#882

September 4, 2019

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

Dear Dr. Zang

RE: GRAS Exemption Claim for Rebaudioside M

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Cargill, Incorporated, 15407 McGinty Road West, M.S. 163, Wayzata, Minnesota, 55391], a Notice of the evaluation, on the basis of scientific procedures, that rebaudioside M, as defined in the enclosed documents and manufactured according to current Good Manufacturing Practices, is GRAS under specific conditions of use as an ingredient in food and beverages, and therefore, is exempt from the premarket approval requirements of the *Federal Food, Drug, And Cosmetic Act*. Information setting forth the basis for the GRAS evaluation, which includes detailed information on the notified substance and a summary of the basis for GRAS status, as well as a consensus opinion of an independent panel of experts in support of the safety of rebaudioside M under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Notice for Rebaudioside M" were scanned for viruses prior to submission and is thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

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

Yours sincerely

Nicole Cuellar-Kingston
Principal Scientist, Scientific & Regulatory Affairs
Cargill, Incorporated

GRAS NOTICE FOR REBAUDIOSIDE M

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Cargill, Incorporated
15407 McGinty Road West, M.S. 163
Wayzata, MN
55391 USA

DATE:
4 September 2019

GRAS Notice for Rebaudioside M

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GRAS Notice for Rebaudioside M

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Cargill, Incorporated (Cargill) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that rebaudioside M (Reb M), manufactured according to current Good Manufacturing Practices (cGMP) is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Cargill's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Cargill, Nicole Cuellar-Kingston hereby certifies that all data and information presented in this Notice represents a complete, representative, and balanced submission, and which considered all unfavorable as well as favorable information known to Cargill and pertinent to the evaluation of the safety and GRAS status of Reb M as an ingredient for addition to food.

Signed,

[redacted] 9/4/2019

Nicole Cuellar-Kingston, M.S. Date
Principal Scientist, Scientific & Regulatory Affairs
Cargill, Incorporated

1.1 Name and Address of Notifier

Nicole Cuellar-Kingston
Cargill, Incorporated
15407 McGinty Road West, M.S. 163
Wayzata, MN
55391 USA

Telephone: 952-742-2113
Email: Nicole_Cuellar-Kingston@cargill.com

1.2 Common Name of Notified Substance

Stevia sweetener

Synonyms: Stevia sweetener (steviol glycosides), steviol glycosides rebaudioside M, Reb M, EverSweet™

1.3 Conditions of Use

Reb M is a purified steviol glycoside product, primarily containing rebaudioside M, obtained from a *Yarrowia lipolytica* (*Y. lipolytica*) production strain and is intended to be used as a general purpose sweetening agent in conventional food and beverage products in accordance with the principles of cGMP, under the same conditions of use as defined for steviol glycosides produced by *Saccharomyces cerevisiae* (*S. cerevisiae*) in GRN 000626 (U.S. FDA, 2016a). The sweetness intensity of Reb M depends on the ratio of individual steviol glycosides present in a preparation and may range from 200 to 350 times sweeter than sucrose (Prakash *et al*., 2014). The use-levels of other high-intensity sweeteners (HIS) that have been approved by the U.S. FDA as general purpose sweeteners (or otherwise received "no questions" letters from the U.S. FDA upon submission of a GRAS Notification) are not restricted to specific foods or use-levels. Instead, the use-levels of HIS are self-limiting based on their organoleptic properties (*i.e.*, sweetness potency). Steviol glycosides have a sweetness profile comparable to aspartame which is 200 times as sweet as sucrose and has been used as a basis for determining the use-levels described in several previous GRAS Notifications for steviol glycosides. As such, the uses and use-levels of Reb M are expected to be similar to those currently permitted for other HIS that are approved for use in the U.S.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a) and (b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2018b), Cargill has concluded that the intended uses of Reb M as described herein are GRAS on the basis of scientific procedures. This GRAS evaluation has used data pertaining to the safety of Reb M which are generally available in the public domain. A panel of experts (GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of Reb M as a component of food was convened and the GRAS Panel concurred with Cargill's evaluation of GRAS status.

The scientific data pertaining to the safety of Reb M is presented herein. All information presented within this notification was reviewed by the GRAS Panel, qualified in their field by scientific training to evaluate the safety of Reb M. The consensus statement of the GRAS Panel is provided in Appendix A entitled "**GRAS Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of Rebaudioside M for Use as a General Purpose Sweetener**".

1.5 Availability of Information

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

Cargill, Incorporated
15407 McGinty Road West, M.S. 163
Wayzata, MN
55391 USA

In addition, should the U.S. FDA have any questions or additional information requests regarding this Notification during or after the Agency's review of the Notice, Cargill will supply these data and information.

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

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

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

2.1 Description

The ingredient that is the subject of this GRAS evaluation is a purified steviol glycoside product, primarily containing rebaudioside M, and referred to as Reb M. Reb M is obtained from a *Y. lipolytica* production strain and contains primarily rebaudioside M in combination with any of the following steviol glycosides: rebaudioside A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and/or dulcoside A. Reb M meets or exceeds the ≥95% steviol glycoside purity definition established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Additional description of the ingredient and information characterizing the identity of the source organism is presented below.

2.1.1 Chemical and Physical Characteristics

Reb M is a white to off-white powder with a characteristic sweet taste, consistent with the description of commercial steviol glycoside preparations published by JECFA in the most recent chemical and technical assessment (CTA) (FAO, 2016).

The molecular structure of steviol glycosides consists of a steviol backbone that is linked to mono-, di-, or oligosaccharide groups (*i.e.,* glucose, xylose, rhamnose, fructose, deoxyglucose, galactose, and arabinose) at the R_1 and R_2 positions on carbons 19 and 13, respectively (see Figure 2.1.1-1). The Chemical Abstracts Service (CAS) numbers, empirical formulae, molecular weights, and R_1 and R_2 groups for the individual steviol glycosides that may be present in Reb M are summarized in Table 2.1.1-1. Given the structural similarities of all steviol glycosides, it is expected that the physiochemical properties of Reb M will be similar to other steviol glycosides, as described in the numerous GRAS Notices previously submitted to the U.S. FDA (see Section 3.1).

Figure 2.1.1-1 Backbone Structure for Steviol Glycosides

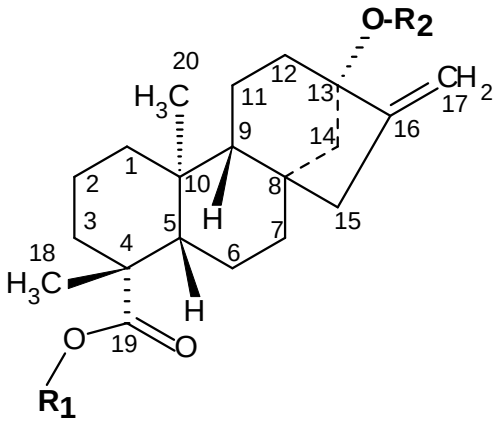


Table 2.1.1-1 Molecular Weight and Formula, and R-Groups in Backbone Structure (see Figure 2.1.1-1)

Steviol Glycoside	CAS Number	Molecular Weight (Da)	Molecular Formula	R-Groups in Backbone Structure	
				R_1	R_2
Rebaudioside M	1220616-44-3	1,291.3	$C_{56}H_{90}O_{33}$	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside D	63279-13-0	1,129.15	$C_{50}H_{80}O_{28}$	B-Glc-β-Glc(2-1)	Glcβ(1-2)[Glcβ(1-3)]Glcβ1
Rebaudioside A	58543-16-1	967.01	$C_{44}H_{70}O_{23}$	β-Glc	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside B	58543-17-2	804.88	$C_{38}H_{60}O_{18}$	H	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside C	63550-99-2	951.02	$C_{44}H_{70}O_{22}$	β-Glc	Rhaα(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside E	63279-14-1	967.01	$C_{44}H_{70}O_{23}$	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside F	438045-89-7	936.99	$C_{43}H_{68}O_{22}$	β-Glc	β-Glc-β-Xyl(2-1)
Stevioside	57817-89-7	804.88	$C_{38}H_{60}O_{18}$	β-Glc	β-Glc-β-Glc(2-1)
Steviolbioside	41093-60-1	642.73	$C_{32}H_{50}O_{13}$	H	β-Glc-β-Glc(2-1)
Rubusoside	64849-39-4	642.73	$C_{32}H_{50}O_{13}$	β-Glc	β-Glc
Dulcoside A	64432-06-0	788.88	$C_{38}H_{60}O_{17}$	β-Glc	β-Glc-α-Rha(2-1)

CAS = Chemical Abstracts Service; Glc = Glucose; Rha = Rhamnose; Xyl = Xylose.

2.1.2 Equivalence of Reb M Produced by Fermentation to Steviol Glycosides Extracted from the Leaf

To demonstrate the equivalence of rebaudiosides M and D present in Reb M obtained from fermentation to rebaudiosides M and D extracted from the leaves of *Stevia rebaudiana* (*S. rebaudiana*) Bertoni, high-performance liquid chromatography (HPLC) analyses were conducted. Cargill utilized its ultra-high-performance liquid chromatography (UHPLC) method, further described in Section 2.3.5, to compare the retention times of rebaudiosides M and D derived from the *Y. lipolytica* production strain to rebaudiosides M and D extracted from the leaves of *S. rebaudiana* Bertoni. For rebaudiosides M and D from *Y. lipolytica* (Reb M Lot No.), 2 major peaks were identified in the chromatogram at retention times of 7.050 and 6.183 minutes. These peaks corresponded to rebaudiosides M and D, respectively, based on the retention times of the rebaudioside M and rebaudioside D standards from *S. rebaudiana* Bertoni that were overlaid on the same chromatogram. These data demonstrate that rebaudioside M and rebaudioside D produced by fermentation have the same HPLC retention times as rebaudioside M and rebaudioside D from *S. rebaudiana* Bertoni and establish that steviol glycosides from these 2 sources are chemically identical.

2.2 Manufacturing

2.2.1 Production Microorganism

2.2.1.1 Parental Strain

Three parental strains of *Y. lipolytica* (strains ATCC 76861, ATCC 76982, and ATCC 201249) were obtained directly from the American Type Culture Collection (ATCC) and used to generate 2 starting strains. Strain construction initiated with 2 strains (strains ML326 and ML350) that had opposite mating types to allow for subsequent mating and natural polymorphic variation.

2.2.1.2 Production Strain

Both starting strains (ML326 and ML350) were engineered with the steviol glycoside production pathways according to general transformation procedures, as described in further in Section 2.2.1.3. After several modifications to each strain, the strains were mated to produce diploids, and said diploids were sporulated to produce haploid progeny. A single haploid progeny was further modified by transformation to improve production. The spores were screened for high steviol glycoside production and the production strain was derived from one of these spores. Antibiotic resistance markers (kanamycin, hygromycin, and nourseothricin) were transiently used in the process. Marker systems were rendered non-functional restoring antibiotic sensitivity to the strain, which was confirmed using polymerase chain reaction (PCR) analysis and by verifying that the strain was sensitive to the relevant antibiotics.

2.2.1.3 Construction of the Production Strain

The genes used to generate the production strain code for enzymes required to synthesize, transport, and improve the overall production efficiency of steviol glycosides. The parental strains of *Y. lipolytica* were initially modified to over-express the genes responsible for the production of steviol glycosides (*i.e.,* Reb M). Most of the genes originated from the plant *S. rebaudiana* Bertoni or other edible plants but were produced by gene synthesis and adapted with respect to codon usage for optimal expression in the yeast. *S. rebaudiana* Bertoni is the current botanical source of steviol glycosides. In addition to enzymes specific to the steviol glycoside pathway, native genes from *Yarrowia* were overexpressed to increase the flow of carbon into the steviol glycoside pathway and transport of steviol glycosides.

Yarrowia strains of both mating types were engineered for steviol glycoside production. These strains were mated, the diploid sporulated, and spores with steviol glycoside production were selected. One of these spores was further developed for the production of steviol glycosides. Strain ML10371 (MAT - A, lysl -, ura3 -, leu2 -) was transformed with defined DNA fragments using a lithium acetate/PEG fungal transformation protocol method and transformants were selected on minimal medium. Antibiotic resistance markers nourseothricin and hygromycin (HPH hygromycin resistance gene) were used during integrations and were rendered non-functional in commercial strains. Linear fragments contain a construct for the overexpression of genes of interest linked to *Yarrowia* promoters and terminators together flanked by lox sites (Güldener *et al*., 1996; Lambert *et al*., 2007). The introduced DNA sequences are integrated partly in predefined loci (targeted integration) but mostly randomly integrated. The yeast *Y. lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences, therefore both random and targeted introduction of DNA sequences will not lead to an increased risk due to unintended pleiotropic effects. The final production strain is sensitive to kanamycin, nourseothricin, and hygromycin. The production strain is not toxigenic or pathogenic and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens. The incorporated DNA is either synthetic or sourced from biosafety level 1 organisms and is not associated with any known allergens or toxins.

The identity of the production strain is confirmed through PCR analysis of the inserted genes. Additionally, as homologous recombination is used for the genetic transformation of the yeast, the genetic elements introduced are stable. The cell line stability is demonstrated by using secondary and tertiary cell banks and comparing productivities to primary cell banks. Whole genome sequencing of the production strain can also be used to confirm genetic stability. Extended seed trains also are typically tested to ensure retention of phenotype over many generations.

2.2.2 Raw Materials, Processing Aids, and Equipment Specifications

Information regarding the raw materials, processing aids, and equipment used during the manufacture of Reb M is provided in Table 2.2.2-1. All raw materials, processing aids, and equipment used in the manufacture of Reb M are food-grade quality, are permitted by U.S. regulation, or have GRAS status for their respective uses and are used consistent with cGMP requirements.

Table 2.2.2-1 Regulatory Status of Raw Materials, Processing Aids, and Equipment Used in the Manufacture of Reb M

Raw Material	Use	Regulatory Status	
		21 CFR (U.S. FDA, 2018a)	Approved Uses
Dextrose	Fermentation Medium	§184.1857	No limitation other than cGMP.
Sucrose	Fermentation Medium	§184.1854	No limitation other than cGMP.
Glycerin	Fermentation Medium	§182.1320	No limitation other than cGMP.
Ammonium sulfate	Fermentation Medium	§184.1143	GRAS; standard materials used within enzyme industry.
Potassium phosphate	Fermentation Medium		GRAS; standard materials used within enzyme industry.
Magnesium sulfate	Fermentation Medium	§184.1443	No limitation other than cGMP as flavor enhancer, nutrient supplement, and processing aid.
Potassium sulfate	Fermentation Medium	§184.1643	GRAS; standard materials used within enzyme industry.
Sodium sulfate	Fermentation Medium	§186.1797	GRAS; standard materials used within enzyme industry.
Biotin	Fermentation Medium	§182.8159	No limitation other than cGMP.
Calcium pantothenate	Fermentation Medium	§184.1212	Used as a nutrient supplement with no limitation other than cGMP.
Niacin (nicotinic acid)	Fermentation Medium	§184.1530	Used as a nutrient supplement with no limitation other than cGMP.
Thiamine	Fermentation Medium	§184.1875	Used as a flavoring agent and nutrient supplement with no limitation other than cGMP.
Pyridoxine	Fermentation Medium	§184.1676	Used as a nutrient supplement with no limitation other than cGMP.
para-Aminobenzoic acid	Fermentation Medium		EAFUS listed.
Myo-inositol	Fermentation Medium	§184.1370	Used as a nutrient supplement with no limitation other than cGMP.
Sodium Hydroxide	Fermentation Medium; Ion-exchange regeneration	§184.1763	pH control agent and processing aid with no limitation other than cGMP.
Sodium EDTA	Fermentation Medium	§172.135	Permitted in a number of foods as a food additive at specified levels.
Zinc sulfate	Fermentation Medium	§182.8997	Used as a nutrient supplement with no limitation other than cGMP.
Manganese chloride	Fermentation Medium	§184.1446	Used as a nutrient supplement with no limitation other than cGMP.
Manganese sulfate	Fermentation Medium	§184.1461	Used as a nutrient supplement with no limitation other than cGMP.
Copper sulfate	Fermentation Medium	§184.1261	Used as a nutrient supplement and processing aid with no limitation other than cGMP.

Table 2.2.2-1 Regulatory Status of Raw Materials, Processing Aids, and Equipment Used in the Manufacture of Reb M

Raw Material	Use	Regulatory Status	
		21 CFR (U.S. FDA, 2018a)	Approved Uses
Calcium chloride	Fermentation Medium	§184.1193	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.
Ferrous sulfate	Fermentation Medium	§184.1315	Used as a nutrient supplement and processing aid with no limitation other than cGMP.
Potassium iodide	Fermentation Medium	§172.375	As a source of iodine.
		§184.1634	Used as a nutrient supplement and in table salt (0.01%).
Ammonium hydroxide	Fermentation Medium	§184.1139	Used as a leavening agent, pH control agent, surface- finished agent, and boiler water additive with no limitation other than cGMP.
Citric acid	Fermentation Medium	§184.1033	pH control agent and processing aid with no limitation other than cGMP.
Phosphoric acid	Fermentation Medium	§182.1073	pH control agent and processing aid with no limitation other than cGMP.
Potassium hydroxide	Fermentation Medium	§184.1631	pH control agent and processing aid with no limitation other than cGMP.
Sulfuric acid	Fermentation Medium	§184.1095	pH control agent.
Antifoam	Fermentation Medium	§173.340	Secondary direct food additive, defoaming agent.
Boiler chemicals	Fermentation Medium	§173.310	Boiler water additives.
Yeast extract	Seed Cultures	§184.1983	Used as a flavoring agent and adjuvant at levels not to exceed 5% in food.
Potassium sorbate	Preservative	182.3640	GRAS when used in accordance with cGMP.
Sodium benzoate	Preservative	§184.1733	Used as an anti-microbial agent at levels not to exceed GMP (typically 0.1% in food).
Microfiltration/ Ultrafiltration	Purification		Used in accordance with §177.2910.
Adsorption resin	Purification		Used in accordance with §173.65.
Ion-exchange resin	Purification		Used in accordance with §173.25.
Hydrochloric acid	Ion-exchange regeneration	§182.1057	GRAS when used in accordance with cGMP.
Activated carbon	Decolorizing agent		GRAS; standard material used within food industry.
Ethanol[a]	Elution solvent Crystallization	§184.1293	GRAS when used in accordance with cGMP.
Methanol[b]	Crystallization		GRAS when used in accordance with cGMP.

CFR = Code of Federal Regulations; cGMP = Current Good Manufacturing Practices; EAFUS = Everything Added to Foods in the United States; EDTA = ethylenediaminetetraacetic acid; GRAS = Generally Recognized as Safe.

[a] JECFA specifications for steviol glycosides specify a level of not more than 5,000 ppm for ethanol residues.

[b] JECFA specifications for steviol glycosides specify a level of not more than 200 ppm for methanol residues.

2.2.3 Reb M Manufacturing Process

Dextrose or sucrose, salts, trace metals, and water are steam-sterilized (121°C for 30 minutes) and mixed with the filter-sterilized vitamins, yeast extract, and filtered deionized water to create the fermentation medium. The final medium is mixed with the yeast inoculum, which has been grown sequentially from the original glycerol stock solution using dextrose or glycerin and yeast extract as nutrition sources, and allowed to ferment under aerobic conditions. The pH of the fermentation is maintained at pH 4.5 to 6.5 using potassium hydroxide, phosphoric acid, or ammonium hydroxide. The fermentation broth undergoes heat treatment (75 to 95°C, 5 minutes to 1 hour) to stop fermentation and kill the yeast cells. Optionally, the pH of the broth is adjusted to 4.2 with phosphoric acid, hydrochloric acid, sulfuric acid, or citric acid and the yeast biomass is subsequently removed from the dissolved product by any combination of centrifugation, microfiltration, or clarification. The product stream may be purified by ultrafiltration to further remove dissolved proteins and other nitrogen-containing compounds. Preservatives such as potassium sorbate and sodium benzoate may be added to the filtrate and the pH of the filtrate may be lowered to about 4.2 to minimize microbial contamination downstream. The filtrate then undergoes typical purification processes used for steviol glycosides extracted from *S. rebaudiana* leaves. Additionally, the optional drying steps described below can be utilized to vary the percentages of the individual steviol glycosides in the final product.

The filtrate may be passed through an adsorption resin, retaining steviol glycosides, thus separating them from other constituents that may be present in the filtrate. The resin is subsequently washed with ethanol to elute steviol glycosides. The eluate undergoes evaporation to remove ethanol. Further treatment of the steviol glycoside-rich eluate may be passed through ion-exchange resins and optional pH adjustment and activated carbon treatment removes any additional impurities and colored substances from the eluate. The eluate is concentrated by evaporation to initiate crystallization in water or mixed with aqueous ethanol or methanol to start crystallization. A second crystallization may be conducted depending upon the desired steviol glycoside composition in final product. Optionally, the eluate may be dried prior to crystallization in the presence of aqueous ethanol or methanol. The mother liquor is separated from the solids and retained for further processing. The crystals are rinsed with water or ethanol (wash added to mother liquor) and optionally evaporated prior to drying. The product is then checked for its final composition using HPLC and released as the final steviol glycoside product (Reb M). Additionally, the final product may be blended with Reb M from other production lots that meet the specifications outlined in Section 2.3.1.

As mentioned above, the mother liquor separated from the steviol glycoside crystals is further dried or the liquid concentrated by evaporation to isolate any remaining steviol glycosides. The concentrate or solids are re-dissolved in water or aqueous ethanol or methanol to crystallize steviol glycosides. The crystals are separated, rinsed with water or ethanol, optionally evaporated, and dried. Following drying, the steviol glycosides may be designated as the final product, or mixed with Reb M produced previously.

A schematic overview of the manufacturing process for Reb M is provided as a flowchart in Figure 2.2.3-1.

Figure 2.2.3-1 Schematic Overview of the Manufacturing Process for Reb M

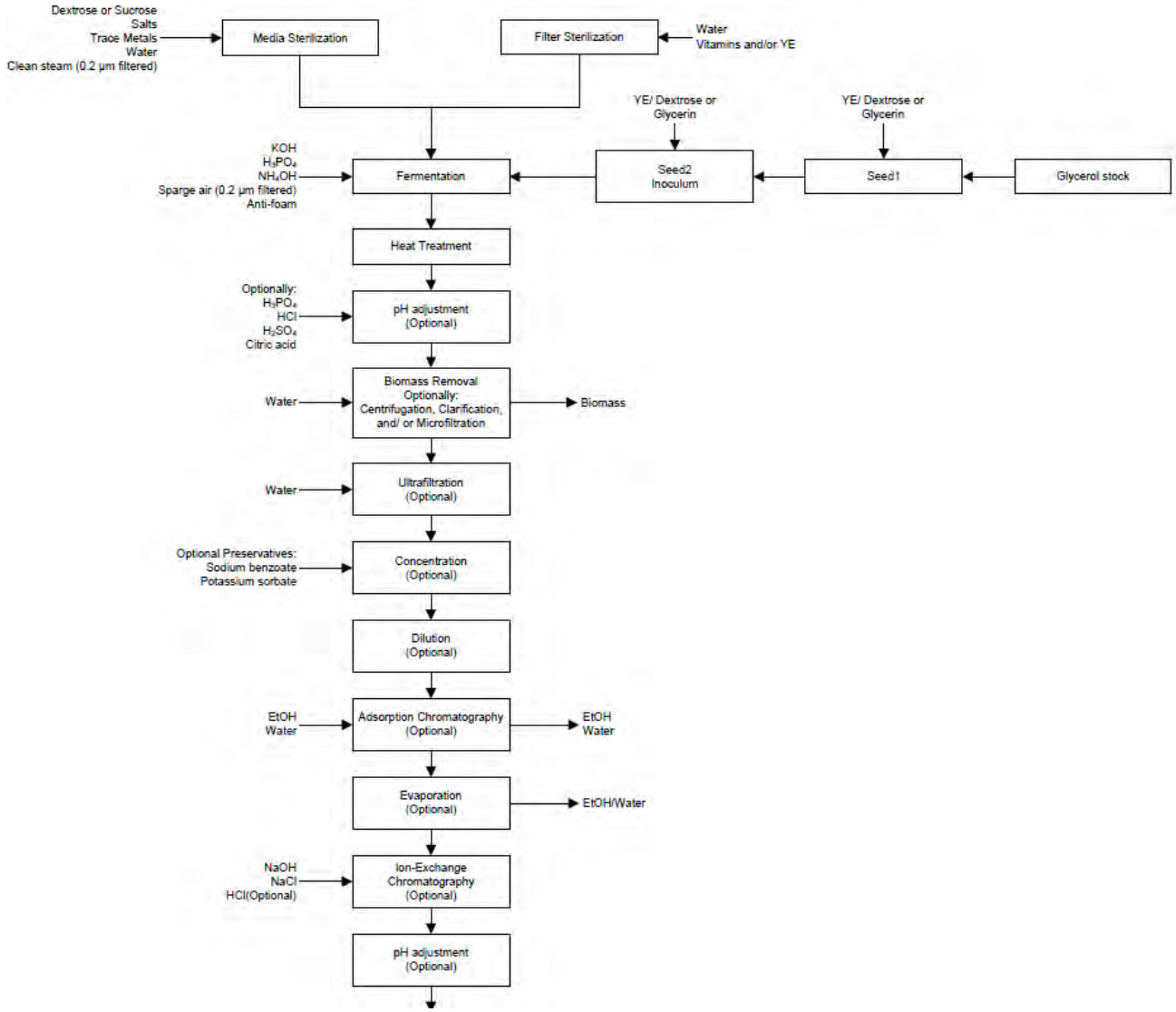
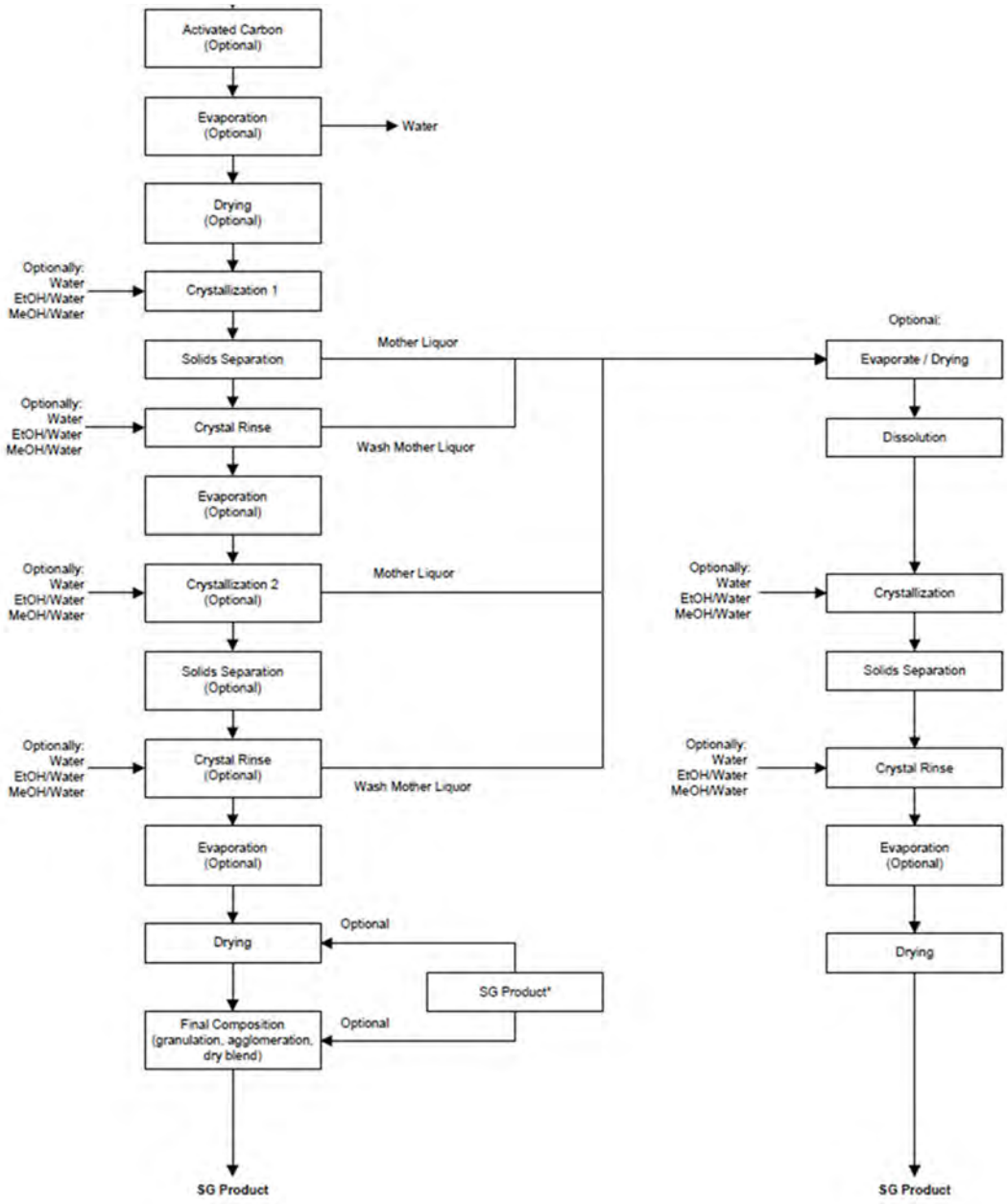


Figure 2.2.3-1 Schematic Overview of the Manufacturing Process for Reb M



2.3 Product Specifications and Batch Analyses

2.3.1 Physical and Chemical Specifications

The physical and chemical specifications for Reb M are presented in Table 2.3.1-1 and were established based on those published by JECFA for "Steviol glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a) and by the Food Chemicals Codex (FCC) for steviol glycosides (FCC, 2018). Specifications for similar steviol glycoside products, steviol glycosides from *S. cerevisiae* and steviol glycosides consisting primarily of rebaudioside M produced in *Y. lipolytica*, are published in GRAS Notifications GRN 000626 and 000759, respectively, to which the U.S. FDA responded with "no questions" letters regarding the GRAS status of the ingredients for use in food (Cargill, 2016; U.S. FDA, 2016a; DSM, 2018; U.S. FDA, 2018b). For comparison, the specifications for steviol glycosides from *S. rebaudiana* Bertoni set by JECFA and the specifications for steviol glycosides from *S. cerevisiae* published in GRN 000626 are also listed in Table 2.3.1-1.

Table 2.3.1-1 Physical and Chemical Specifications for Reb M

Specification Parameter	Units	Specification			Method Used by Cargill
		Steviol Glycosides from *Y. lipolytica* (Reb M)	**Steviol Glycosides from *S. cerevisiae* (GRN 000626)**	**Steviol Glycosides from *S. rebaudiana* Bertoni (JECFA, 2017a)**	
Total steviol glycosides	%	NLT 95.0	NLT 95 (wt/wt) (on an anhydrous basis)	NLT 95 (on dried basis)	ERT-017-3
Identity					
Appearance	NA	Conforms to standard	Conforms to standard	White to light yellow powder	ERT-039-4
Solubility	NA	Slightly soluble in a mixture of ethanol and water (50:50)	NS	Freely soluble in a mixture of ethanol and water (50:50)	ERT-047-01
Odor	NA	Conforms to standard	Conforms to Standard	Odorless or having slight characteristic odor	ERT-039-4
pH	NA	4.5 to 7	NS	4.5 to 7	ERT-006-1
Purity					
Ash	%	NMT 1.0	NMT 1 (wt/wt)	NMT 1	AOAC 945.46
Loss on drying	%	NMT 6.0	NMT 10 (KF)	NMT 6	ERT-027-1
Residual ethanol	%	NMT 0.5	NMT 0.5	NMT 0.5	ERT-046-1
Residual methanol	%	NMT 0.02	NMT 0.02	NMT 0.02	ERT-046-1
Lead	ppm	NMT 1	NMT 1	NMT 1	EPA 3050/6020 USP 730
Cadmium	ppm	NMT 1	NS	NS	EPA 3050/6020 USP 730
Arsenic	ppm	NMT 1	NMT 0.2	NMT 1	EPA 3050/6020 USP 730
Mercury	ppm	NMT 1	NS	NS	EPA 3050/6020 USP 730

KF = Karl Fischer; NA = not applicable; NLT = not less than; NMT = not more than; NS = not specified.

2.3.2 Microbiological Specifications

The microbiological specifications for Reb M are presented in Table 2.3.2-1 and were established based on those published by JECFA for "Steviol glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a) and by the FCC for steviol glycosides (FCC, 2018). Microbiological specifications for the similar steviol glycoside product, steviol glycosides from *S. cerevisiae,* were published in GRN 000626. For comparison, the specifications for steviol glycosides from *S. rebaudiana* Bertoni set by JECFA and the specifications for steviol glycosides from *S. cerevisiae* published in GRN 000626 are also listed in Table 2.3.2-1.

Table 2.3.2-1 Microbiological Specifications for Reb M

Specification Parameter	Units	Specification			Method Used by Cargill
		Steviol Glycosides from *Y. lipolytica* (Reb M)	**Steviol Glycosides from *S. cerevisiae* (GRN 000626)**	**Steviol Glycosides from *S. rebaudiana* Bertoni (JECFA, 2017a)**	
Aerobic plate count	CFU/g	NMT 1,000	NMT 1,000	NMT 1,000	AOAC 966.23
Yeast	CFU/g	NMT 100	NMT 100	NMT 200	AOAC 997.02
Mold	CFU/g	NMT 100	NMT 100		AOAC 997.02
Coliforms	/g	NMT 3	NMT 10	NS	FDA-BAM 7th ed.
Salmonella	/25 g	Negative	Negative	Negative	AOAC-RI100201
E. coli	/g	Negative	Negative	Negative	USP 62

CFU = colony forming units; NMT = not more than; NS = not specified.

2.3.3 Physical and Chemical Batch Analyses

Analysis of 3 non-consecutive lots of Reb M demonstrates that the manufacturing process as described in Section 2.2 produces a consistent product that meets the defined specifications. A summary of the physical and chemical analysis for the 3 lots of Reb M is presented in Table 2.3.3-1.

Table 2.3.3-1 Summary of the Physical and Chemical Product Analysis for 3 Lots of Reb M

Specification Parameter	Limit	Manufacturing Lot		
Total steviol glycosides (%)	NLT 95.0	100.4	99.57	100.14
Identity				
Appearance	Conforms to standard	Pass	Pass	Pass
Solubility	Slightly soluble in a mixture of ethanol and water (50:50)	Pass	Pass	Pass
Odor	Conforms to standard	Pass	Pass	Pass
pH	4.5 to 7	5.0	4.7	4.7
Purity				
Ash (%)	NMT 1.0	<0.04	<0.04	<0.04
Loss on drying (%)	NMT 6.0	0.65	1.09	1.45
Residual ethanol (%)	NMT 0.5	0.4682	0.3722	0.3611
Residual methanol (%)	NMT 0.02	<0.00001	<0.00001	<0.00001
Lead (ppm)	NMT 1	<0.01	<0.01	<0.01
Cadmium (ppm)	NMT 1	<0.001	<0.001	<0.001

Table 2.3.3-1 Summary of the Physical and Chemical Product Analysis for 3 Lots of Reb M

Specification Parameter	Limit	Manufacturing Lot		
Arsenic (ppm)	NMT 1	<0.01	<0.01	<0.01
Mercury (ppm)	NMT 1	<0.005	<0.005	<0.005

NLT = not less than; NMT = not more than.

2.3.4 Microbiological Batch Analyses

Analysis of 3 non-consecutive lots of Reb M demonstrates that the manufacturing process as described in Section 2.2 produces a product that is free of microbiological contamination that meets the defined specifications. A summary of the microbiological analysis for the 3 lots of Reb M is presented in Table 2.3.4-1.

Table 2.3.4-1 Summary of the Microbiological Product Analysis for 3 Lots of Reb M

Specification Parameter	Limit	Manufacturing Lot		
Aerobic place count (CFU/g)	NMT 1,000	<10	<10	<10
Yeast (CFU/g)	NMT 100	<10	<10	<10
Mold (CFU/g)	NMT 100	<10	<10	<10
Coliforms (/g)	NMT 3	<3.0	<3.0	<3.0
Salmonella (/25 g)	Negative	Negative	Negative	Negative
E. coli (/g)	Negative	Negative	Negative	Negative

CFU = colony forming units; NMT = not more than.

2.3.5 Steviol Glycoside Composition

Reb M is primarily comprised of rebaudioside M and may contain a mixture of the following additional glycosides in various concentrations, such that the total steviol glycoside content is no less than 95%: rebaudiosides A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and dulcoside A. The distribution of steviol glycosides present in Reb M will vary depending on the production process and final product formulation, as described in Section 2.2. In order to determine the steviol glycoside composition, Cargill developed a UHPLC method utilizing gradient elution with ultraviolet light detection, similar to the updated HPLC method described in the specifications for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). Similar to the JECFA 2017 HPLC method, Cargill's method allows for the improved separation of steviol glycosides, especially rebaudioside M and rebaudioside D, in comparison to the isocratic elution method previously utilized by JECFA (JECFA, 2010), and thereby, improves the quantification of steviol glycosides with similar run times.

The steviol glycoside distribution of 3 non-consecutive commercial lots of Cargill's Reb M (Lot #) was determined using HPLC. As presented in Table 2.3.5-1, the final purified Reb M product consists primarily of rebaudiosides M (86.4 to 96.1%) and rebaudioside D (3.1 to 11.9%), with small amounts of rebaudioside A (0.86 to 0.92%) and rebaudioside B (0.24 to 0.39%). Total steviol glycoside content in these 3 commercial lots of Reb M was between 99.6 to 100.4%.

Table 2.3.5-1 Steviol Glycoside Composition for 3 Lots of Reb M

Steviol Glycoside (% dry weight)	Manufacturing Lot		
Rebaudioside A	0.857	0.895	0.924
Rebaudioside B	0.238	0.393	0.325
Stevioside	0	0	0
Rebaudioside C	0	0	0
Rebaudioside D	3.134	11.923	8.181
Rebaudioside E	0	0	0
Rebaudioside F	0	0	0
Rebaudioside M	96.144	86.357	90.714
Rubusoside	0	0	0
Dulcoside A	0	0	0
Steviolbioside	0	0	0
Total steviol glycosides	100.373	99.568	100.144

2.3.6 Residual Protein Analysis

To confirm the success of the purification steps in the manufacturing process (*e.g.,* ion exchange chromatography, adsorption chromatography, and crystallization) and to confirm the absence of residual protein in Reb M, samples from the same non-consecutive lots of final product (Lot) were assayed by the bicinchoninic acid (BCA) protein assay and Protein Dot Blot method. Analytical standards of high purity (>95%) rebaudioside M and rebaudioside D were also tested. All samples were prepared at a concentration of 1 mg/mL and protein content in the solution was measured against a standard curve of bovine serum albumin (BSA): BSA 5 µg/mL to 250 µg/mL for the BCA assay and BSA 0.125 µg/mL to 2 µg/mL for the Protein Dot Blot method. All assessments were carried out in triplicate. Overall, no residual protein was detected in any of the Reb M test samples above the limit of detection for the BCA assay (25 ppm) or above 1.5 ppm using the Protein Dot Blot method.

2.3.7 Residual DNA Analysis

To confirm the absence of residual DNA in Reb M, the same 3 non-consecutive lots of final product (Lot) were assayed by PCR. Primer design and PCR conditions were designed to amplify a specific fragment of one of the genes inserted in the production strain. Genomic DNA extracted from the production strain was used as a positive control. Reb M samples were prepared in water and to determine the matrix-dependent limit of detection samples were spiked with positive control genomic DNA from the production strain (92 ng to 92 fg). Each Reb M test sample was also assayed without a DNA spike. Genomic DNA was extracted from the test samples and the PCR reaction targeting the specific DNA sequence was carried out. In the spiked Reb M samples, positive control genomic DNA down to 10 ng/g product was detected (*i.e.,* matrix-dependent limit of detection), whereas in all the Reb M samples without a DNA spike, no genomic DNA was detected above 10 ng/g. These results confirm the absence of residual genomic DNA in the Reb M final product above the analytical limit of detection of 10 ng/g.

2.4 Stability

2.4.1 General Stability of Steviol Glycosides

At the 68th meeting of JECFA, the Committee evaluated the stability of steviol glycosides under conditions simulating their use in foods (JECFA, 2007a). JECFA noted that steviol glycosides do not undergo browning or caramelization when heated, and are reasonably stable under elevated temperatures used in food processing. Based on the findings from the studies submitted for review, as well as additional publicly available stability studies, JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions. In particular, high-purity steviol glycosides (90 to 94%) are stable for at least 180 days when stored at temperatures up to 24°C in acidic solutions (pH 2 to 4). However, when solutions of steviol glycosides were exposed to elevated temperatures (80°C in water, 8 hours) at pH 4.0 or 3.0, 4 and 8% decomposition, respectively, was observed, indicating that the stability 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 or 3.0, respectively) were observed.

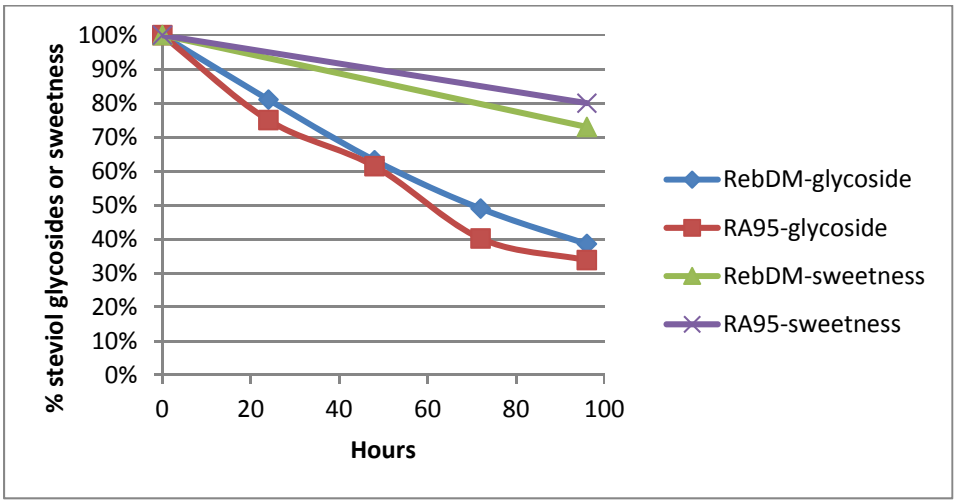
2.4.2 Stability of Steviol Glycosides Produced by Fermentation

Stability studies have not yet been conducted with the Reb M preparation that is the subject of this Notification (*i.e.,* steviol glycosides produced by *Y. lipolytica*). Several stability investigations, however, have been carried out with a steviol glycoside preparation produced by fermentation that has a similar individual steviol glycoside profile as Reb M (*i.e., s*teviol glycosides produced by *S. cerevisiae,* GRN 000626, primarily containing rebaudioside M), the results of which are presented below.

In the first study, the degradation of steviol glycosides produced by *S. cerevisiae* (GRN 000626; ≥95% steviol glycosides) was compared to rebaudioside A extracted from *S. rebaudiana* (RA95, 95% rebaudioside A) under accelerated conditions (see Figure 2.4.2-1). Samples of the steviol glycoside product produced by fermentation (Lot [redacted]) and rebaudioside A were prepared in a citric acid-based environment (pH 3.0), representative of typical beverage conditions. The beverages were manufactured using a hot-fill process (185°F for 2 minutes) and maintained at elevated temperatures to accelerate degradation. Samples were collected at different time points throughout the study and analyzed for steviol glycoside content in accordance with the JECFA HPLC method (2010), modified to include rebaudiosides M and D. Additionally, sensory analyses were conducted to determine how the degradation of the ingredients affected the taste profile.

In comparison to rebaudioside A, steviol glycosides produced by *S. cerevisiae* showed an equivalent degradation profile when analyzed for steviol glycoside content using HPLC and the sweetness degradation also was similar between the 2 steviol glycoside products (see Figure 2.4.2-1 below). The similarities among the degradation of rebaudioside A from *S. rebaudiana* and steviol glycosides from *S. cerevisiae* confirms that the stability of Reb M is expected to be similar to that of individual steviol glycosides and the stability conclusions made by JECFA, that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions, may be extended to include Reb M.

Figure 2.4.2-1 Accelerated Stability Testing of Steviol Glycosides Produced by Fermentation (Lot [redacted]; GRN 000626) Compared to Rebaudioside A, 95% from *Stevia rebaudiana* [pH 3.0]



RA95 = rebaudioside A 95%; RebDM = Steviol glycosides described in GRN 000626

The second set of stability studies includes conventional shelf-life (Table 2.4.2-1) and accelerated stability assessments (Table 2.4.2-2), both of which are currently ongoing. For both studies, 3 non-consecutive lots of steviol glycosides produced by *S. cerevisiae,* GRN 000626, (Lot No. [redacted]) are being analyzed in duplicate for moisture content and steviol glycoside content, and in the conventional study microbial parameters (aerobic plate count, yeast, and mold) are also being assessed. The conventional study is being conducted in a controlled environment at 25°C and 60% relative humidity and testing is scheduled to occur at baseline, 3, 6, 9, 12, 18, 24, and 36 months. The accelerated study is being conducted in a controlled environment at 40°C and 75% relative humidity and testing is scheduled to occur at baseline, 1, 2, 3, 6, and 9 months. Initial results from both studies are provided in the tables below and support the stability of steviol glycosides produced by fermentation under conventional shelf-life and accelerated stability conditions for up to 3 months.

Table 2.4.2-1 Preliminary Results from a Conventional Stability Study on 3 Commercial Lots of Steviol Glycosides Produced by Fermentation, GRN 000626 (study currently on-going)

Parameter	Lot No. [redacted]		Lot No. [redacted]		Lot No. [redacted]	
	Baseline	3 months	Baseline	3 months	Baseline	3 months
Rebaudioside M (%)	86.8	85.65	82.65	83.2	86.25	TBM
Rebaudioside A (%)	0.7625	0.7245	0.722	0.7155	1.1	TBM
Rebaudioside B (%)	1.03	1.07	1.135	1.15	1.01	TBM
Rebaudioside C (%)	<0.107	<.1	<0.107	<.1	<0.107	TBM
Rebaudioside D (%)	8.665	8.62	10.75	10.8	8.1	TBM
Rebaudioside F (%)	<0.107	<.1	<0.107	<.1	<0.107	TBM
Rubusoside (%)	<0.107	<.1	<0.107	<.1	<0.107	TBM
Stevioside (%)	<0.107	<.1	<0.107	<.1	0.211	TBM
Steviolbioside (%)	<0.107	<.1	<0.107	<.1	<0.107	TBM
Dulcoside A (%)	<0.107	<1	<0.107	<.1	<0.107	TBM
Total steviol glycosides (%)	97.25	96.1	95.25	96	96.65	TBM

Table 2.4.2-1 Preliminary Results from a Conventional Stability Study on 3 Commercial Lots of Steviol Glycosides Produced by Fermentation, GRN 000626 (study currently on-going)

Parameter						
	Baseline	**3 months**	**Baseline**	**3 months**	**Baseline**	**3 months**
Moisture initial (%)	6.11	5.3	6.26	4.99	6.22	TBM
Moisture after equilibration (%)	5.3	5.9	4.73	6.49	6.27	TBM
Aerobic plate count (CFU/g)	NM	<10	NM	<10	NM	TBM
Yeast (CFU/g)	NM	<10	NM	<10	NM	TBM
Mold (CFU/g)	NM	<10	NM	10	NM	TBM

CFU = colony forming units; NM = not measured; TBM = to be measured.

Table 2.4.2-2 Preliminary Results from an Accelerated Stability Study on 3 Commercial Lots of Steviol Glycosides Produced by Fermentation, GRN 000626 (study currently on-going)

Parameter	Lot No.				Lot No.				Lot No.			
	Baseline	1 month	2 months	3 months	Baseline	1 month	2 months	3 months	Baseline	1 month	2 months	3 months
Rebaudioside M (%)	86.8	85.7	85.15	85	82.65	82.9	82.9	81.95	86.25	85.9	85.5	TBM
Rebaudioside A (%)	0.7625	0.9765	0.744	0.754	0.722	0.8705	0.718	0.705	1.1	1.11	1.12	TBM
Rebaudioside B (%)	1.03	1.165	1.22	1.32	1.135	1.2	1.25	1.28	1.01	1.13	1.21	TBM
Rebaudioside C (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.1	<0.100	<0.1	<0.107	<0.100	<0.107	TBM
Rebaudioside D (%)	8.665	8.94	8.64	8.8	10.75	10.7	10.6	10.8	8.1	8	7.98	TBM
Rebaudioside F (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.100	<0.100	<0.100	<0.107	<0.100	<0.107	TBM
Rubusoside (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.100	<0.100	<0.100	<0.107	<0.100	<0.107	TBM
Stevioside (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.100	<0.100	<0.100	0.211	0.22	0.22	TBM
Steviolbioside (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.100	<0.100	<0.100	<0.107	<0.100	<0.107	TBM
Dulcoside A (%)	<0.107	<0.100	<0.100	<.1	<0.107	<0.100	<0.100	<0.100	<0.107	<0.100	<0.107	TBM
Total steviol glycosides (%)	97.25	96.75	95.8	95.9	95.25	95.7	95.5	94.75	96.65	96.4	96	TBM
Moisture initial (%)	6.11	5.9	5.5	6.1	6.26	5.12	6.92	4.88	6.22	5.94	5.97	TBM
Moisture after equilibration (%)	5.3	5.53	5.97	6.24	4.73	6.04	5.37	6.64	6.27	6.37	6.26	TBM

TBM = to be measured.

Part 3. § 170.235 Dietary Exposure

3.1 History of Consumption of Steviol Glycosides

Officially discovered in the West in 1887 by Antonio Bertoni (a South American natural scientist), *S. rebaudiana* leaves have been used by the native peoples of Brazil and Paraguay for hundreds of years as both a food ingredient and as a tea (Blumenthal, 1995; Geuns *et al*., 2003). The native Indians of the Guarani Tribe also have been documented to use stevia leaves as a sweetener since pre-Columbian times (Ferlow, 2005). Extracts of *S. rebaudiana*, including stevioside, were already being used as sweeteners in several parts of the world, including Japan, South Korea, Brazil, and China (Geuns *et al*., 2003) prior to JECFA's allocation of an acceptable daily intake (ADI) for steviol glycosides, and subsequent approvals of steviol glycosides in various jurisdictions. In Japan, for example, stevioside has been used as a sweetener for more than 40 years, and its use has been reported to be safe, without the occurrence of adverse effects (Ferlow, 2005). According to the PureCircle Stevia Institute (2019), stevia glycosides are currently used as food additives/sweetening agents in dozens of countries in North America, South America, Asia, Africa, and Europe.

Stevia became a popular herbal tea ingredient in the U.S. in the 1980s and in 1995 and was cleared for use as a dietary supplement (Blumenthal, 1995; Geuns *et al*., 2003; Schoenhals, 2003; Ferlow, 2005). The use of powdered stevia leaves and its leaf-refined extracts in dietary supplement products have been notified to the U.S. FDA under the New Dietary Ingredient Notification (NDIN) requirements of the Dietary Supplement Health and Education Act of 1994 (DSHEA) (DSHEA, 1994; Geuns, 2003; Schoenhals, 2003). As such, stevia is used in a variety of energy bars and beverages in the U.S. that have been labeled and are marketed as dietary supplements (Schoenhals, 2003). Over 50 steviol glycoside preparations have been the subject of GRAS Notifications submitted to the U.S. FDA, to which the Agency has responded with "no questions" letters regarding their use as general purpose sweeteners in food and beverage products. This includes a steviol glycoside preparation produced by *S. cerevisiae* (GRN 000626 – U.S. FDA, 2016a) that is similar in composition to the steviol glycoside preparation produced by *Y. lipolytica* (*i.e.,* Reb M) that is the subject of this evaluation, as well as 2 other GRAS Notifications for rebaudioside A and rebaudioside M produced by a similar strain of *Y. lipolytica* (GRN000632 and GRN000759) (U.S. FDA, 2016b, 2018b).

There have been limited reports of adverse effects following the consumption of steviol glycosides (Lee *et al*., 1979; Ferlow, 2005; Urban *et al*., 2015). Two cases from Japan and 1 case from the U.S. have been reported wherein allergic and hypersensitivity reactions, respectively, to stevia sweeteners occurred (Kimata, 2007; Esmail and Kabadi, 2012). In these cases, the purity of the stevia sweeteners was suspected by the study authors to not meet the JECFA specification of ≥95% total steviol glycosides. Thus, impurities present in the final product have been hypothesized to be the cause of the few reported adverse reactions that have occurred following consumption of stevia products.

3.2 Estimated Consumption of Reb M from Proposed Food Uses

Based on production data, the *per capita* consumption of caloric sweeteners in the U.S. is up to 141 g/day (USDA/ARS, 2016). Assuming that Reb M would replace all sugar consumption, has a sweetness equivalency of between 200 and 350 times that of sucrose, and is primarily comprised of the individual steviol glycoside rebaudioside M, this would correspond to a Reb M intake expressed as steviol equivalents of 1.4 to 2.5[1] mg/kg body weight/day (average body weight of 70 kg assumed). This estimate of Reb M intake is highly conservative since it is unlikely that Reb M would entirely replace sugar consumption.

An alternative approach to estimating intakes for steviol glycosides is to substitute intakes of other known intense sweeteners with Reb M. For example, the intake of rebaudioside A was estimated by Renwick (2008) using published data on dietary exposures to approved intense sweeteners, such as aspartame, from post-market surveillance studies. The exposure from those sweeteners evaluated within the post-market surveillance studies were adjusted for their relative sweetness intensities, assuming a relative sweetness for rebaudioside A of 200 times that of sucrose. The data used in these analyses were primarily from studies that used specifically designed food diaries combined with actual use-levels or approved levels in different foods and beverages (Renwick, 2008). These data were pooled in order to provide a realistic, but conservative estimate of potential consumption of rebaudioside A, ranging from 0.4 to 1.7 mg/kg body weight per day steviol equivalents.

Utilizing the Renwick (2008) paradigm, Cargill generated a consumption estimate for Reb M assuming the complete replacement of the other intense sweeteners included in the Renwick (2008) assessment, a sucrose equivalency of 200 to 350 times sweeter, and a composition of predominantly rebaudioside M (yielding a steviol conversion factor of 0.25). The consumption estimates for Reb M are presented in Table 3.2-1. The mean intake of Reb M on a steviol equivalent basis is predicted to range from 0.18 mg/kg body weight/day for non-diabetic adults to 0.84 mg/kg body weight/day for diabetic children. Predicted intakes for heavy consumers on a steviol equivalent basis ranged from 0.48 mg/kg body weight/day for non-diabetic adults to 1.24 mg/kg body weight/day for non-diabetic children. The predicted intakes of Reb M, expressed as steviol equivalents, are all below the current ADI defined by the JECFA for steviol glycosides of 0 to 4 mg/kg body weight/day as steviol (JECFA, 2009, 2017b).

[1] Calculations: 141,000 mg / 70 kg / 350 = 5.76 mg/kg bw/day x 0.25 = 1.44 mg/kg bw/day steviol equivalents
141,000 mg / 70 kg / 200 = 10.07 mg/kg bw/day x 0.25 = 2.52 mg/kg bw/day steviol equivalents

Table 3.2-1 Intakes of Intense Sweeteners from Renwick (2008) and Predicted Intakes of Reb M

Population Group	Intakes of Intense Sweeteners (as sucrose equivalents)[a] (mg/kg bw/day)		Predicted Intakes of Reb M[b] (mg/kg bw/day)		Predicted Intakes of Reb M (as steviol equivalents)[c] (mg/kg bw/day)	
	Average Consumer	Heavy Consumer	Average Consumer	Heavy Consumer	Average Consumer	Heavy Consumer
Non-diabetic Adults	255	675	0.73 to 1.28	1.93 to 3.38	0.18 to 0.32[d]	0.48 to 0.85
Diabetic Adults	280	897	0.80 to 1.40	2.56 to 4.49	0.20 to 0.35	0.64 to 1.12
Non-diabetic Children	425	990	1.21 to 2.13	2.83 to 4.95	0.30 to 0.53	0.71 to 1.24
Diabetic Children	672	908	1.92 to 3.36	2.59 to 4.54	0.48 to 0.84	0.65 to 1.14

bw = body weight.

[a] Source: Renwick AG (2008). The use of a sweetener substitution method to predict dietary exposures for the intense sweetener rebaudioside A. Food Chem Toxicol 46(Suppl. 7):S61-S69. DOI:10.1016/j.fct.2008.05.009.

[b] Determined based on the sucrose equivalency of 200 to 350 times for steviol glycosides.

[c] Calculated based on the assumption that Reb M is primarily comprised of rebaudioside M (1,291.30 g/mol) and a conversion factor of 0.25 based on a molecular weight of 318.45 g/mol for steviol.

[d] Example calculation of range: 0.73 mg/kg bw/day x 0.25 = 0.18 mg/kg bw/day; 1.28 mg/kg bw/day x 0.25 = 0.32 mg/kg bw/day.

The JECFA Committee recently re-assessed the dietary exposure to steviol glycosides at their 82nd meeting using sugar/intense sweetener substitution methods as described above (JECFA, 2017b). The Committee included mixtures of steviol glycosides in their evaluation and applied a range of conversion factors from 0.2 to 0.7 to account for the different molecular weights of individual steviol glycosides and assumed the most conservative sucrose equivalence of 200. Substituting sugar/intense sweetener consumption data from different global jurisdictions (*e.g.,* U.S., Australia) for steviol glycosides generated a range of consumption estimates from 0.4 to 7.2 mg/kg body weight/day, expressed as steviol equivalents. The Committee noted that, "*Sugar substitution methods were generally overestimates of dietary exposure, as not all sugar in food products would be replaced be intense sweeteners, and a number of intense sweeteners are used in the marketplace*".

Part 4. § 170.240 Self-Limiting Levels of Use

Reb M has a sweetness potency of approximately 200 to 350 times greater than sucrose and under its intended use as a general-purpose sweetener, the use of Reb M in food and beverages is limited by the desired amount of sweetness. Thus, the use of Reb M is self-limiting based on its organoleptic properties when used as a general-purpose sweetener.

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

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

6.1 Introduction

The safety of steviol glycosides has been extensively evaluated and is supported by conclusions from several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, the European Commission's Scientific Committee on Food, the European Food Safety Authority (EFSA), Food Standards Australia/New Zealand (FSANZ), and Health Canada. The data examined in these evaluations included the comparative metabolism and pharmacokinetics of steviol glycosides in animals and humans, acute, 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 of various purities 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 high-purity rebaudioside A (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). Several studies available in the public domain conducted with high purity stevia extracts have demonstrated the shared metabolic fate of all steviol glycosides. Following ingestion, steviol glycosides are hydrolyzed to steviol by members of the *Bacteroidaceae* family residing in the colon. The common metabolite steviol is absorbed from the lower gastrointestinal tract, conjugated to glucuronic acid, and excreted primarily *via* the urine in humans. Steviol glycosides, whether produced by fermentation or extracted from the *S. rebaudiana* plant, are metabolized and biologically handled in an identical manner following oral administration. Because of this shared metabolic fate, the safety database that exists for individual steviol glycosides can therefore be extended to include all glycosylated derivates of the aglycone steviol, including steviol glycosides produced by fermentation.

The safety of Cargill's purified steviol glycoside product primarily containing rebaudioside M and obtained from a *Y. lipolytica* production strain, referred to as Reb M, is based on scientific procedures under the conditions of its intended use as a general purpose sweetening agent in conventional food and beverage products. A discussion on the metabolic fate of steviol glycosides, including a study demonstrating that steviol glycosides produced by fermentation are metabolized by human fecal homogenates in the same manner as steviol glycosides extracted from *S. rebaudiana*, is provided in Section 6.2. Since steviol glycosides produced by *Y. lipolytica* are identical to steviol glycosides extracted from *S. rebaudiana,* the extensive safety database that exists for steviol glycosides from *S. rebaudiana* may be applied to establish the safety of Reb M. Summaries of the conclusions from authoritative scientific and regulatory bodies on the safety of steviol glycosides extracted from *S. rebaudiana* are presented in Section 6.3. Safety data for steviol glycoside preparations published in the scientific literature were previously summarized by Cargill and reviewed by the U.S. FDA in GRAS Notification GRN 000768 in 2017/2018, therefore these data are incorporated by reference while new safety data pertaining to the safety of steviol glycosides published between 01 July 2017 and 28 March 2019 are summarized in Section 6.4. Lastly, an evaluation of the safety of the steviol glycoside production organism is presented in Section 6.5. All information used to establish the safety of Reb M is available in the public domain and, as such, there are no data that are exempt from disclosure under the Freedom of Information Act.

6.2 Metabolism of Steviol Glycosides

Data specific to the pharmacokinetics, metabolism, and elimination of numerous steviol glycosides has already been reviewed by many scientific bodies and regulatory authorities, including the U.S. FDA; therefore, individual studies are not discussed in detail. Instead, this section provides a discussion regarding the common metabolic fate of steviol glycosides.

In vitro and *ex vivo* studies have confirmed that steviol glycosides are not hydrolyzed by digestive enzymes of the upper gastrointestinal tract 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 (Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon 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; Purkayastha *et al.,* 2014*,* 2015, 2016). Specifically, Koyama *et al*. (2003a) investigated the degradation of a stevia mixture containing rebaudioside A, stevioside, rebaudioside C, and dulcoside A (purities not reported) in the presence of human fecal homogenates under anaerobic conditions. Similar to results of studies conducted with individual steviol glycosides (*e.g.*, stevioside or rebaudioside A), the stevia mixture was degraded completely to steviol within 24 hours of incubation. Nikiforov *et al.* (2013) conducted a similar *in vitro* study using rat cecal contents and reported that rebaudioside D was hydrolyzed to stevioside and steviol over a 90-minute period, which was comparable to the hydrolysis of rebaudioside A. *In vitro* metabolism studies have also been conducted with crude pectinase from *Aspergillus niger,* as pectinolytic bacteria are known to reside in the human intestine (Jensen and Canale-Parola, 1985), and likewise, steviol was detected following incubation of rebaudioside E with pectinase (Chaturvedula and Prakash, 2013).

Given the large collection of *in vitro* steviol glycoside metabolism studies with human fecal homogenates, Purkayastha *et al.* (2016) recently re-assessed the existing data to allow for improved comparison of results between different studies. Published studies that compared individual steviol glycoside metabolism (dulcoside A, rebaudiosides B, C, D, E, F, M, and steviolbioside) to that of rebaudioside A at similar test concentrations (0.2 or 2.0 mg/mL, depending on solubility) and incubation times (up to 48 hours) were collected and compared. Assessment of the data in parallel demonstrated that steviol glycosides, irrespective of the type of sugar moiety (*e.g.,* glucose, rhamnose, xylose) or the number of sugar moieties attached to the steviol backbone, were metabolized to steviol at generally similar rates of hydrolysis. The authors noted that while subtle differences may exist in the degradation rates of individual glycosides, it is unlikely that the absorption rate of steviol *in vivo* would be significantly impacted, particularly when compared to rebaudioside A (Purkayastha *et al.,* 2016).

The metabolism of steviol glycosides has also been extensively studied *in vivo* in both rodents and humans. A number of metabolic studies have demonstrated that steviol glycosides (stevioside and rebaudioside A) are not readily absorbed from the upper gastrointestinal tract, and as reported *in vitro*, are hydrolyzed by the colonic flora to steviol (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Gardana *et al.*, 2003; Koyama *et al.*, 2003b; Wang *et al.*, 2004; Geuns *et al.,* 2006*,* 2007; Wheeler *et al.,* 2008; Roberts *et al.,* 2016). Steviol is readily absorbed from the colon to the portal vein and distributed to a number of organs and tissues, including the liver, spleen, adrenal glands, and fat. Following absorption from the colon, steviol primarily undergoes conjugation with glucuronic acid to steviol glucuronide in the liver. Pharmacokinetic studies demonstrated that steviol glucuronide is excreted in rats primarily *via* the bile (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002; Roberts and Renwick, 2008), whereas in humans steviol glucuronide is cleared primarily *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004;

Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). The difference in the route of elimination in rats and 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.

To more accurately characterize the pharmacokinetic/toxicokinetic differences in the production of steviol/steviol glucuronide following oral consumption of steviol glycosides between rats and humans, Roberts *et al.* (2016) recently conducted comparative studies in rats and humans. Male Sprague-Dawley rats and healthy male human volunteers were orally administered a single dose of stevioside (40 mg/kg body weight; equivalent to 16 mg steviol equivalents/kg body weight) and plasma samples collected over the following 72 hours were analyzed for steviol and steviol glucuronide by liquid chromatography-tandem mass spectrometry. Although peak plasma concentrations (C_{max}) of steviol and steviol glucuronide occurred slightly later in humans in comparison to rats, C_{max} values of plasma steviol were similar between rats and humans (~72 to 77 ng/mL). C_{max} values for steviol glucuronide, however, were approximately 25-fold higher in humans than rats (~4,400 ng/mL *vs.* 180 ng/mL). Systemic exposure was determined based on the area-under-the-curve (AUC) of the concentration *vs.* time data, and steviol and steviol glucuronide exposure were reported to be 2.8-fold higher (~1,650 ng*h/mL *vs.* 590 ng*h/mL) and 57-fold higher (~136,000 ng*h/mL *vs.* 2,400 ng*h/mL), respectively, in humans compared to rats. As stated by Roberts *et al.* (2016), these data indicate that the extent of glucuronidation is higher in humans than in rats.

6.2.1 Microbial Metabolism of Steviol Glycosides Produced by Fermentation

As described above, to demonstrate that different individual steviol glycosides share the same metabolic fate as the major steviol glycoside rebaudioside A, several microbial degradation studies with human fecal homogenates have been conducted *in vitro*. Although microbial metabolism studies have not yet been conducted with the Reb M preparation that is the subject of this application (*i.e.,* Reb M produced by *Y. lipolytica*), such studies have been carried out with a Reb M preparation that has a similar individual steviol glycoside profile as Reb M (*i.e., s*teviol glycosides produced by *S. cerevisiae,* GRN 000626, primarily containing rebaudioside M), the results of which are presented below.

Human fecal homogenate samples were prepared based on the pooling of fecal samples from 2 healthy male and 2 healthy female volunteers. Steviol glycosides produced by fermentation (Lot No. [redacted]; GRN 000626) was mixed and incubated in 3 (n=2 pooled) adult male and adult female pooled fecal homogenate samples at concentrations of 0.2 mg/mL under anaerobic conditions at 37°C for 4 to 48 hours in triplicate. To demonstrate the complete metabolic hydrolysis of steviol glycosides produced by fermentation, the formation of steviol, the final stable metabolite, was assayed at each timepoint using an established liquid chromatography-mass spectrometry (LC-MS) method. The mean steviol metabolite concentration was used to determine the percent steviol metabolite formed based on the chemical composition of the study material (*i.e.*, 85.1% rebaudioside M, 9.2% rebaudioside D) and the molar equivalent metabolic conversion of rebaudioside M and rebaudioside D to steviol based on their molecular weights of 1,290 and 1,128 g/mol, respectively. Rebaudioside A from *S. rebaudiana* Bertoni, a steviol glycoside known to be completely metabolized to steviol in the presence of human fecal homogenates, was studied as a metabolic activity positive control in parallel to ensure that the experimental incubation conditions were satisfactory, and to allow for comparison of the hydrolysis rate and degree between the 2 materials. Steviol was also included as a stability control.

A summary of the mean steviol metabolite concentrations formed and the percent steviol glycoside metabolized to steviol in adult male and adult female fecal homogenates is presented in Table 6.2.1-1, and for comparison the data for rebaudioside A from *S. rebaudiana* Bertoni are provided in Table 6.2.1-2. These data indicate that near complete deglycosylation of steviol glycosides (*i.e.,* rebaudiosides M and D) produced by fermentation occurred within an incubation period of 48 hours in pooled fecal homogenates, at which point the mean percent steviol glycoside metabolized to steviol was 103.4% in male samples and 89.8% in female samples. The positive control rebaudioside A from *S. rebaudiana* Bertoni had a similar rate and overall degree of hydrolysis as steviol glycosides produced by fermentation. These data demonstrate that steviol glycosides produced by *S. cerevisiae* (GRN 000626), comprised of primarily rebaudioside M and D, in the presence of human fecal homogenates is metabolized completely to steviol within 48 hours and confirms that these glycosides share the same metabolic fate as steviol glycosides, such as rebaudioside A, from *S. rebaudiana* Bertoni.

Table 6.2.1-1 Hydrolysis of Steviol Glycosides Produced by Fermentation (GRN 000626) in Human Fecal Homogenates

Gender	Time Point (hour)	Mean Steviol Concentration (µg/mL)	Standard Deviation	% Steviol Glycoside Metabolized to Steviol
Male	0	<LLOQ	NA	NA
	4	<LLOQ	NA	NA
	12	10.4	0.5	19.7
	24	35.1	2.0	66.3
	48	54.8	10.2	103.4
Female	0	<LLOQ	NA	NA
	4	<LLOQ	NA	NA
	12	34.4	2.9	65.0
	24	47.9	3.9	90.5
	48	47.6	0.8	89.9

LLOQ = lower limit of quantification, 0.2 µg/mL; NA = not applicable.

Table 6.2.1-2 Hydrolysis of Rebaudioside A from *Stevia rebaudiana* Bertoni in Human Fecal Homogenates

Gender	Time Point (hour)	Mean Steviol Concentration (µg/mL)	Standard Deviation	Mean % Molar Equivalent Reb A Metabolized to Steviol	Standard Deviation
Male	0	<LLOQ	NA	NA	NA
	4	<LLOQ	NA	NA	NA
	12	20.4	2.4	31.0	3.6
	24	53.7	4.1	81.5	6.2
	48	68.8	3.4	104.0	5.2
Female	0	<LLOQ	NA	NA	NA
	4	<LLOQ	NA	NA	NA
	12	52.0	4.9	79.0	7.4
	24	64.0	2.9	97.1	4.4
	48	64.6	1.4	98.1	2.1

LLOQ = lower limit of quantification, 0.2 µg/mL; NA = not applicable.

6.2.2 Summary and Conclusions

Collectively, the results of the degradation and pharmacokinetic studies on steviol glycosides confirm the common metabolic pathway for all steviol glycosides: steviol glycosides are extensively hydrolyzed to steviol, steviol is absorbed and conjugated with glucuronic acid, and steviol glucuronide is excreted primarily *via* the urine in humans. Steviol glycosides, whether produced by fermentation or extracted from the *S. rebaudiana* plant, share this same metabolic fate. This is consistent with the fact that except for having different numbers and types of sugar moieties, steviol glycosides, regardless of source, share the same structural backbone steviol. Considering the common pathway of metabolism, and the fact that systemically, exposure only occurs to steviol following consumption of steviol glycosides, the safety data and conclusions drawn for individual steviol glycosides from *S. rebaudiana* can therefore be extended to include all purified steviol glycosides including those derived from yeast, such as *Y. lipolytica* and *S. cerevisiae* production strains.

6.3 Summary of Safety Opinions by Scientific and Regulatory Authorities

The safety of steviol glycosides has been extensively reviewed by JECFA at several meetings of the Committee (JECFA, 1999, 2006, 2007b, 2009, 2017b). JECFA concluded that the metabolic fate of steviol glycosides is similar in humans and rats, such that steviol glycosides are converted to steviol through the successive removal of glucose units by intestinal bacteria. Steviol is then absorbed from the colon, rapidly converted to steviol glucuronide, and excreted *via* the urine in humans. The Committee also concluded that steviol glycosides are not mutagenic and that steviol is not mutagenic *in vivo*. Studies conducted in humans demonstrated that steviol glycosides, meeting the established purity specifications, did not cause any adverse effects when consumed at doses of up to 4 mg steviol equivalents/kg body weight/day, including individuals with type-2 diabetes mellitus for up to 16 weeks and individuals with normal or low-normal blood pressure for 4 weeks. Based on the above findings, JECFA calculated an ADI for steviol glycosides of 0 to 4 mg/kg body weight, expressed as steviol equivalents. The ADI was determined by applying a 100-fold safety factor for inter-and intra-species differences to the no-observed-adverse-effect level (NOAEL) of 970 mg stevioside/kg body weight/day (equivalent to 383 mg steviol equivalents/kg body weight/day) determined from a carcinogenicity study conducted with stevioside in rats evaluated at the 51st meeting (Toyoda *et al*., 1997). Initial specifications established by JECFA (2010) stipulated that the purity of steviol glycoside preparations was to be not less than 95% of the 9 named steviol glycosides (stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside). JECFA recently re-assessed the safety of steviol glycosides at the 82nd meeting by reviewing all new data that had become available since the previous evaluation, and the ADI for steviol glycosides was confirmed. Based on the new data, a specification was established for "Steviol glycosides from *Stevia rebaudiana* Bertoni" which defines steviol glycosides as "*a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of S. rebaudiana Bertoni*" (JECFA, 2017a). The inclusion of all steviol glycosides within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all glycosides are hydrolyzed to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general. The Committee also evaluated data on a novel yeast-derived steviol glycoside product resulting in the issuance of new specifications for "Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica*", with a purity definition of no less than 95% rebaudioside A (JECFA, 2016).

Similar to JECFA's conclusions, other regulatory authorities including EFSA, FSANZ, and the Health Canada Food Directorate have conducted their own evaluations on the safety of steviol glycosides and also have established an ADI of 4 mg/kg body weight, expressed as steviol equivalents (FSANZ, 2008, 2015; EFSA, 2010; Health Canada, 2012a).

EFSA (2010) evaluated the safety of steviol glycosides[2] for use in food in the European Union at the request of the European Commission as part of the authorization process for food additives. EFSA evaluated the available data and allocated an ADI of 4 mg/kg body weight, expressed as steviol equivalents, for steviol glycosides. Following this safety opinion, the European Commission permitted the use of steviol glycoside as a sweetening agent under Commission Regulation (EU) No 1131/2011 (EU, 2011). In a subsequent Scientific Opinion, EFSA expanded the definition of steviol glycosides to include rebaudiosides D and M and concluded that, "*Extending the current specifications to include* [2 additional steviol glycosides], *rebaudiosides D and M, as alternatives to rebaudioside A in the predominant components of steviol glycosides would not be of safety concern*" and that, "*The ADI of 4 mg/kg body weight/day can also be applied where total steviol glycosides comprise more than 95% of the material*" (EFSA, 2015). In a recent evaluation of a proposed amendment to the specifications of steviol glycosides, EFSA did not agree to expand the definition of steviol glycosides to include all individual steviol glycosides because of uncertainties on the rate and extent of the metabolism of the different steviol glycosides to steviol (EFSA, 2018a). Similarly, EFSA also concluded in a recent evaluation of glucosylated steviol glycosides that the data provided was not sufficient to assess the safety of these glycosides due to the limited evidence on the complete hydrolysis of glucosylated steviol glycosides to steviol, and responded that metabolic fate data from parent steviol glycosides cannot be used in a read-across approach (EFSA, 2018b).

FSANZ has recently approved a request to amend the definition of steviol glycosides in the Food Standards Code to include "*all minor steviol glycosides*" extracted from the *S. rebaudiana* Bertoni leaf in addition to the 10 steviol glycosides (stevioside, rebaudioside A, B, C, D, F, and M, dulcoside A, rubusoside, and steviolbioside) which were approved previously (FSANZ, 2008, 2015, 2017). As part of the approval process, FSANZ performed a risk assessment in which it considered *in vitro* biotransformation studies of several steviol glycosides, the results of which demonstrated that steviosides, rebaudiosides, and dulcosides are biotransformed to steviol and are consistent with previously-approved steviol glycosides. Based on the outcome of the safety assessment, FSANZ concluded that the ADI for steviol glycosides from *S. rebaudiana* Bertoni leaf of 0 to 4 mg/kg body weight (as steviol) is "*applicable to all steviol glycosides in stevia leaf*" which FSANZ recognizes includes at least 40 different steviol glycosides (FSANZ, 2017). FSANZ issued specifications for steviol glycosides from *S. rebaudiana* with a total steviol glycoside content of no less than 95% on the dried basis, which expands the definition to include all individual steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni. Most recently, FSANZ evaluated an alternative manufacturing process for steviol glycosides, enzymatic conversion, whereby rebaudioside M is produced from purified stevia leaf extract using enzymes derived from a bioengineered yeast (*i.e., Pichia pastoris*). Consequently, in early 2019 FSANZ updated the specification for "Steviol glycosides from *S. rebaudiana* Bertoni" to include enzymatic conversion of purified stevia leaf extract as an alternative method to produce rebaudioside M. This method uses protein engineered enzymes that contain both uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase components that are sourced from strains of *Pichia pastoris* (FSANZ, 2019).

[2] Consisting of stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside (EFSA, 2010)

Health Canada reviewed the safety of steviol glycosides and similar to other scientific and regulatory authorities, established an ADI of 4 mg steviol equivalents/kg body weight (Health Canada, 2012a). Steviol glycosides as initially defined by JECFA were approved by Health Canada for use as sweetening agents at levels of up to 0.35% calculated as steviol equivalents (Health Canada, 2012b). In addition to the 9 steviol glycosides initially considered by JECFA, Health Canada expanded the purity definition of steviol glycosides to include rebaudioside M as being 1 of the 10 steviol glycosides that may be present alone or in combination in finished preparations to reach the total steviol glycoside content of at least 95% purity (Health Canada, 2016). Health Canada has since received a request to expand the use of the food additive 'steviol glycosides' to include all steviol glycosides in the *S. rebaudiana* Bertoni plant (Health Canada, 2017). Following a safety assessment in which no safety concerns were identified, Health Canada decided to expand the steviol glycoside food additive description as requested. Most recently, Health Canada conducted a detailed safety assessment on 'Steviol glycosides from *Saccharomyces cerevisiae* CD15380 and CD15407', and since no safety concerns were raised during the evaluation, Health Canada has enabled their use in a variety of foods (Health Canada, 2019).

6.4 New Safety Data on Steviol Glycosides

Cargill's most recent GRAS Notification for steviol glycosides, GRAS Notification GRN 000768 for stevia leaf extract preparations that received a "no questions" letter from the U.S. FDA, contained safety data up to July 2017 and is incorporated by reference into the safety discussion of this GRAS dossier (U.S. FDA, 2018c). New safety data related to steviol glycosides published after July 2017 was obtained through a comprehensive and detailed search of the scientific literature published between 01 July 2017 and 28 March 2019. The literature search was completed using ProQuest and included searches of the following databases for pertinent literature on the safety of steviol glycosides: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Due to the purity criteria laid down in several specifications, studies were excluded if the test article investigated had a purity of less than 95% steviol glycosides. The studies identified in the updated literature search included 2 repeated-dose studies, 1 genotoxicity study, 2 reproductive toxicity studies, and 2 human studies and provide further support for the safety of steviol glycosides. The details of these studies are discussed in the following Sections.

6.4.1 Repeated-Dose Studies

Barrios-Correa *et al*. (2018) investigated the brain of mice for changes in the JAK2/STAT3 signaling pathway and changes in appetite and body composition following chronic intake of commercial sweeteners. Adult BALB/c mice (9/sex/group) were provided with one of the following drinking water formulations for 6 weeks: sucrose (10% dilution of sucrose per 100 mL purified water), sucralose (one 1 g packet of commercial sucralose sweetener Splenda®, equivalent to 0.012 g sucralose, per 100 mL water), or steviol glycosides (one 1 g packet of commercial steviol glycoside sweetener Svetia®[3], equivalent to 0.025 g rebaudioside A, per 100 mL water; approximately 15 mg/kg body weight/day steviol equivalents). The control mice were given purified water and all the animals were provided food and water *ad libitum*. Following the 6-week study period, mice were terminated, and the brains were removed. Food and water intakes were measured daily throughout the study period. Body weight was measured at study start and weekly thereafter, and energy intake was determined at study end. Utilizing these data, an approximate exposure to rebaudioside A present in the Svetia® sweetener was calculated to be 15 mg/kg body

[3] Svetia® is a co-crystalized blend of cane sugar and high purity rebaudioside A and is twice as sweet as sugar. The blend is formulated with 2.5% rebaudioside A (http://www.svetia.us/home/#nutrimental-table).

weight/day steviol equivalents, which is about 4 times higher than the upper limit of the steviol glycoside ADI. Body composition and expression of total and phosphorylated JAK2, STAT3, and Akt, as well as SOCS3 and ObRb in the brain tissue were measured. Male mice provided with steviol glycosides showed significantly decreased energy intake, adiposity, downregulation of feeding behavior, and decreased weight gain, compared with controls. Increased expression of pJAK2 and pSTAT3 in the brain were also observed in male mice supplemented with steviol glycosides when compared to the controls. On the other hand, JAK2 and pJAK2 expression was upregulated in female mice supplemented with steviol glycosides, compared to the controls. The authors concluded that alterations in brain activity with regards to signaling pathways that control appetite and energy balance occurred following repeated steviol glycoside intake; however, since the administered dose was calculated to be about 4 times higher than the upper limit of the ADI for steviol glycosides, the relevance of these data to human exposure to steviol glycosides in food is limited.

Han *et al.* (2019) investigated the effects of stevioside (97% purity) on feed intake and digestibility in goats in a replicated 3 x 3 Latin square design. Male Xiangdong Black goats (n=3/group) were provided a diet containing 0, 400, or 800 mg stevioside/kg forage (rice straw) for 20 days (approximately 3.7 and 7.2 mg/kg body weight/day steviol equivalents). The forage was provided with a feed concentrate twice daily and consumed *ad libitum*. Fecal samples collected on Days 12 to 17 were analyzed for nutrient digestibility and chemical composition, and total tract digestibility was calculated. On Day 18, feeding behavior (including eating, ruminating, and resting) was analyzed over a 24-hour period by visual examination. Serum metabolites, glucose, total protein, albumin, globulin, triglyceride, and total cholesterol were examined from blood samples taken from each animal on Day 19. Rumen fluid was analyzed for pH, volatile fatty acid concentrations, and ammoniacal nitrogen (NH_3-N) concentration from samples collected on Days 19 and 20. The following results were reported following exposure to stevioside in the forage: statistically significant linear increase in dry matter intake of forage and total diet; statistically significant quadratic increase in rumen pH; statistically significant quadratic decrease in total volatile fatty acids; statistically significant quadratic response of stevioside on rumen isobutyrate and isovalerate, with an increase from 0 to 400 mg/kg stevioside and a decrease from 400 to 800 mg/kg stevioside; and statistically significant linear and quadratic increases in neutral and acid detergent fiber digestibility, with an increase in digestibility from 0 to 400 mg/kg stevioside but a decrease in digestibly from 400 to 800 mg/kg stevioside. The authors suggested that the addition of stevioside may have increased the palatability of the forage. The addition of stevioside in the forage had no significant effects on serum parameters. The authors concluded that the addition of stevioside to the feed of goats increased dry matter intake and increased the digestibility of neutral and acid detergent fiber.

6.4.2 Genotoxicity

In a chromosome aberration test and micronucleus test, the genotoxic potential of stevia in human lymphocytes obtained from the venous blood of healthy adult donors (2 males and 2 females) was investigated by Uçar *et al*. (2018). The lymphocytes were cultured for 24 and 48 hours at 37°C and exposed to 0, 1, 2, 4, 8, and 16 µg/mL stevia (steviol glycoside purity of 99%) in duplicate. Mitomycin C (0.2 µg/mL) was used as the positive control. The cells were cultured for a total of 72 hours and then collected, fixed onto slides, and assessed by scoring 400 metaphases for each treatment for chromosome aberrations and assessed by scoring a total of 4,000 binucleated cells per concentration for micronuclei formations. The micronucleus test was conducted with the same test concentrations, culture conditions, and times as the chromosome aberration assay. There were no significant differences in the number of chromosome aberrations or micronucleations at any test concentration compared to the negative control. The authors concluded a lack of genotoxicity of steviol glycosides in human lymphocytes.

6.4.3 Long-term Toxicity and Carcinogenicity

The chronic toxicity and carcinogenicity of steviol glycosides has been previously addressed in the safety evaluations by the scientific bodies and regulatory agencies described in Section 6.3. No new data were identified in relation to this endpoint.

6.4.4 Reproductive and Developmental Toxicity

The effects of *S. rebaudiana* extract (purity not reported) on reproductive function in diabetes-induced healthy adult male rats (albino Wistar) were examined by Ghaheri *et al.* (2018). Diabetes mellitus was induced with an intraperitoneal injection of 50 mg streptozotocin/kg body weight. The rats that reached fasting glucose levels greater than 250 mg/dL after 72 hours were selected for the study. The animals (7/group) were administered *via* gavage 5, 50, or 100 mg stevia extract/kg body weight daily for 28 days. The non-commercial stevia extract was prepared by hot water extraction, but the purity of the final extract was not reported. A diabetic and non-diabetic control group received 2 mL of distilled water only. Sexual behaviors were recorded for 30 minutes every 2 weeks for 1 month, including mount latency, intromission latency, mount frequency, intromission frequency, ejaculation latency, the mount latency post ejaculation, and ejaculation frequency. Serum testosterone concentration was measured at the end of the study period. Histological examination was carried out on the right testis and epididymis. In diabetic low-dose animals, significantly increased frequency of intromission was observed compared to diabetic controls, along with significantly increased frequency of ejaculation when compared to diabetic controls and high-dose animals. Significantly decreased latency in ejaculation was observed in low-dose animals compared to high-dose animals; this effect was not significantly different between the treated animals and the controls. No statistically significant differences in the other sexual behavior parameters were observed. Significantly reduced numbers of Leydig cells were noted in high-dose animals *versus* the non-diabetic control group; however, this effect was not statistically significantly different compared to the diabetic controls. It is also likely that the high-dose exposure to stevia is above the upper limit of the steviol glycoside ADI, limiting the relevance of this finding with respect to human exposure. No changes in organ weights and serum testosterone levels were reported between groups. The results of this study demonstrate that a non-commercial aqueous stevia extract may reduce some of the adverse reproductive effects reported in rats with streptozotocin-induced diabetes.

Jiang *et al.* (2018) evaluated the effects of daily consumption of rebaudioside A (obtained from Aladdin Co., Ltd., China) on the ovarian cycle and steroidogenesis in weanling rats. Female weanling Sprague-Dawley rats (body weight 42.3 ± 4.1 g; 6/group) received 0.5 or 2.5 mM rebaudioside A in drinking water for 48 consecutive days (equivalent to approximately 76 and 486 mg/kg body weight/day steviol equivalents, assuming 100% purity). The control rats received normal water, and all animals were provided with rat chow and water *ad libitum*. Food and water intake, and body weight were measured every third day in the morning. The day of vaginal opening was recorded (from tightly closed to open) and vaginal smears were taken daily to monitor the estrous cycle. Following the study period, blood samples and ovaries on diestrus-2 were collected. Serum progesterone levels were detected using a radioimmunoassay. The ovaries were examined *via* H&E staining, Western blot, and immunohistochemistry. A significant decrease in body weight was observed in high-dose animals from Day 18 until Day 30, when body weights returned to similar weights as that found in the control group. Water intake during the first 3 weeks of the study was significantly increased in high-dose rebaudioside A-treated animals, compared to the controls. During the last 3 weeks of the study, water intake was significantly higher in the high-dose animals compared to the low-dose animals. Serum progesterone levels were significantly decreased in treated rats compared to controls. Increased expression of taste receptor type 2 subunit 38 (T2R38) was observed in low- and high-dose groups, while lower expression of other proteins (T1R3, Gα, StAR, CYP11A1, 3β-HSD, CYP17A1,

17β-HSD, and CYP19A1) in the ovaries was observed, compared to controls. In addition, a lower expression of T1R3 and Gα proteins in rebaudioside A-treated groups was observed. Given that the doses of rebaudioside A utilized in this study on a steviol equivalent basis are well above the upper limit of the steviol glycoside ADI (about 19 to 120 times higher), the relevance of these data to human exposure to steviol glycosides in food is limited.

6.4.5 Human Studies

In a randomized, crossover placebo-controlled clinical study, Al-Dujaili *et al.* (2017) investigated the effects of stevia (100% stevia powder, purchased at Boots, Ltd. UK) on blood pressure, stress hormone levels, and anthropometric parameters in healthy subjects. Male and female subjects [8/group; mean age 27.75 ± 13.75 years; body mass index (BMI) 26.33 ± 5.26 kg/m^2] received either stevia (0.2 g) or sucrose placebo (5 g) and were instructed to take the dose 3 times daily for 7 days (approximate exposure of 8 mg stevia body weight/day) and have the substance preferably dissolved in a hot drink. A 3-day washout period was carried out before and after each treatment arm, and during this time the subjects were instructed to avoid the intake of other sweeteners and sugars, as well as throughout the study. The following parameters were measured at baseline and on Day 7 of each study arm: 24-hour urine samples and saliva samples in triplicate (morning, afternoon, evening), blood pressure, weight, height, and BMI. No significant effects on weight or BMI were observed following exposure to stevia, however, statistically significant elevations in systolic and diastolic blood pressure were observed compared to baseline, which were within the expected reference range. A slightly significant increase was observed in salivary cortisol levels; however, this elevation was only observed in measurements taken in the morning compared to baseline and was not apparent in measurements taken at other times of the day (afternoon and evening). Significant increases and decreases were observed in urinary concentrations of free cortisol and cortisone, compared to baseline. The authors noted several limitations with the study design, including small sample size, potential for recognition by subjects of difference between placebo and stevia test article, lack of validation of dosage consumed, and a short washout period and as a result, concluded that further research is needed to understand the significance of the observed effects.

In a randomized single-blinded, crossover, placebo-controlled clinical study, Ahmad *et al.* (2018) investigated the effects of a single dose of stevia leaf powder (prepared from dried stevia leaves; steviol glycoside content not reported) on blood glucose and related parameters in healthy subjects. Males and females (10/group; mean age 24.1 ± 1.33 years; BMI 22.09 ± 3.88 kg/m^2) were fasted overnight and provided with a single dose of either a placebo cookie (made from 100% wheat flour) or cookie containing stevia leaf powder (3% w/w; approximately equivalent to 4.2 g stevia) in the morning. A 1- to 2-week washout period was carried out before and after each treatment period. The subjects were instructed to avoid vigorous physical activity prior to each study visit and to maintain the same dietary patterns in the evening prior to each visit. At baseline and following each treatment, fasting blood glucose concentration, appetite, hunger levels, and gastrointestinal discomfort were measured. Palatability, blood pressure, weight, height, and BMI were also measured. A decrease in appetite was observed in the stevia group compared to the control group, and the effect was only significant at 30 minutes following intake. In addition, the stevia cookies had a lower rating for texture based on the palatability testing when compared to the control cookies. No other significant differences were observed in the palatability parameters, and the stevia-containing cookies did exceed the score required to be considered acceptable. The results also demonstrated no significant effects on any of the anthropometric parameters, blood glucose response, or gastrointestinal discomfort. The authors concluded that consumption of stevia leaf powder in cookies decreased hunger when compared to cookies without stevia leaf powder.

6.5 Safety of Production Organism

The *Y. lipolytica* production strain used to produce Reb M is a strain of non-pathogenic and non-toxicogenic yeast of the *Y. lipolytica* species. The steviol glycoside production strain contains no known pathogenicity-related proteins, toxins, allergens, or genes; the incorporated DNA is either synthesized or sourced from biosafety level 1 organisms. The majority of genes used to create the production strain originate from the plant *S. rebaudiana* Bertoni or other edible plants and were produced by gene synthesis. Several steps are undertaken during the manufacturing process to inactivate (*i.e.,* heat treatment) and remove (*i.e.,* ion exchange chromatography, adsorption chromatography, and crystallization) the microorganism. The final Reb M product has been tested and shown to be absent of residual protein above the limit of detection of 25 ppm using the BCA assay or above 1.5 ppm using the Protein Dot Blot method, and absent of recombinant DNA above the limit of detection of 10 ng/g for the PCR assay, supporting that DNA inserted in the production organism is of no safety concern.

Y. lipolytica has been extensively studied and is customarily classified as a biosafety class 1 microorganism (Groenewald *et al*., 2014). It has a long history of safe use in the production of food (*e.g.*, cheese ripening) and food ingredients (*e.g.*, citric acid, γ-decalactone). Steviol glycosides (*e.g.,* rebaudioside A and rebaudioside M) obtained from *Y. lipolytica* expressing steviol glycoside biosynthesis pathway genes, similar to the production organism that is described in this application for Reb M, are GRAS for use as table top sweeteners and as general purpose non-nutritive sweeteners in foods in the U.S. (GRN 000632 and 000759 – U.S. FDA, 2016c, 2018b). In addition, under 21 CFR §173.165, *Y. lipolytica* (identified by its prior classification of *Candida lipolytica*) is permitted for use as a secondary direct food additive for fermentation production of citric acid in the U.S. (U.S. FDA, 2018a). In 2011, the U.S. FDA received 2 GRAS Notices for the production of an eicosapentaenoic acid (EPA)-rich triglyceride by *Y. lipolytica* (GRN 355) and for erythritol produced *via* biotransformation by a strain of *Y. lipolytica* (GRN 382), and responded with "no questions" letters regarding the GRAS status of both ingredients (U.S. FDA, 2011a,b). EFSA has granted Qualified Presumption of Safety (QPS) status for *Y. lipolytica* and therefore has deemed it safe to derive genetically modified strain lineages to use in the production of food additives and enzymes (EFSA, 2018c).

6.5.1 Potential Toxicity of the Inserted Gene Sequences

Although no residual protein (*i.e.,* above the limit of detection of 25 ppm using the BCA assay or above 1.5 ppm using the Protein Dot Blot method) or DNA (*i.e.,* above the limit of detection of 10 ng/g for the PCR assay) is present in the final Reb M product, to confirm that the proteins expressed in the *Y. lipolytica* production strain are not associated with any toxic potential, the Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information (NCBI) was used to conduct sequence alignment queries of the full-length FASTA protein sequences of the inserted gene sequences against curated databases maintained by UniProt containing (a) venom proteins and toxins (UniProtKB/Swiss-Prot Tox-Prot[4]); and (b) virulence factors (UniProtKB/Swiss-Prot/TrEMBL[5]).

[4] The UniProtKB/Swiss-Prot Tox-Prot database is available at: http://www.uniprot.org/uniprot/?query=taxonomy%3A%22Metazoa+[33208]%22+AND+%28keyword%3Atoxin++OR+annotation%3A%28type%3A%22tissue+specificity%22+AND+venom%29%29+AND+reviewed%3Ayes&sort=score.

[5] The UniProtKB/Swiss-Prot/TrEMBL database is available at: http://www.uniprot.org/uniprot/?query=keyword:KW-0843.

The BLAST searches identified sequence matches with 17 to 68% identity with various animal venom proteins and toxins and virulence factors, and associated E-values ranging from $3x10^{-60}$ to 10.0. E-values greater than $1x10^{-7}$ suggest that proteins are unlikely to share structural homology (Hileman *et al.*, 2002). The sequence alignments with low E-values (in the range of 10^{-9} to 10^{-60}) shared approximately 21 to 31% identity with cytochrome P450 monooxygenases. It should be noted that cytochrome P450 monooxygenase is a native enzyme involved in endogenous reactions in humans. Based on the available data it is anticipated that the inserted genes do not encode for proteins that are homologs of any animal venom protein or toxins or virulence factors. It is understood that the amino acid sequence of the enzyme is an important determinant of the 3-dimensional structure and motif which dictate the toxic function of the protein (Dunker *et al.,* 2008; Hammond *et al.,* 2013; Negi *et al.,* 2017). Given the low structural homology between the inserted gene sequences with known animal venom proteins and toxins and virulence factors (*i.e.,* sequence identities of no more than 68% and associated E- values of greater than $1x10^{-7}$), it is expected that these enzymes do not share the protein domains necessary for toxic function. Furthermore, it should be noted that the production strain does not contain any putative pathogenicity-associated proteins, toxins, allergens, or genes, and the DNA incorporated into the production strain's genome is either synthesized, sourced from biosafety level 1 organisms, or obtained from plant sources with a long history of safe consumption. Evolutionary changes resulting in amino acid substitutions are conservative in which the stability of the protein is maintained; as such, enzymes retain the 3-dimensional structure and functional characteristics of the enzyme family from which they were derived and exhibit similar variation in amino acids than what occurs through natural sequence variation (Pariza and Cook, 2010; Hammond *et al.,* 2013). As confirmed by bioinformatics analysis using the amino acid sequences of the proteins encoded by the inserted genes, no toxic or pathogenic potential is anticipated with the Reb M products produced by the *Y. lipolytica* production strain.

6.5.2 Potential Allergenicity of the Inserted Gene Sequences

Although no residual protein (*i.e.,* above the limit of detection of 25 ppm using the BCA assay or above 1.5 ppm using the Protein Dot Blot method) or DNA (*i.e.,* above the limit of detection of 10 ng/g for the PCR assay) is present in the final Reb M product, an allergenicity screen of the heterologous gene sequences inserted in the production strain was conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001) and the Codex Alimentarius (2009) in order to confirm the lack of potential for allergenic cross-reactivity of the inserted gene sequences in the production strain. This screen for relevant matches to known putative allergens was carried out using the AllergenOnline database version 19 (available at http://www.allergenonline.org; updated 10 February 2019) that is maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2019). A FASTA 35.04 overall search of AllergenOnline was conducted using default settings (E-value/score cut-off = 1 and maximum alignments of 20). Searches were conducted using the full-length amino acid sequence and an 80-amino acid 'sliding window' (segments 1–80, 2–81, 3–82, *etc*.) in accordance with the Codex Alimentarius criterion for use in flagging proteins that might be of some concern of cross-reactivity for genetically engineered plants (Codex Alimentarius, 2003, 2009). Significant homology is defined as an identity match of greater than 35% (Codex Alimentarius, 2009), and in such instances, cross-reactivity with the known allergen must be considered a possibility. Using this search strategy, no identity matches of greater than 35% were identified.

6.6 Expert Panel Evaluation

Cargill has concluded that the purified steviol glycoside product Reb M, primarily containing rebaudioside M and obtained from a *Y. lipolytica* production strain, manufactured consistent with cGMP is GRAS for use as a general purpose sweetener in conventional food and beverage products, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on data generally available in the public domain pertaining to the safety of Reb M, as discussed herein, and on a unanimous opinion among a panel of independent experts ("the GRAS Panel"), who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Michael W. Pariza (University of Wisconsin-Madison), Dr. I. Glenn Sipes (University of Arizona), and Dr. Stanley M. Tarka Jr. (Pennsylvania State University College of Medicine).

The GRAS Panel, convened by Cargill, independently and critically evaluated all data and information presented herein, and concluded that Reb M is GRAS for use as a general purpose sweetener in conventional food and beverage products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel and evaluation of such data as it pertains to the proposed GRAS uses of Reb M is presented in Appendix A.

6.7 Conclusion

Based on the above data and information presented herein, Cargill has concluded that the purified steviol glycoside product Reb M, primarily containing rebaudioside M and obtained from a *Y. lipolytica* production strain, is GRAS on the basis of scientific procedures, for use in conventional food and beverage products as described in Section 1.3. General recognition of Cargill's GRAS conclusion is supported by the unanimous consensus rendered by an independent GRAS Panel, qualified by experience and scientific training, to evaluate the use of Reb M in conventional food and beverage products, who similarly concluded that the intended use of Reb M in conventional food and beverage products as described herein is GRAS.

Reb M therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

Part 7. § 170.255 List of Supporting Data and Information

The following generally-available data were cited in this notification and were used to provide the basis for the GRAS status of Cargill's Reb M in conventional food and beverage products:

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

Part	Section §	Section Title
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172—Food Additives Permitted for Direct Addition to Food for Human Consumption	172.135	Disodium EDTA
	172.375	Potassium iodide
173—Secondary Direct Food Additives Permitted in Food for Human Consumption	173.165	Candida lipolytica
	173.310	Boiler water additives
	173.340	Defoaming agents
182—Substances Generally Recognized as Safe	182.1057	Hydrochloric acid
	182.1073	Phosphoric acid
	182.1320	Glycerin
	182.3640	Potassium sorbate
	182.8159	Biotin
	182.8997	Zinc phosphate
184—Direct Food Substances Affirmed as Generally Recognized as Safe	184.1033	Citric acid
	184.1095	Sulfuric acid
	184.1139	Ammonium hydroxide
	184.1143	Ammonium sulfate
	184.1193	Calcium chloride
	184.1212	Calcium pantothenate
	184.1261	Copper sulfate
	184.1293	Ethyl alcohol
	184.1315	Ferrous sulfate
	184.1370	Inositol
	184.1443	Magnesium sulfate
	184.1446	Manganese chloride
	184.1461	Manganese sulfate
	184.1530	Niacin
	184.1631	Potassium hydroxide

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

Part	Section §	Section Title
	184.1634	Potassium iodide
	184.1643	Potassium sulfate
	184.1676	Pyridoxine hydrochloride
	184.1733	Sodium benzoate
	184.1763	Sodium hydroxide
	184.1854	Sucrose
	184.1857	Corn sugar
	184.1875	Thiamine hydrochloride
	184.1983	Bakers yeast extract
186— Indirect food substances affirmed as generally recognized as safe	186.1797	Sodium sulfate

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GRAS Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of Rebaudioside M for Use as a General Purpose Sweetener

13 May 2019

INTRODUCTION

Cargill, Incorporated (Cargill) intends to market a purified steviol glycoside product, primarily containing rebaudioside M (herein referred to as Reb M), obtained from a *Yarrowia lipolytica* (*Y. lipolytica*) production strain, for use in conventional food and beverage products as a general purpose sweetening agent in the United States (U.S.). Steviol glycosides represent a group of over 40 natural constituents present in the leaves of the *Stevia rebaudiana* (*S. rebaudiana*) Bertoni plant. Typically, these steviol glycosides can be obtained by extracting stevia leaves with hot water, followed by solvent purification of the water-soluble extract. Cargill has developed an alternative manufacturing process wherein steviol glycosides are produced by fermentation of simple sugars using a *Y. lipolytica* production strain.

At the request of Cargill, a panel of independent scientists (the "GRAS Panel"), qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened on 13 May 2019 to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether Reb M under the conditions of intended use as a general purpose sweetener would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley Tarka, Ph.D. (The Pennsylvania State University College of Medicine; The Tarka Group, Inc.).

The GRAS Panel, independently and collectively, critically examined a comprehensive package of publicly available scientific information and data compiled from the literature and other published sources based on previous steviol glycoside GRAS notifications and searches of the scientific literature. In addition, the GRAS Panel evaluated other information deemed appropriate or necessary, including data and information provided by Cargill. The data evaluated by the GRAS Panel included information pertaining to the methods of manufacture of Reb M and construction of the production strain, product specifications and supporting analytical data, consumption estimates for the intended use of Reb M, and comprehensive literature on the safety of steviol glycosides and the production strain, as described in the supporting dossier "*Documentation Supporting the Evaluation of Rebaudioside M as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener*".

Following independent evaluation of such data and information, the GRAS Panel unanimously concluded that under the conditions of intended use described herein, Reb M, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the GRAS Panel's conclusion, excluding confidential data and information, is provided below.

COMPOSITION, MANUFACTURING, AND SPECIFICATIONS

The ingredient that is the subject of this GRAS evaluation is a steviol glycoside product, primarily containing rebaudioside M, and referred to as Reb M. Reb M is obtained from a *Y. lipolytica* production strain and the purified steviol glycoside ingredient primarily contains rebaudioside M in combination with any of the following steviol glycosides: rebaudioside A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and/or dulcoside A. Reb M meets or exceeds the ≥95% steviol glycoside purity definition established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). High-performance liquid chromatography (HPLC) data provided by Cargill demonstrate that both rebaudioside M and rebaudioside D produced by fermentation have the same HPLC retention times as rebaudioside M and rebaudioside D from *S. rebaudiana* Bertoni and establishes that steviol glycosides from these 2 sources are chemically identical.

The GRAS Panel reviewed information provided by Cargill describing the chemistry and manufacturing of Reb M. The manufacturing process for Reb M is consistent with other commercial methods used to produce steviol glycosides and follows the principles of cGMP. Reb M is produced by fermentation of dextrose or sucrose using a production strain of *Y. lipolytica.* The *Y. lipolytica* production organism has been genetically modified to express several proteins and enzymes involved in the making of steviol glycosides, similar to the synthesis pathways naturally present in the leaves of *S. rebaudiana* Bertoni. The majority of genes used to create the production strain originated from the plant *S. rebaudiana* Bertoni and were produced by gene synthesis. Following fermentation, the yeast is removed by heat treatment and by any combination of centrifugation, microfiltration, and/or clarification. The filtrate undergoes typical purification processes used for steviol glycosides extracted from *S. rebaudiana* leaves, yielding the final highly purified steviol glycoside ingredient Reb M (≥95% steviol glycosides).

The physical, chemical, and microbial specifications for Reb M are based on those published by JECFA (2017a) and the Food Chemicals Codex (FCC) for steviol glycosides (FCC, 2018). These specifications are also similar to those established in previous GRAS Notifications GRN No. 000626 (Rebaudioside M produced by *S. cerevisiae*) and GRN No. 000759 (Rebaudioside M produced by *Y. lipolytica*) to which the U.S. Food and Drug Administration (FDA) issued "no questions" letters to the notifiers (U.S. FDA, 2016, 2018). Routine analyses of Cargill's Reb M were carried out to verify compliance with the established product specifications. The absence of residual protein (*i.e.,* above the limit of detection of 25 ppm using the bicinchoninic acid (BCA) assay or above 1.5 ppm using the Protein Dot Blot method) and residual DNA (*i.e.,* above the limit of detection of 10 ng/g for the polymerase chain reaction [PCR] assay) arising from the use of the *Y. lipolytica* production strain in the manufacturing process were confirmed in the final Reb M product. The stability of steviol glycosides has been previously established by JECFA under conditions of storage and use in foods and beverages across a range of pH values (2.0 to 8.0) and temperatures (5 to 56°C) (JECFA, 2007). According to JECFA, the compositional similarity among steviol glycosides indicates that Reb M would have comparable stability under normal production and storage conditions; stability data provided by Cargill for the similar steviol glycoside product rebaudioside M produced by *S. cerevisiae* supports this conclusion.

INTENDED USE AND ESTIMATED EXPOSURE

Reb M is proposed for use as a general purpose sweetener that will be added to a variety of conventional food and beverage products, consistent with the current uses of other related high-intensity sweeteners that are already in the market, including steviol glycosides. Dietary exposure data published by Renwick (2008) for other approved high intensity sweeteners in the U.S. were adjusted for the relative sweetness intensity of Reb M in order to determine the predicted intake range of Reb M. Using these data, the mean intake of Reb M on a steviol equivalent basis was predicted to range from 0.18 mg/kg body weight/day for non-diabetic adults to 0.84 mg/kg body weight/day for diabetic children. Predicted intakes for heavy consumers on a steviol equivalent basis ranged from 0.48 mg/kg body weight/day for non-diabetic adults to 1.24 mg/kg body weight/day for non-diabetic children. The predicted intakes of Reb M, expressed as steviol equivalents, are all below the current acceptable daily intake (ADI) defined by the JECFA for steviol glycosides of 0 to 4 mg/kg body weight/day as steviol (JECFA, 2009, 2017b).

DATA PERTAINING TO SAFETY

The GRAS Panel reviewed the available data to support the safety of Reb M that included a detailed discussion of the metabolic fate of steviol glycosides and history of safe use, a summary of the conclusions made by global scientific and regulatory authorities regarding the safety of steviol glycosides, an update on the safety data published in the most recent scientific literature, and information related to the safety of the *Y. lipolytica* production strain.

Absorption, Distribution, Metabolism, and Excretion

Following ingestion, steviol glycosides pass through the stomach and upper gastrointestinal tract intact (Hutapea *et al*., 1997; Geuns *et al*., 2003, 2007; Koyama *et al*., 2003). Once these steviol glycosides enter the colon, they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Renwick and Tarka, 2008). The data on the metabolic fate of steviol glycosides were recently assessed by Purkayastha *et al.* (2016) who concluded that steviol glycosides are metabolized to steviol at generally similar hydrolysis rates, irrespective of the types and number of sugar moieties attached to the steviol backbone. *In vitro* microbial degradation studies conducted by Cargill with a Reb M preparation produced by fermentation (*i.e.,* rebaudioside M produced by *S. cerevisiae*; GRN 000626) that has a similar individual steviol glycoside profile as Reb M produced by *Y. lipolytica*, confirm that in the presence of human fecal homogenates Reb M is metabolized completely to steviol within 48 hours. These data confirm that steviol glycosides produced by fermentation share the same metabolic fate as steviol glycosides, such as rebaudioside A, from *S. rebaudiana* Bertoni.

In metabolic fate studies in rats, the aglycone produced in the colon has been shown to be absorbed systemically *via* the portal vein then transported to the liver where it is metabolized to steviol glucuronide before being excreted in the feces *via* the bile (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002; Roberts and Renwick, 2008). Human data present a similar metabolic fate of steviol glycosides with the exception that steviol glucuronide is eliminated *via* the urine due to the lower molecular weight threshold for biliary excretion in rats as compared to humans (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Renwick, 2007; Wheeler *et al*., 2008; Roberts *et al.*, 2016). This difference in the route of elimination is of no toxicological significance due to the fact that the water-soluble phase II metabolites are rapidly cleared in both rats and humans. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans.

History of Safe Use in Foods and Beverages

The *S. rebaudiana* plant and its steviol glycoside components have been consumed as sweeteners in foods and beverages by humans in countries such as Brazil and Paraguay, as well as by indigenous peoples for decades to hundreds of years (Blumenthal, 1995; Geuns, 2003; Ferlow, 2005). Stevioside has been reported to be in commercial use in Asia since at least 1995, and for more than 30 years in Japan (International Sugar Organization, 2001; Ferlow, 2005). There have been no reports of adverse effects following the use of *S. rebaudiana* as a sweetener (Lee *et al.*, 1979; Ferlow, 2005). Very few allergic or hypersensitivity reactions from the use of stevia sweeteners have been reported (Kimata, 2007; Esmail and Kabadi, 2012). These reactions were likely due to impurities present in stevia sweeteners which did not meet the purity specification (≥95%) set by JECFA.

Safety Opinions by Scientific and Regulatory Authorities

The safety of steviol glycosides has been extensively evaluated and is supported by conclusions from several scientific bodies and regulatory agencies, including the U.S. FDA, 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. The data examined during these evaluations included the comparative metabolism and pharmacokinetics of steviol glycosides in animals and humans, acute, short-, and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies. JECFA established an ADI of 0 to 4 mg/kg body weight, as steviol equivalents, based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al*., 1997) and a safety factor of 100, to account for intra- and inter-species differences (JECFA, 2009). Initial specifications established by JECFA (2010) stipulated that the purity of steviol glycoside preparations was to be not less than 95% of the 9 named steviol glycosides (stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside). Following the review of new data that was presented in the 82nd JECFA meeting, an updated specification was established for "Steviol glycosides from *Stevia rebaudiana* Bertoni" which defines steviol glycosides as "*a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of S. rebaudiana Bertoni*" (JECFA, 2017a). The Committee also evaluated data on a novel yeast-derived steviol glycoside product resulting in the issuance of new specifications for "Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica*", with a purity definition of no less than 95% rebaudioside A (JECFA, 2016). Similar to JECFA's conclusions, other regulatory authorities including EFSA, FSANZ, and the Health Canada Food Directorate have also established a steviol glycoside ADI of 0 to 4 mg/kg body weight, expressed as steviol equivalents (FSANZ, 2008, 2015; EFSA, 2010; Health Canada, 2012). Most recently, in early 2019 FSANZ updated the specification for "Steviol glycosides from *S. rebaudiana* Bertoni" to include enzymatic conversion of purified stevia leaf extract as an alternative method to produce rebaudioside M, whereby rebaudioside M is produced from purified stevia leaf extract using enzymes derived from a bioengineered yeast (*i.e., Pichia pastoris*).

New Safety Data

New safety data published subsequent to Cargill's most recent GRAS evaluation for steviol glycosides, GRAS Notification GRN 000768 for stevia leaf extract preparations that received a "no questions" letter from the U.S. FDA, was evaluated by the GRAS Panel. These studies included 2 repeated-dose studies, 1 genotoxicity study, 2 reproductive toxicity studies, and 2 human studies. Barrios-Correa *et al*. (2018) investigated the brain of mice for changes in the JAK2/STAT3 signaling pathway and changes in appetite and body composition following 6 weeks of exposure to rebaudioside A (approximately 15 mg/kg body weight/day steviol equivalents) and concluded that alterations in brain activity with regards to signaling pathways that control appetite and energy balance occurred following repeated rebaudioside A intake; however, since the dose was calculated to be about 4 times higher than the upper limit of the ADI for steviol glycosides, the relevance of these data to human exposure to steviol glycosides in food is limited. Han *et al.* (2019) investigated the effects of aqueous stevia obtained from *S. rebaudiana* leaves on feed intake and digestibility in goats and reported that the addition of stevioside to the feed of goats increased dry matter intake and increased the digestibility of neutral and acid detergent fiber. In a chromosome aberration test and micronucleus test, the genotoxic potential of stevia in human lymphocytes was investigated and stevia was confirmed to not be genotoxic (Uçar *et al*., 2018). The effects of a non-commercial aqueous *S. rebaudiana* extract on reproductive function in rats with streptozotocin-induced diabetes were examined by Ghaheri *et al.* (2018), who reported that some of the adverse reproductive effects observed in rats with streptozotocin-induced diabetes may be reduced by exposure to stevia leaf extract. Jiang *et al.* (2018) evaluated the effects of the daily consumption of extremely high doses of rebaudioside A (approximately 76 and 486 mg/kg body weight/day steviol equivalents) on the ovarian cycle and steroidogenesis in weanling rats. Although the authors reported a disruption in steroidogenesis following rebaudioside A exposure, the doses utilized in this study were about 19 to 120 times higher than the upper limit of the steviol glycoside ADI on a steviol equivalent basis, and therefore the relevance of these findings to the safety of human exposure to steviol glycosides in food is limited. In a randomized, crossover placebo-controlled clinical study, Al-Dujaili *et al.* (2017) investigated the effects of a pure stevia powder (individual steviol glycoside percentages were not quantified) on blood pressure, stress hormone levels, and anthropometric parameters in healthy subjects. Significant increases and decreases were observed in urinary concentrations of free cortisol and cortisone compared to baseline. The authors noted that the small sample size was a limitation of the study and concluded that further research is needed to understand the significance of the observed effects. In a randomized single-blinded, crossover, placebo-controlled clinical study, Ahmad *et al.* (2018) investigated the effects of a single dose of stevia leaf powder (purity nor individual steviol glycoside percentages were reported) on blood glucose and related parameters in healthy subjects. The study reported no significant effects on any of the anthropometric parameters, blood glucose response, or gastrointestinal discomfort. The GRAS Panel notes that the findings from these studies provide no new information that would indicate any safety concerns which would impact the overall safety of steviol glycosides for use as general purpose sweeteners in conventional food and beverage products.

Safety of the Production Organism

The *Y. lipolytica* production strain is a non-pathogenic and non-toxicogenic yeast of the *Y. lipolytica* species. The steviol glycoside production strain contains no known pathogenicity-related proteins, toxins, allergens, or genes; the incorporated DNA is either synthesized or sourced from biosafety level 1 organisms. Bioinformatic analyses of the proteins encoded by the inserted gene sequences confirm that the expressed proteins are not associated with any toxic or allergenic potential. The GRAS Panel notes that *Y. lipolytica* has a long history of safe use in the production of food (*e.g.*, cheese ripening) and food ingredients (*e.g.*, citric acid, γ-decalactone). Steviol glycosides (*e.g.,* rebaudioside A and rebaudioside M) obtained from *Y. lipolytica* expressing steviol glycoside biosynthesis pathway genes, similar to the production organism for

Cargill's Reb M, are GRAS for use as table top sweeteners and as general purpose non-nutritive sweeteners in foods in the U.S. (GRN 000632 and 000759 – U.S. FDA, 2016, 2018). EFSA has granted Qualified Presumption of Safety (QPS) status for *Y. lipolytica* and therefore has deemed it safe to derive genetically modified strain lineages to use in the production of food additives and enzymes (EFSA, 2018). Furthermore, several steps are undertaken during the manufacturing process to inactivate (*i.e.,* heat treatment) and remove (*i.e.,* chromatography and crystallization) the microorganism. The final Reb M product has been tested and shown to be absent of residual protein (above the limit of detection of 25 ppm using the BCA assay or above 1.5 ppm using the Protein Dot Blot method), and absent of recombinant DNA (above the limit of detection of 10 ng/g for the PCR assay). The GRAS Panel concludes that the production organism is derived from a safe strain lineage and that the inserted DNA is of no safety concern.

SUMMARY

Reb M consists of not less than 95% steviol glycosides and is intended for use as a general purpose sweetener in conventional food and beverage products in the U.S. Reb M is manufactured in accordance with cGMP and is obtained from a *Y. lipolytica* production strain, which is a species of yeast that has been determined to be safe for the production of food ingredients. Reb M is expected to be used at rates consistent with other steviol glycosides already permitted for use in the U.S., and the predicted intakes of Reb M (as steviol equivalents) are below the upper limit of the steviol glycoside ADI of 4 mg/kg body weight/day (as steviol equivalents) established by JECFA (2007). Steviol glycosides, whether produced by fermentation or extracted from the *S. rebaudiana* plant, are metabolized and biologically handled in an identical manner following oral administration: steviol glycosides are rapidly hydrolyzed to steviol, steviol is absorbed and conjugated with glucuronic acid, and steviol glucuronide is excreted primarily *via* the urine in humans. The safety data and conclusions drawn for individual steviol glycosides from *S. rebaudiana* Bertoni, therefore, can be extended to include all purified steviol glycosides including Reb M derived from a *Y. lipolytica* production strain. The scientific evidence examined by the GRAS Panel demonstrates that under the conditions of intended use, Cargill's Reb M obtained from a *Y. lipolytica* production strain would not produce any adverse health effects.

CONCLUSION

We, the undersigned independent qualified members of the Generally Recognized as Safe (GRAS) Panel, have independently and collectively, critically evaluated the data and information summarized above that is pertinent to the safety of the proposed use of Reb M obtained from a *Y. lipolytica* production strain as a general purpose sweetener in conventional food and beverage products. We unanimously conclude that the proposed use of Reb M, produced in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting the appropriate specifications as presented in the supporting dossier *"Documentation Supporting the Evaluation of Rebaudioside M as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener"* is safe.

We further unanimously conclude that the proposed use of Reb M, produced in a manner that is consistent with cGMP and meeting the appropriate specifications as presented in the supporting dossier, is GRAS based on scientific procedures under the conditions of intended use in foods and beverages specified herein.

It is our professional opinion that other qualified experts would also concur with this conclusion.

[redacted]

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