as defined in the product specifications, and the potential allergenicity of the heterologous gene sequences inserted in the production strain does not present a health concern.

(10) Conclusions

Based on a critical evaluation and analysis of the information available on steviol glycosides reb M produced by fermentation summarized above, it is concluded that there is reasonable certainty that steviol glycosides reb M produced by fermentation is safe under the intended conditions of use as a general-purpose sweetener and is also Generally Recognized as Safe (GRAS). Information and data on the toxicology and other relevant properties of steviol glycosides reb M produced by fermentation are available in the public scientific literature and indicate it is safe for use as a general-purpose sweetener. Amyris's steviol glycosides reb M produced by fermentation has been reviewed extensively by an expert committee qualified by education and training to

evaluate the safety of such products and they have independently concluded that Amyris's steviol glycosides reb M produced by fermentation is GRAS based on scientific procedures for use as a general-purpose sweetener.

Part 7 – List of supporting data and information

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GRAS EXPERT PANEL REPORT

The Generally Recognized as Safe (GRAS) Status of the Proposed Uses of Steviol Glycosides Rebaudioside M Produced by Fermentation

August 2018

We, an independent panel of experts, qualified by scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), were specially convened by Keller and Heckman LLP, on behalf of their client, Amyris Inc., to evaluate the safety and "Generally Recognized As Safe" ("GRAS") status of the proposed uses of steviol glycosides rebaudioside ("reb") M produced by fermentation, which is manufactured using a strain of *S. cerevisiae* and composed of ≥ 95% reb M in addition to other steviol glycosides.

Amyris's steviol glycosides reb M produced by fermentation with a genetically modified strain of *Saccharomyces cerevisiae* is proposed for use as a general-purpose sweetening agent, excluding infant formulas and meat and poultry products. Similar to most other high-intensity sweeteners that have been approved by the FDA as general-purpose sweeteners, use of Amyris's steviol glycosides reb M produced by fermentation is not restricted to specific foods or use-levels, but is controlled by technological properties (e.g., sweetness potency). Amyris's steviol glycosides reb M produced by fermentation is characterized by a sweetness intensity comparable to other high-intensity sweeteners and is approximately 200-300 times sweeter than sucrose. Further, the uses and use-levels of Amyris's steviol glycosides reb M produced by fermentation reflects those currently permitted for other high-intensity sweeteners in the United States.

The Expert Panel critically evaluated the GRAS Notification prepared by Keller and Heckman LLP that summarizes the characteristics of Amyris's steviol glycosides reb M produced by fermentation, the manufacturing process, proposed uses, and safety information. Given the metabolic fate of steviol glycosides, the safety of steviol glycosides reb M produced by fermentation can be established based on the conclusions of the steviol glycoside safety reviews, and on the publicly available scientific literature related to the safety of steviol glycosides, including the reviews conducted by the U.S. FDA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the European Commission's Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA), Food Standards Australia New Zealand (FSANZ), and Health Canada.

Following its critical evaluation of all the information submitted and other information deemed appropriate, the Expert Panel unanimously concluded that the proposed uses of Amyris's steviol glycosides reb M produced by fermentation, manufactured consistent with current good

manufacturing practices (cGMPs) and meeting appropriate food-grade specifications presented in the GRAS Notification, are safe and suitable, and GRAS based on scientific procedures.

Our signatures confirm no conflict of interest with our work on this panel, including no ownership or equity in Amyris, no compensation for services to Amyris outside of our work on this panel, no research funding from Amyris, no debt relationship with Amyris, and no role as a director, officer, trustee, general partner, or employee of Amyris or Keller and Heckman. We further confirm no such conflict of interest with respect to a spouse, child, general partner, or prospective employer.

It is the opinion of this Expert Panel that other qualified experts would concur with these conclusions.

(b) (6)

Date: 10 September 2018

Joseph F. Borzelleca, Ph.D.
Emeritus Professor Pharmacology & Toxicology
School of Medicine
Virginia Commonwealth University
Richmond, VA
USA

(b) (6)

Date: 05 Sept 2018

John A. Thomas, Ph.D.
Adjunct Professor
School of Medicine
Indiana University
Indianapolis, IN
USA

(b) (6)

Date: 04 Sept 2018

Michael W. Pariza, Ph.D.
Emeritus Professor, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison
Madison, WI
USA

Perrier, Judith

To: Perrier, Judith
Subject: FW: GRN 812 - Rebaudioside M From Saccharomyces Cerevisiae
Attachments: 2018-10-16_Acknowledgement_Letter_0812_Transmittal_.pdf; Steviol Glycoside Reb M. Amyris GRASN .pdf; GRAS Notice Cover Letter.pdf

Hello Judy,

We see that Amyris' GRAS Notice 812 has been posted on the GRAS Notice Inventory at https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=812.
Thank you!

The client has asked that I reach out to request that the ingredient name be reflected as "Steviol Glycosides Rebaudioside M" as it is reflected in the GRAS Notice and Cover Letter (both attached) instead of just "Rebaudioside M". Please let us know if this change can be made.

Many thanks,
Eve

Evangelia C. Pelonis
Partner
tel: +1 202.434.4106 | fax: +1 202.434.4646 | pelonis@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

From: Pelonis, Evangelia C.
To: Perrier, Judith
Subject: RE: GRN 812 - Rebaudioside M From Saccharomyces Cerevisiae
Date: Monday, October 29, 2018 5:38:12 PM

Dear Ms. Perrier,

Thanks for your email. Please let us know if it would it be possible to use the name "Steviol Glycosides Rebaudioside M" in the GRAS Inventory so it reflects the title of the GRAS Notice.

Regards,
Eve

Evangelia C. Pelonis
Partner
tel: +1 202.434.4106 | fax: +1 202.434.4646 | pelonis@khlaw.com
1001 G Street NW, Suite 500 West | Washington, DC 20001

APPENDIX I:
GRAS EXPERT PANEL REPORT

GRAS EXPERT PANEL REPORT

The Generally Recognized as Safe (GRAS) Status of the Proposed Uses of Steviol Glycosides Rebaudioside M Produced by Fermentation

August 2018

We, an independent panel of experts, qualified by scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), were specially convened by Keller and Heckman LLP, on behalf of their client, Amyris Inc., to evaluate the safety and "Generally Recognized As Safe" ("GRAS") status of the proposed uses of steviol glycosides rebaudioside ("reb") M produced by fermentation, which is manufactured using a strain of *S. cerevisiae* and composed of ≥ 95% reb M in addition to other steviol glycosides.

Amyris's steviol glycosides reb M produced by fermentation with a genetically modified strain of *Saccharomyces cerevisiae* is proposed for use as a general-purpose sweetening agent, excluding infant formulas and meat and poultry products. Similar to most other high-intensity sweeteners that have been approved by the FDA as general-purpose sweeteners, use of Amyris's steviol glycosides reb M produced by fermentation is not restricted to specific foods or use-levels, but is controlled by technological properties (e.g., sweetness potency). Amyris's steviol glycosides reb M produced by fermentation is characterized by a sweetness intensity comparable to other high-intensity sweeteners and is approximately 200-300 times sweeter than sucrose. Further, the uses and use-levels of Amyris's steviol glycosides reb M produced by fermentation reflects those currently permitted for other high-intensity sweeteners in the United States.

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Following its critical evaluation of all the information submitted and other information deemed appropriate, the Expert Panel unanimously concluded that the proposed uses of Amyris's steviol glycosides reb M produced by fermentation, manufactured consistent with current good

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manufacturing practices (cGMPs) and meeting appropriate food-grade specifications presented in the GRAS Notification, are safe and suitable, and GRAS based on scientific procedures.

Our signatures confirm no conflict of interest with our work on this panel, including no ownership or equity in Amyris, no compensation for services to Amyris outside of our work on this panel, no research funding from Amyris, no debt relationship with Amyris, and no role as a director, officer, trustee, general partner, or employee of Amyris or Keller and Heckman. We further confirm no such conflict of interest with respect to a spouse, child, general partner, or prospective employer.

It is the opinion of this Expert Panel that other qualified experts would concur with these conclusions.

(b) (6)

Date: 29 August 2018

Joseph F. Borzelleca, Ph.D.
Emeritus Professor Pharmacology & Toxicology
School of Medicine
Virginia Commonwealth University
Richmond, VA
USA

(b) (6)

Date: 30 Aug. 2018

John A. Thomas, Ph.D.
Adjunct Professor
School of Medicine
Indiana University
Indianapolis, IN
USA

Michael W. Pariza, Ph.D.

Digitally signed by Michael W. Pariza, Ph.D.
DN: cn=Michael W. Pariza, Ph.D., o=Michael W. Pariza Consulting LLC, ou=Member, email=mwpariza@gmail.com, c=US
Date: 2018.08.29 14:53:58 -05'00'

Date:

Michael W. Pariza, Ph.D.
Emeritus Professor, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison
Madison, WI
USA

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