ORIGINAL SUBMISSION

626

GRN 000626

February 1, 2016

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Exemption Claim for Steviol Glycosides from *Saccharomyces cerevisiae* Expressing Steviol Glycoside Biosynthesis Pathway

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 Rd W, Wayzata, MN 55391], a Notice of the determination, on the basis of scientific procedures, that steviol glycoside extract preparations, produced by Cargill, Incorporated, as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, 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 determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of steviol glycosides from *Saccharomyces cerevisiae* expressing steviol glycoside biosynthesis pathway under the intended conditions of use, also are enclosed for review by the agency.

The enclosed electronic files for the Notice entitled, "GRAS Exemption Claim for Steviol Glycosides from *Saccharomyces cerevisiae* Expressing Steviol Glycoside Biosynthesis Pathway" were scanned for viruses prior to submission and is thus certified as being virus-free using McAfee VirusScan 8.8.

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

Sincerely,

(b) (6)

Alex Eapen, Ph.D., DABT
Principal Scientist – Toxicology and Regulatory
Cargill, Incorporated

Cargill

February 1, 2016

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
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5100 Paint Branch Parkway
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Alex Eapen, Ph.D., DABT
Principal Scientist – Toxicology and Regulatory
Cargill, Incorporated

GRAS Exemption Claim for Steviol Glycosides from *Saccharomyces cerevisiae* Expressing Steviol Glycoside Biosynthesis Pathway

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

Cargill, Incorporated
15407 McGinty Rd W
Wayzata, MN 55391
USA

February 1, 2016

GRAS Exemption Claim for Steviol Glycosides from *Saccharomyces cerevisiae* Expressing Steviol Glycoside Biosynthesis Pathway

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List of Figures and Tables

I. GRAS Exemption Claim

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR § 170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

A purified steviol glycoside mixture produced from *Saccharomyces cerevisiae* (*S. cerevisiae*) expressing steviol glycoside biosynthesis pathway genes (herein referred to as RebDM) has been determined by Cargill, Incorporated (Cargill hereafter) to be Generally Recognized as Safe (GRAS) under the conditions of intended use in food as described in Section I.D., consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food. Therefore, the use of RebDM in food as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

01-Feb-2016

Alex Eapen
Cargill, Incorporated

Date

I.B Name and Address of Notifier

Alex Eapen
Principal Scientist – Toxicology and Regulatory
Cargill, Incorporated
15407 McGinty Rd W
Wayzata, MN 55391
USA

I.C Common Name of the Notified Substance

RebDM; EverSweet; Steviol glycosides; Rebaudiosides D and M; Reb D and M.

I.D Conditions of Intended Use in Food

RebDM is intended for use as a general purpose sweetening agent, in accordance with current Good Manufacturing Practices (cGMP). In the United States (U.S.), most existing high-intensity sweeteners (HIS), including aspartame, have been approved by the U.S. Food and Drug Administration (FDA) as general purpose sweeteners without any further restrictions on their

use beyond good manufacturing practice. The conditions of use of HIS such as aspartame are therefore largely self-limiting and controlled by technological properties (*e.g.*, sweetness potency). Similarly, RebDM will be used at levels not to exceed those necessary to perform its technological function. Considering however that RebDM is characterized by a sweetness potency of approximately 200 times that of sugar, which is largely comparable to that of aspartame, RebDM is likely to be used in a similar manner as aspartame, with a few minor exceptions. The use-levels for RebDM will be lower than those of aspartame for baked goods, cereal and granola bars, as a result of stability issues with aspartame and the technical requirement to add an overage to provide appropriate sweetening capability. Increased use-levels of RebDM are required for chewing gum, breath freshening micro sweets, soft candy, nougats, and marzipans. Only one new category of use, soybean-based beverages, has been proposed.

The intake of aspartame primarily results from uses in beverages and table-top products. The introduction of RebDM use in soybean-based beverages (soy milks) will not have any impact on total intake considering that this use is a minor use category. The small proposed increases in chewing gum, breath freshening micro mints, and soft candy use levels for purified steviol glycosides, will be compensated for by the decreases in baked goods, cereal and granola bars. Overall, these changes will have little impact on the intake of steviol glycosides in comparison to aspartame as a general purpose sweetener.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2015a), the proposed uses of RebDM as an ingredient has been determined by Cargill to be GRAS on the basis of scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Cargill, Incorporated
15407 McGinty Rd W
Wayzata, MN 55391
USA

Attention:
Alex Eapen

Should the FDA have any questions or additional information requests regarding this notification, Cargill will supply these data and information.

II. Detailed Information on Identity and Manufacturing of RebDM

II.A Identity

RebDM is a mixture of up to 11 steviol glycosides, which are natural constituents of the *S. rebaudiana* plant: rebaudiosides A, B, C, D, E, F, M, stevioside, steviolbioside, rubusoside, and dulcoside A. The distribution of steviol glycosides present in RebDM will vary depending on the production process and final product formulation, as described in Section II.B.3. Considering, the structural similarities among steviol glycosides, it is expected that the physiochemical properties of a mixture of steviol glycosides will be similar as those of individual steviol glycosides, like rebaudioside A and stevioside.

Common or Usual Name:

Steviol glycosides, RebDM; Rebaudiosides D and M; Reb D and M

Trade Name:

EverSweet

Chemical Name:

The chemical names for the 11 steviol glycosides that may be present in RebDM are listed below.

Rebaudioside A	13-[(2-O-β-D-glucopyranosyl-3-O-β-Dglucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rebaudioside B	13-[(2-O-β–D-glucopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid
Rebaudioside C	13-[(2-O-α–L-rhamnopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rebaudioside D	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester
Rebaudioside E	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester

Rebaudioside F	13[(2-O-β-D-xylofurananosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Rebaudioside M	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl -β-D-glucopyranosyl ester
Stevioside	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Steviolbioside	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid
Rubusoside	13-β-D-glucopyranosyloxykaur-16-en-18-oic acid, β-D-glucopyranosyl ester
Dulcoside A	13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester

Chemical Abstracts Service (CAS) Number:

The CAS Numbers for the 11 steviol glycosides, as well as the aglycone steviol, are summarized in Table II.A-1.

Empirical Formula:

The empirical formulae for the 11 steviol glycosides, as well as the aglycone steviol, are summarized in Table II.A-1.

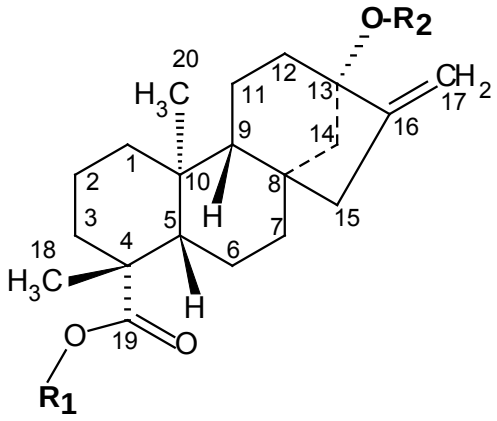
Molecular Weight:

The molecular weights for the 11 steviol glycosides, as well as the aglycone steviol, are summarized in Table II.A-1.

Structural Formula:

All of the potential 11 steviol glycosides that may be present in the final steviol glycoside product share the same backbone structure (see Figure II.A-1) and differ only with respect to the type and number of glycoside units at positions R_1 and R_2.

Figure II.A-1 Backbone Structure for the 11 Steviol Glycosides Identified in Steviol Glycoside Products



The CAS numbers, empirical formulae, molecular weights, and R_1 and R_2 groups for the 11 related steviol glycosides naturally identified in the *S. rebaudiana* plant, as well as the aglycone steviol, are summarized in Table II.A-1.

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

Steviol Glycoside	CAS Number	Molecular Weight	Molecular Formula	R-Groups in Backbone Structure	
				R_1	R_2
Rebaudioside A	58543-16-1	967.01	$C_{44}H_{70}O_{23}$	β-Glc	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside B	58543-17-2	804.88	$C_{38}H_{60}O_{18}$	H	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside C	63550-99-2	951.02	$C_{44}H_{70}O_{22}$	β-Glc	β-Glc-α-Rha(2→1) | β-Glc(3→1)
Rebaudioside D	63279-13-0	1,129.15	$C_{50}H_{80}O_{28}$	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1) | β-Glc(3→1)
Rebaudioside E*	63279-14-1	967.01	$C_{44}H_{70}O_{23}$	β-Glc-β-Glc(2→1)	β-Glc-β-Glc(2→1)
Rebaudioside F	438045-89-7	936.99	$C_{43}H_{68}O_{22}$	β-Glc	β-Glc-β-Xly(2→1) | β-Glc(3→1)
Rebaudioside M	1220616-44-3	1,291.3	$C_{56}H_{90}O_{33}$	β-Glc-β-Glc(2→1) | β-Glc(3→1)	β-Glc-β-Glc(2→1) | β-Glc(3→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

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

Steviol Glycoside	CAS Number	Molecular Weight	Molecular Formula	R-Groups in Backbone Structure	
				R_1	R_2
Dulcoside A	64432-06-0	788.88	$C_{38}H_{60}O_{17}$	β-Glc	β-Glc-α-Rha(2→1)
Steviol	471-80-7	318.46	$C_{20}H_{30}O_3$	H	H

Glc = Glucose; Rha = Rhamnose; Xyl = Xylose
* Currently only included in the European Commission's specifications for steviol glycosides.

II.B Method of Manufacture

II.B.1 Production Microorganism

Parental Strain

A wild-type *S. cerevisiae* is used as the parental microorganism, herein referred to as the parental strain, to construct the steviol glycoside-producing yeast. Genetically speaking, the parental strain is highly related to *S. cerevisiae* Meyen ex E.C. Hansen (S288C; *S. cerevisiae* Hansen, teleomorph, ATCC 204508). The wild-type yeast was made auxotrophic for histidine, leucine, and uracil by the partial deletion of *HIS3*, *LEU2*, and *URA3* using the antibiotic resistance markers natMX, hphMX, and kanMX, respectively. These antibiotic resistance markers are subsequently removed in the final production strain with full restoration of prototrophy by repairing *HIS3*, *LEU2* and *URA3*.

Production Strain

S. cerevisiae was converted into a steviol glycoside-producing yeast, herein referred to as production strain, by a series of site-specific genomic integrations of DNA constructs, in non-essential stable regions of the genome (including, but not limited to loci ECM3, YORWΔ22, KIN1, YPRCΔ15, ddp1, MPT5 and PRP5), introduced into the yeast genome by homologous recombination. The genes used to generate the production strain code for enzymes required to synthesize, transport, and improve the overall production efficiency of steviol glycosides. All promoters and terminators used for the incorporated genes are native to *S. cerevisiae* or *Ashbya gossypii,* a yeast-like fungus, the genomes of which share a high level of synteny. Table II.B.1-1 provides a summary of the representative enzymes and their functions incorporated into the production strain. As noted previously, the antibiotic resistance markers are removed and not present in the final production strain. The production strain is not toxigenic or pathogenic and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens. Additionally, the incorporated DNA is either synthetic or sourced from biosafety level 1 organisms and is not associated with any known allergens or toxins.

Table II.B.1-1 Summary of Enzymes and Respective Functions Introduced into the Production Strain

Enzyme	Function
Cytochrome P450 reductases (*A. thaliana, S. rebaudiana-1, S. rebaudiana-8*)	Works with P450 enzyme(s) in pathway
UDP-Glucosyl Transferase 74G1	Adds a glucose to C19 of steviol or steviol glycosides
UDP-Glucosyl Transferase 76G1	Adds a glucose in beta 1,3 positions of primary glucose in steviol glycosides
UDP-Glucosyl Transferase 91D2	Adds a glucose in *beta* 1,2 positions of primary glucose molecules in steviol glycosides
Copalyl diphosphate synthase	Converts GGPP to CDP
Kaurene Synthase	Converts CDP to kaurene
Kaurene Oxidase	Converts kaurene to kaurenoic acid
UDP-Glucosyl Transferase	Adds a glucose in *beta* 1,2 positions of primary glucose molecules in steviol glycosides
Geranylgeranyl pyrophosphate synthase	Converts prenyl phosphates to GGPP
Kaurenoic acid hydroxylase	Converts kaurenoic acid to steviol
UDP-Glucosyl Transferase 85C2	Adds a glucose to C13- of steviol or steviol glycosides
Beta-isopropylmalate dehydrogenase	Complements LEU2 auxotrophy in *S. cerevisiae*
Histidinol phosphate transaminase	Complements HIS3 auxotrophy in *S. cerevisiae*
Orotidine-5'-phosphate decarboxylase	Complements URA3 auxotrophy in *S. cerevisiae*

Construction of Production Strain

Using the methodology described in Shao *et al.* (2009), the constructs consist of genomic DNA upstream and downstream of the desired integration site, a selection marker, and a series of open reading frames flanked on each side with a promoter and terminator. Prior to transforming the wild-type *S. cerevisiae*, the constructs are excised from the plasmid backbones by endonuclease mediated restriction digestion. In some cases, the DNA constructs are combined in the same spore clone by mating and dissection. Constructs also are inserted by yeast transformation and homologous recombination using amino acid or antibiotic-resistance markers. Subsequently, all antibiotic-resistance markers are removed from the yeast by Cre-lox recombination, along with the respective promoters and terminators. Prototrophy is restored in the production strain with the appropriate amino acid auxotrophic markers.

Additionally, the production strain has been rendered HO^- (haploid) to prevent switching of mating types during the production of steviol glycosides and will not undergo mating events/ unwanted genetic rearrangement (Jensen *et al.*, 1983). The HO gene was a partial deletion spanning the active site of the endonuclease. The growth rate of the strain is slightly altered due to expression of a large number of heterologous genes; however, no other phenotypic changes are present in the production strain.

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.

II.B.2 Raw Material, Processing Aids, and Equipment Specifications

All raw materials, processing-aids, and additives used to manufacture RebDM are food-grade ingredients permitted by U.S. regulation, or have been previously determined to be GRAS for their respective uses (Table II.B.2-1).

Table II.B.2-1 Regulatory Status of Raw Materials, Processing Aids, and Equipment Used in the Manufacture of RebDM

Raw Material	Use	Regulatory Status	
		21 CFR	Approved Uses
95% Dextrose	Fermentation Medium	184.1857	No limitation other than cGMP
Ammonium sulfate	Fermentation Medium		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		GRAS; standard materials used within enzyme industry
Sodium sulfate	Fermentation Medium		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

Table II.B.2-1 Regulatory Status of Raw Materials, Processing Aids, and Equipment Used in the Manufacture of RebDM

Raw Material	Use	Regulatory Status	
		21 CFR	Approved Uses
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
Copper sulfate	Fermentation Medium	184.1261	Used as a nutrient supplement and processing aid with no limitation other than cGMP
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
Antifoam	Fermentation Medium	173.340	Secondary direct food additive, defoaming agent
Chemtex B-825 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)
Ethanol[a]	Elution solvent Crystallization	182.1	GRAS when used in accordance with cGMP
Methanol[b]	Crystallization	182.1	GRAS when used in accordance with cGMP
Hydrochloric acid	Ion-exchange regeneration	182.1	GRAS when used in accordance with cGMP
Activated carbon	Decolorizing agent		GRAS; standard material used within food industry
Ion-exchange resin	Purification		Used in accordance with §173.25

Table II.B.2-1 Regulatory Status of Raw Materials, Processing Aids, and Equipment Used in the Manufacture of RebDM

Raw Material	Use	Regulatory Status	
		21 CFR	Approved Uses
Adsorption resin	Purification		Used in accordance with §173.25
Microfilter	Purification		

CFR = Code of Federal Regulations (U.S. FDA, 2015a); cGMP = current Good Manufacturing Practices; EAFUS = Everything Added to Food in the United States (EAFUS, 2011); GRAS = Generally Recognized as Safe; JECFA = Joint FAO/WHO Expert Committee on Food Additives

[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

II.B.3 Manufacturing Process

Dextrose, 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 and yeast extract as nutrition sources, and allowed to ferment for 90 to 140 hours at 28 to 32°C under aerobic conditions. The pH of the fermentation is maintained at pH 4.5 to 6.5 using potassium hydroxide, phosphoric acid, sulfuric acid, hydrochloric 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 citric acid and subsequently centrifuged and/or microfiltered (0.1 or 0.2 µm) to remove the yeast biomass. 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.

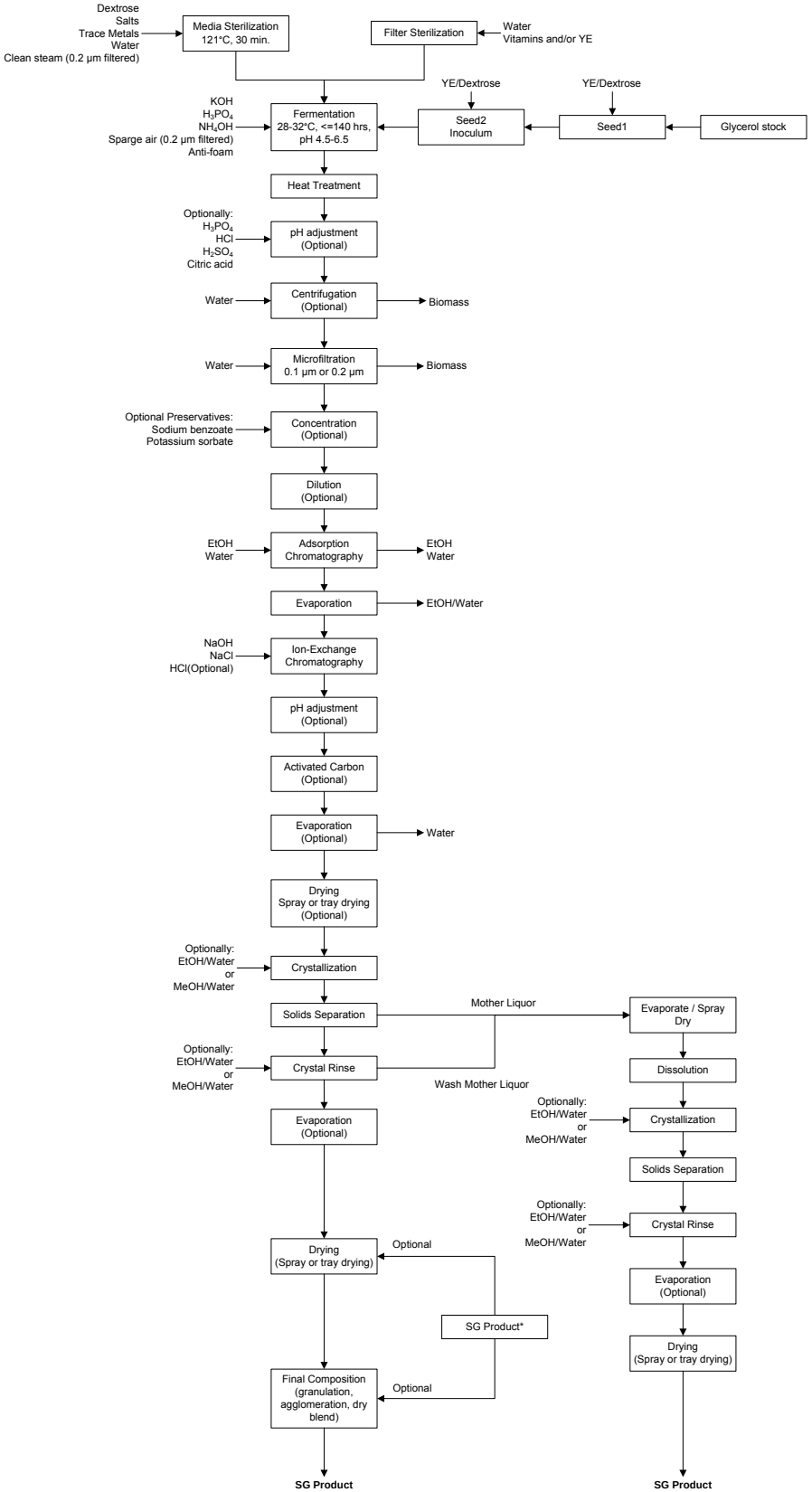
The filtrate is passed through an adsorption resin, retaining steviol glycosides, thus separating them from other constituents that may be present in the filtrate. The resin is subsequently washed with ethanol to elute steviol glycosides. The eluate undergoes evaporation to remove ethanol. Further treatment of the glycoside-rich eluate through ion-exchange resins and with optional pH adjustment and activated carbon treatment removes any additional impurities and colored substances from the eluate. The eluate is concentrated by evaporation before being mixed with aqueous ethanol or methanol to start crystallization. Optionally, the eluate may be dried using a spray dryer, vacuum dryer, or tray drying 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 ethanol (wash added to mother liquor) and

optionally evaporated prior to drying (spray or tray). The product is then checked for its final composition and released as the final steviol glycoside product (RebDM). Additionally, the final product may be blended with RebDM from other production lots that meet the specifications outlined in Section II.C.

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

A schematic overview of the manufacturing process for steviol glycosides is provided in Figure II.B.3-1, below.

Figure II.B.2-1 Schematic Overview of the Production Process for RebDM



* Represents the optional blending with additional RebDM product that meets product specifications.

II.C Specifications and Analytical Data for Food-Grade Material

II.C.1 Physical and Chemical Specifications

The physical and chemical specifications for RebDM, as presented in Table II.C.1-1, were established based on those published by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2010) and the European Commission. With the exception of the identification parameter of having stevioside or rebaudioside A as the main steviol glycoside (≥75%) in the final product as required by the European Commission, the inclusion of rebaudioside M in the definition of steviol glycoside purity, the measurement of pH in a 1% solution, and the lower specification limit for arsenic, all remaining parameters and specification limits are consistent with those defined by JECFA and the European Commission. It should be noted, however, that the European Food Safety Authority (EFSA) recently concluded that the purity definition of steviol glycosides could be expanded to include rebaudioside M on the basis of the shared metabolic pathway of steviol glycosides and that the removal of having stevioside or rebaudioside A representing at least 75% of the finished material poses no safety concern (EFSA, 2015).

The analytical method to assay the steviol glycosides present in RebDM is based on the JECFA high performance liquid chromatography (HPLC) method, that has been optimized to account for the steviol glycosides with a higher degree of glycosylation (*e.g.*, rebaudiosides D and M) present in the final product. As such, experimentally determined correction factors for rebaudiosides D and M are used instead of using molecular weight correction factors, as in the case of the JECFA method. Standard solutions of purified rebaudioside D and rebaudioside M are used to compare the response factors (area/standard concentration) for the 2 glycosides to the analytical standard (rebaudioside A) so as to experimentally determine the correction factor [response factor (rebaudioside A)/response factor (rebaudioside D or M)].

Table II.C.1-1 Physical and Chemical Specifications for RebDM

Specification Parameter	Specification	Method
Appearance	Conforms to standard	STV-003-01
Odor	Conforms to standard	STV-003-02
Assay (Total 11 steviol glycoside content)[a]	≥95%(wt/wt) (on an anhydrous basis)	STV-002-06
Moisture	Not more than 10.0% (KF)	STV-006-01
Ash	Not more than 1.0% (wt/wt)	STV-007-01
Lead (as Pb)	Not more than 1 ppm	ERT-009-1
Arsenic	Not more than 0.2 ppm	ERT-009-1
Solvent residues		
Ethanol	Not more than 0.5% (wt/wt)	STV-009-01
Methanol	Not more than 0.02% (wt/wt)	STV-009-01

[a] Based on the 9 steviol glycosides listed within JECFA's specification (rebaudiosides A, B, C, D, and F, stevioside, rubusoside, steviolbioside, and dulcoside A), rebaudioside E (as per the European Commission's specification) and rebaudioside M.

II.C.2 Microbiological Specifications

Standard microbial tests appropriate for food ingredients are employed for RebDM to ensure safety of its in food. The tests and limits are presented in Table II.C.2-1.

Table II.C.2-1 Microbiological Specifications for RebDM

Specification Parameter	Specification	Method
Aerobic Plate Count	Not more than 1,000 CFU	AOAC 966.23
Yeast	Not more than 100 CFU	FDA-BAM, 7th ed.
Mold	Not more than 100 CFU	FDA-BAM, 7th ed.
Coliform	Not more than 10 CFU	FDA-BAM, 8th ed.

CFU, Colony Forming Unit

II.C.3 Physical and Chemical Analysis of RebDM

Analysis of 4 non-consecutive lots of RebDM verified that the process as described in Section II.B.3 produces a consistent product that meets the rigorous specifications set by the manufacturer. A summary of the physical and chemical analysis for the 4 lots is presented in Table II.C.3-1.

Table II.C.3-1 Summary of Physical and Chemical Analyses for Four (4) Lots of RebDM

Specification Parameter	Specification	Manufacturing Lot			
		150417-B1	150817-B1	150901-B1	141203-B1
Appearance	Conforms to standard	White/Off white	White/Off white	White/Off white	White/Off white
Odor	Conforms to standard	Characteristic	Characteristic	Characteristic	Characteristic
Assay (Total 11 steviol glycoside content)[a]	≥95%(wt/wt) (on an anhydrous basis)	95.1	100	99.5	99.9
Moisture	Not more than 10.0% (KF)	3.4	3.6	4.4	2.7
Ash	Not more than 1.0% (wt/wt)	<0.04	0.009	0.03	<0.04
Lead (as Pb)	Not more than 1 ppm	< 0.01	<0.01	0.1	0.01
Arsenic	Not more than 0.2 ppm	< 0.01	<0.01	<0.01	<0.01
Solvent residues					
Ethanol	Not more than 0.5% (wt/wt)	0.13	0.33	0.26	0.27
Methanol	Not more than 0.02% (wt/wt)	ND	0	0	0

ND = not detected
[a] Based on the 9 steviol glycosides listed within JECFA's specification (rebaudiosides A, B, C, D, and F, stevioside, rubusoside, steviolbioside, and dulcoside A), rebaudioside E (as per the European Commission's specification) and rebaudioside M

II.C.4 Microbiological Analysis

The analyses from 4 non-consecutive lots of RebDM shows that a consistent product is produced that conforms to the microbial specification limits. A summary of the microbial analysis for the 4 lots of RebDM in presented in Table II.C.4-1. Additionally, periodic testing for the presence of *Salmonella* sp., and *Escherichia coli* confirm the absence of the contaminants in RebDM.

Table II.C.4-1 Summary of the Microbiological Analyses for Four (4) Lots of RebDM

Specification Parameter	Specification	Manufacturing Lot			
		150417-B1	150817-B1	150901-B1	141203-B1
Aerobic Plate Count	Not more than 1,000 CFU	10/g	<10/g	<10/g	<10/g
Yeast	Not more than 100 CFU	<10/g	<10/g	<10/g	<10/g
Mold	Not more than 100 CFU	<10/g	10/g	<10/g	<10/g
Coliform	Not more than 10 CFU	<10/g	<10/g	<10/g	<10/g

CFU = Colony Forming Unit

II.C.5 Additional Chemical Characterization

Steviol Glycoside Composition

As described in the chemical specifications, RebDM may consist of a mixture of up to 11 individual glycosides in any combination. In order to determine the steviol glycoside composition, Cargill developed an ultra-high performance liquid chromatography (UHPLC) method with ultra violet light detection that is optimized utilizing gradient elution. This method allows for the improved separation of steviol glycosides in comparison to the isocratic elution method utilized by JECFA, and thereby, improves the separation and quantification of similar steviol glycosides.

Utilizing UHPLC, RebDM consists primarily of rebaudioside D and M, with small amounts of rebaudioside A and B (Table II.C.5-1). Additionally, small amounts (2 to 3.3%) of other glycosides were identified in the finished product. These glycosides were confirmed as having a steviol backbone using tandem liquid chromatography and mass spectrometry (m/z approximately 317) and also by nuclear magnetic resonance (NMR). Further confirmation that these other glycosides are steviol glycosides is provided by the *in vitro* fermentation study conducted by Cargill described in Section IV.B. These other glycosides were completely degraded to steviol within 24 hours of incubation with human fecal homogenates under anaerobic conditions.

Table II.C.5-1 Steviol Glycoside Composition

Steviol Glycosides	Manufacturing Lot			
	150417-B1	150817-B1	150901-B1	141203-B1
Total Steviol Glycosides (%)	99.3	98.8	99.2	99.9
Rebaudioside D (%)	6.9	17	18.6	25.6
Rebaudioside M (%)	88.3	78.4	76.3	72.5
Rebaudioside A (%)	0.4	1.2	1.6	1
Rebaudioside B (%)	0.4	0.2	0.30	0.4
Other glycosides	3.3	2	2.4	2

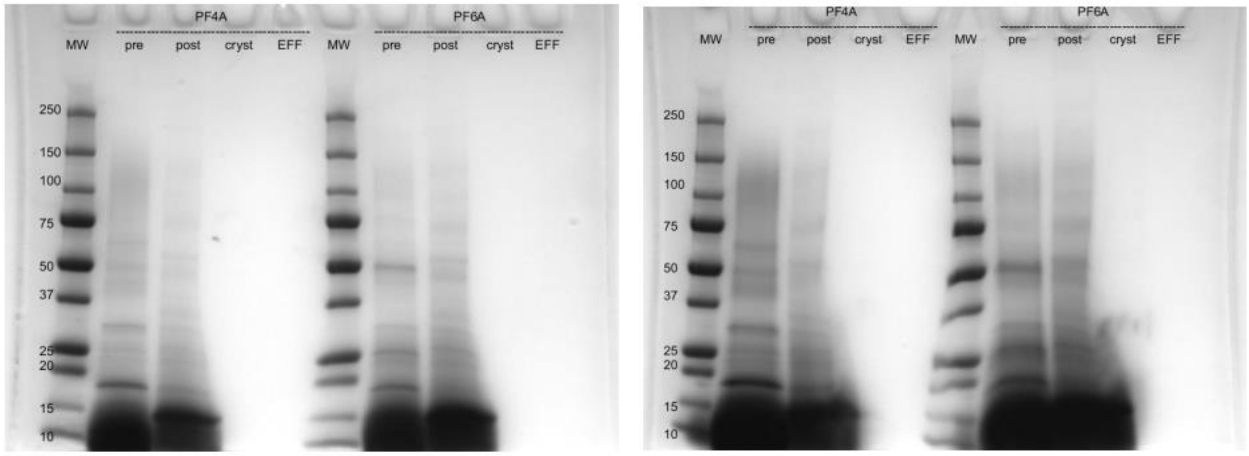
Protein Analysis

To confirm the success of the purification techniques (ion exchange chromatography, adsorption chromatography, and crystallization) and confirm the absence of proteins in RebDM, samples from 2 pilot lots (Lot #140505-B1 and #140825-B1) were analyzed at 4 points of the manufacturing process by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples (10 and 25 µL) obtained from the end of fermentation (pre), after heat treatment (post), after crystallization (cryst), and ion exchange effluent (EFF) were stained with 5x protein loading dye and loaded onto a 4 to 15% SDS gradient gel. As demonstrated in Figure II.C.5-1, protein was detected in samples from the end of fermentation and after heat

treatment; however, no visible protein bands were observed in the samples after purification. At the low molecular weights, the smearing effects are likely due to the overflow of the samples when loading and the large amount of material in the broth samples at the bottom of the gels.

Figure II.C.5-1 Protein Analysis of Two (2) Lots of RebDM Using SDS-PAGE

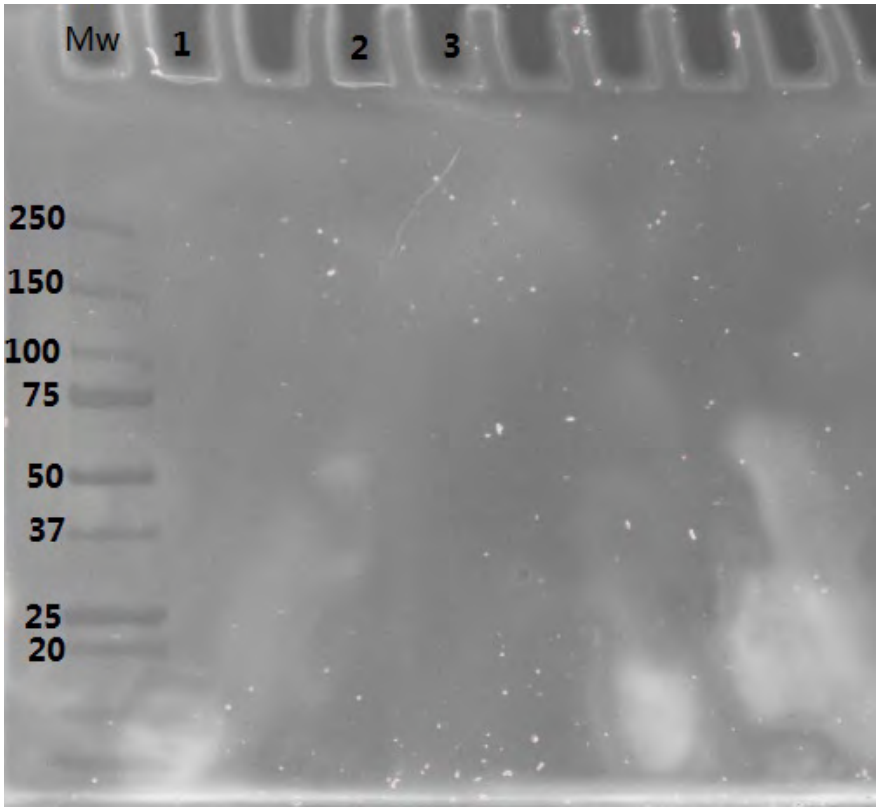
A (10 µL sample) B (25 µL sample)



PF4A = Lot #140505-B1; PF6A = Lot #140825-B1
Dectection limit is 8 to 28 ng/band

Using the same conditions for SDS-PAGE as described above, 3 additional lots of RebDM pilot samples (Lot #150417-B1, Lot #150817-B1, Lot #150901-B1) were analyzed for residual protein following crystallization of the final products. Consistent with the previous analyses, protein was not detected in any of the 3 lots (see Figure II.C.5-2).

Figure II.C.5-2 Protein Analysis of Three (3) Pilot Lots of RebDM Using SDS-PAGE



1 = Lot #150417-B1; 2 = Lot #150817-B1; 3 = Lot #150901-B1

Residual DNA Analysis

To confirm the absence of residual DNA in RebDM, a quantitative gel-based polymerase chain reaction (PCR) method was developed and validated by Eurofins Genescan GmbH (Freiburg, Germany). Primers were designed to amplify the gene coding for kaurenoic acid hydroxylase (KAH) activity. This gene represents the heterologous DNA with the highest copy number in the production strain. Genomic DNA extracted from the production strain was used as a template in the positive control reaction while genomic DNA extracted from the parent strain was used as a template in the negative control reaction. Four (4) lots of RebDM (Lot 150417-B1, 150817-B1, 150901-B1, and 141203-B1) were evaluated for the presence of residual KAH DNA. Each sample was processed using a standard DNA extraction method followed by PCR with KAH specific primers. The positive control showed amplification of a product consistent with the incorporated KAH gene, while the negative control and all lots did not show a detectable PCR product, demonstrating a lack of detectable heterologous DNA in the final steviol glycoside product.

II.D Stability of RebDM

II.D.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 and 3.0, decomposition levels of 4 and 8% 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 and 3.0, respectively) were observed.

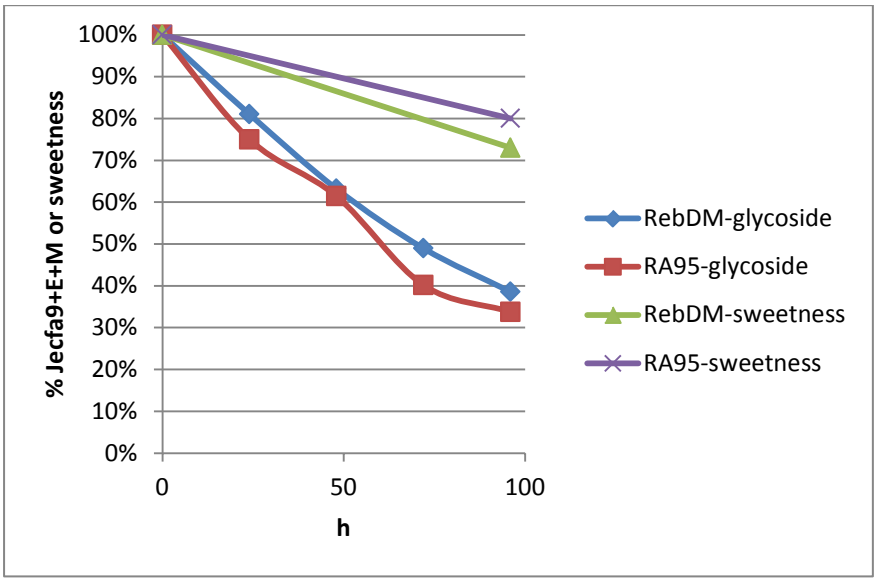
The stability of the rebaudioside A product described in GRN 000253 (≥97% rebaudioside A), tested under conditions simulating the proposed conditions of use, also was summarized therein (U.S. FDA, 2008a). Studies assessing the bulk stability of the rebaudioside A product (dry), as well as the stability of the ingredient in representative food matrices (real food/beverages at both room and elevated temperatures) were summarized within the notice. The photostability of the rebaudioside A product also was examined under dry and aqueous conditions. Collectively, the results from stability studies conducted with rebaudioside A demonstrate its stability in foods representing a broad spectrum of pH and temperature conditions, corroborating the findings by JECFA at their 68th meeting. Given the structural similarities of steviol glycosides as previously described in Section 2.4, it is expected that the stability characteristics of Cargill's finished product would be very similar to those observed for rebaudioside A.

II.D.2 Degradation of RebDM

To confirm the stability of RebDM to other related steviol glycosides, as discussed above, the degradation of RebDM under accelerated conditions was conducted to confirm the similarities between the finished ingredient and rebaudioside A obtained from *S. rebaudiana*. Samples of the final product (Lot #140825-B1) 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 throughout the study and analyzed for steviol glycoside content in accordance with the JECFA method (HPLC). Additionally, sensory analyses were conducted with steviol glycosides and rebaudioside A to determine how the degradation of the ingredients affects the taste profile.

In comparison to rebaudioside A, RebDM showed an equivalent degradation profile when analyzed using HPLC (Figure II.D.2-1). The sweetness degradation also was similar between products (Figure II.D.2-1). The similarities among the degradation of steviol glycosides further confirms that the stability of the finished steviol glycoside product is as expected similar to that of rebaudioside A, as described in Section II.D.1, above.

Figure II.D.2-1 Degradation of RebDM (Lot #140825-B1) under Accelerated Conditions Analyzed Using High Performance Liquid Chromatography (JECFA Method)



III. Self-Limiting Levels of Use

No self-limiting levels of use were identified for RebDM.

IV. Detailed Summary of the Basis for the Notifier's GRAS Determination

Cargill has determined that a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes is GRAS for use in conventional food and beverage products. This GRAS determination was based on scientific procedures using generally available data and information on steviol glycosides that were considered and reviewed by several scientific bodies and regulatory agencies, including FDA, the European Commission's Scientific Committee on Food (SCF), EFSA, Foods Standards Australia New Zealand (FSANZ), Health Canada, and JECFA. The data available for these evaluations included a thorough examination of the comparative metabolism and pharmacokinetics of

steviol glycosides in experimental 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, 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. Moreover, several studies available in the public domain conducted with stevia extracts have demonstrated the comparative metabolism of steviol glycosides. In addition studies examining the safety of steviol glycosides have been extensively summarized and discussed within 37 GRAS Notifications concerning purified mixtures of steviol glycosides, individual steviol glycosides, and enzyme modified steviol glycosides, to which the FDA has responded with "no questions" (U.S. FDA, 2008a,b, 2009a-d, 2010a-e, 2011a-i, 2012a-e, 2013a-f, 2014a-c, 2015b-d). A recent updated search of the relevant literature available in the public domain identified additional toxicological studies, including 4 repeated-dose studies and 2 genotoxicity studies. Discussions regarding the metabolic fate of steviol glycosides, the conclusions related to the safety of steviol glycosides from authoritative bodies, and results from recently identified toxicological studies are provided in Sections IV.B to D, respectively. Moreover, discussions regarding the safety of the parental and production strains, as well as the potential allergenicity of the inserted genes, are provided in Sections IV.E to G, respectively.

Finally, the information presented herein was reviewed by the Expert Panel, qualified by scientific training and experience to evaluate the safety of ingredients as components of food. A discussion of the data and information reviewed by the Expert Panel, and conclusion supporting that the intended uses of RebDM in food as a general purpose sweetening agent are safe and suitable and would be GRAS based on scientific procedures, are included in Appendix A.

IV.A Consumption Estimates

IV.A.1 History of Consumption of Steviol Glycosides

*O*fficially discovered in 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. Stevia became a popular herbal tea ingredient in the U.S. in the 1980s and in 1995, was cleared for use as a dietary supplement (Blumenthal, 1995; Geuns

et al., 2003; Schoenhals, 2003; Ferlow, 2005). There have been no reports of adverse effects following the use of these natural sweeteners (Lee *et al*., 1979; Ferlow, 2005). In 1995, the use of stevioside in Asia was reported to be approximately 160,000 metric tons sucrose equivalents (SE), while in 1999, such use reportedly increased to approximately 200,000 metric tons SE (International Sugar Organization, 2001). In Japan, stevioside has been used as a sweetener for more than 30 years, and its use has been reported to be safe, without the occurrence of adverse effects (Ferlow, 2005).

IV.A.2 Estimated Consumption of RebDM from Proposed Food Uses

Several analyses of the intakes of steviol glycosides have been completed to date based on different methodologies, which have involved use of *per capita* commodity data, national consumption surveys, and post-market surveillance data of specific sweeteners (JECFA, 2009; EFSA, 2010). In order to obtain estimates of RebDM consumption under the proposed conditions of use, intakes were generated by assuming (i) replacement of sugar and (ii) existing, well-established high-intensity sweeteners.

Data related to the current consumption of sugar in the U.S. were sought to determine an estimate of RebDM consumption by assuming replacement of caloric sweeteners. Based on production data, the *per capita* consumption of caloric sweeteners in the U.S. is 216.5 g/day (USDA-ERS, 2014). Assuming that RebDM would replace all sugar consumption, and that steviol glycosides are approximately 200 times as sweet as sugar, this corresponds to a RebDM intake expressed as steviol equivalents of 5.4 mg/kg body weight/day (average body weight of 60 kg assumed). However, these estimated intakes are highly conservative since it is unlikely that RebDM would entirely replace sugar consumption.

Since there have been numerous studies in the U.S., Canada, Australia/New Zealand, and countries in the EU that identify the intakes of aspartame and other HIS through post-market surveillance data, a more realistic, but conservative approach is to estimate steviol glycoside intake based on the intake data reported in these published studies. The intake of rebaudioside A was estimated by Renwick (2008) using published data from dietary exposures to approved intense sweeteners, such as aspartame using post-market surveillance studies, with adjustment for their relative sweetness intensities, assuming a relative sweetness for steviol glycosides of 200 times that of sucrose (Renwick, 2008). 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 steviol glycosides.

Consistent with the methodology used to estimate rebaudioside A intake by assuming complete replacement of other intense sweeteners (Renwick, 2008), exposure estimates for RebDM also were calculated based on the published post-market surveillance data of intense sweetener

consumption (as sucrose equivalents) presented in Renwick (2008) and a relative RebDM sweetness of 200 times that of sucrose. The predicted intakes of RebDM resulting from replacement of all other available intense sweeteners are presented in Table IV.A.2-1. The mean intake of RebDM is predicted to range from 1.3 mg/kg body weight/day for non-diabetic adults to 3.4 mg/kg body weight/day for diabetic children, equivalent to 0.40 and 1.03 mg steviol equivalents/kg body weight/day for non-diabetic adults and diabetic children, respectively. Predicted intakes for heavy consumers ranged from 3.4 mg/kg body weight/day for non-diabetic adults to 5.0 mg/kg body weight/day for non-diabetic children, equivalent to 1.03 and 1.52 mg steviol equivalents/kg body weight/day for non-diabetic adults and non-diabetic children, respectively. The predicted intakes of RebDM, expressed as steviol equivalents, are all below the current ADI defined by the JECFA for steviol glycosides (JECFA, 2009) of 0 to 4 mg/kg body weight/day as steviol.

Table IV.A.2-1 Intakes of Intense Sweeteners and Predicted Intakes of RebDM [based on data presented in Renwick (2008)]

Population Group	Intakes of Intense Sweeteners (as sucrose equivalents)		Predicted Intakes of RebDM		Predicted Intakes of RebDM (as steviol equivalents)[a]	
	Mean Intake (mg/kg bw/d)	Heavy Consumer (mg/kg bw/d)	Mean Intake (mg/kg bw/d)	Heavy Consumer (mg/kg bw/d)	Mean Intake (mg/kg bw/d)	Heavy Consumer (mg/kg bw/d)
Non-diabetic Adults	255	675	1.3	3.4	0.40	1.03
Diabetic Adults	280	897	1.4	4.5	0.43	1.37
Non-diabetic Children	425	990	2.1	5.0	0.64	1.52
Diabetic Children	672	908	3.4	4.5	1.03	1.37

bw = body weight
[a] Calculated using the average of the molecular weight of the 4 primary steviol glycosides present in RebDM to that of steviol (0.30).

IV.B Metabolic Fate of RebDM

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*., 2003b; Nikiforov *et al.*, 2013; Purkayastha *et al.*, 2014).

Specifically, Koyama *et al*. (2003b) 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 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. More recently, another related steviol glycoside, rebaudioside D, was observed to be hydrolyzed to stevioside and steviol upon incubation with rat cecal contents over a period of 90 minutes, which was comparable to that observed with rebaudioside A (Nikiforov *et al*., 2013). In addition, Prakash Chaturvedula and Prakash (2013) observed that incubation of rebaudioside E with crude pectinase (from *Aspergillus niger*) resulted in the generation of steviol; pectinolytic bacteria are known to reside in the human intestine (Jensen and Canale-Parola, 1985), further establishing the intestinal metabolism of steviol glycosides.

Recently, the *in vitro* metabolism of several related steviol glycosides, including rebaudioside B, D, and M, as compared to the metabolism of rebaudioside A also was investigated by Purkayastha *et al*. (2014). Results of this study confirm that rebaudioside B, D, and M follow the same common metabolic pathway involving hydrolysis to steviol as identified for other recognized steviol glycosides. Specifically, approximately 71 to 87% and 58 to 90% of rebaudioside B (0.2 mg/mL) was metabolized to steviol after 8 hours in healthy male and female fecal homogenates, respectively. In comparison, complete conversion to steviol was observed with rebaudioside D (0.2 mg/mL), while >88.5% was metabolized with rebaudioside A. By 24 hours, rebaudioside B was almost completely metabolized. At a higher concentration (2.0 mg/mL), >50% of all rebaudioside A, B, and D were converted to steviol by 24 hours and rebaudioside D was completely hydrolyzed by 48 hours. For rebaudioside M, complete metabolism to steviol was observed after 16 hours incubation, while the metabolism of steviolbioside was approximately 77 to 82% after 24 hours. Although it was expected that steviolbioside, an intermediate hydrolysis product, would be more rapidly hydrolyzed than rebaudioside M, the slower metabolism was attributed to a higher dilution factor utilized in the incubation rather than an experimental effect on the metabolic rate.

To confirm the metabolic pathway of the steviol glycosides present in RebDM, rebaudioside A and the mother liquor from the final crystallization step in the purification process (Lot #141022-B1)[1] (2 mg/mL) was incubated with samples of fecal homogenates from 2 male and 2 female healthy human volunteers for periods of 0, 4, 8, 24, and 48 hours under anaerobic conditions (Bonnema and Gaspard, 2014). The content of steviol glycosides and steviol were determined using UHPLC, which was determined by Cargill to be able to separate the numerous isomers of steviol glycosides present in RebDM in comparison to JECFA's isocratic method. All steviol glycosides present in the mother liquor were degraded to steviol within 24 hours, similar to rebaudioside A. Transient increases in the concentrations of the lower-

[1] Includes a number of other steviol glycosides not previously evaluated in other steviol glycoside products.

glycosylated steviol glycosides (*e.g.*, rubusoside, rebaudioside B), suggest that they are degradation products of other steviol glycosides; however, the lower-glycosylated steviol glycosides also were degraded to steviol within 24 hours. Therefore, the results of this study are consistent with previous microbial degradation studies, such that steviol glycosides are hydrolyzed by colonic microbiota to steviol.

The degradation rate of different steviol glycosides varies depending on the complexities of the steviol glycoside structure (Wingard *et al*., 1980; Koyama *et al*., 2003b; Nikiforov *et al.*, 2013; Purkayastha *et al.*, 2014). The hydrolysis of rebaudioside A to steviol appears to be slower than that of stevioside to steviol partly due to the presence of one additional glucose moiety, suggesting that microbes hydrolyze steviol glycosides sequentially by removing one glucose molecule at a time. Stevioside, therefore, is degraded to steviolbioside, steviolmonosides, and finally steviol, with glucose released with each sequential hydrolysis, whereas rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside B (minor pathway) prior to being ultimately degraded to steviol (Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b). This is consistent with the observation of low levels of rebaudioside A present as an *in vitro* hydrolysis product of rebaudioside D, which contains one additional glucose unit than rebaudioside A (Nikiforov *et al.*, 2013). Additionally, the metabolism of differing steviol glycosides appears to be stereoselective such that degradation of rebaudioside C, which has an α(1>2) rhamnose on the 13-position, is faster than rebaudioside A, which has a β(1>2) glucose on the same position (Koyama *et al.*, 2003b).

The aglycone is absorbed systemically *via* the portal vein and distributed to a number of organs and tissues, including the liver for additional metabolism, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Wang *et al*., 2004; Roberts and Renwick, 2008). Peak concentrations of steviol were detected in the plasma of Sprague-Dawley rats within 15 to 30 minutes of oral steviol administration, whereas maximum levels of steviol were attained approximately 8 hours following oral administration of steviol glycosides to rats, including a mixture of rebaudioside A (28.8%), rebaudioside C (25.2%), stevioside (17.0%), and dulcoside A (10.2%) (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Roberts and Renwick, 2008). As confirmed by high levels of radioactivity in the lower gastrointestinal tract for up to several hours after oral administration of radiolabeled steviol glycosides, the delay between the occurrence of radioactivity/steviol levels in the plasma and the time of administration of steviol glycosides is due to the fact that glycosides must be cleaved to steviol by the colonic microbiota before absorption can occur (Koyama *et al*., 2003a).

Following absorption from the colon, steviol primarily undergoes conjugation with glucuronic acid to steviol glucuronide in the liver. In rats, free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) were identified in the plasma 8 hours after oral administration of radiolabeled rebaudioside A or stevioside (Roberts

and Renwick, 2008). Similarly, steviol glucuronide was detected in the plasma following ingestion of stevioside or rebaudioside A in humans, with maximal concentrations detected 8 and 12 hours after administration, respectively (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The differences in the time to reach maximum steviol glucuronide plasma concentrations between stevioside and rebaudioside A are due to the simpler structure and faster bacterial degradation of stevioside (Wheeler *et al.,* 2008). However, significant inter-individual variability in maximum plasma steviol glucuronide levels, and in the time required to reach peak plasma levels, was noted in study participants following stevioside ingestion (Geuns *et al*., 2007). Such variations can likely be attributed to differences in the time required to release steviol from the glycoside in the gut as a result of inter-individual variability in the microflora composition or gastric emptying rates.

In rats, free and conjugated steviol, as well as any unhydrolyzed fraction of the administered glycosides, are excreted primarily in the feces *via* the bile (generally within 48 hours), with smaller amounts appearing in the urine (less than 3%) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002; Roberts and Renwick, 2008). Two (2) steviol conjugates were identified by Nakayama *et al*. (1986) in the bile of Wistar rats, 1 which was hydrolyzed by a weak acid and another which was hydrolyzed by a weak acid and β-glucuronidase; therefore, following the elimination of steviol glucuronide in the bile, steviol may be released from its conjugated form by the microflora and may enter enterohepatic circulation. In contrast, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, occurs *via* the urine in humans (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). Relative to amounts recovered in urine, larger amounts of steviol (unabsorbed steviol released from steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile) also were eliminated in the feces of humans (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008).

Collectively, the available degradation studies on steviol glycosides confirm the common metabolic pathway for all steviol glycosides: steviol glycosides are rapidly hydrolyzed to steviol in a similar manner for all steviol glycosides. This is consistent with the fact that with the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. Moreover, the data demonstrate that rebaudioside A and stevioside have similar metabolism and pharmacokinetics in the rat. Considering the common pathway of metabolism, and the fact that systemically, exposure only occurs to steviol following consumption of steviol glycosides, the results of toxicology studies on any one steviol glycoside can therefore be applied in an assessment of the safety of any other steviol glycoside.

The difference in the route of elimination of systemically absorbed steviol as steviol glucuronide in rats and humans (*via* the bile and in the urine, respectively) 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). Although the primary routes of elimination of steviol glucuronide differ between rats and humans, the similar metabolism and pharmacokinetics of steviol glycosides confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans given the similarities in metabolic fate.

IV.C Current Regulatory Status of and Safety Evaluations on Steviol Glycosides

Data available from the earliest safety evaluations of steviol glycosides conducted by the European Scientific Committee on Food (SCF) and by JECFA during their 51st meeting indicated that stevioside, in rats, is hydrolyzed to the aglycone steviol prior to absorption from the gut, which is subsequently conjugated and excreted in the bile and ultimately in the feces (JECFA, 1999; SCF, 1999). Although limited data were available to adequately assess the safety of stevioside and establish an acceptable daily intake (ADI) during early evaluations, JECFA subsequently re-evaluated the safety of steviol glycosides at their 63rd, 68th, and 69th meetings (JECFA, 2006a,b, 2007b,c, 2009).

During their 63rd, 68th, and 69th meetings (JECFA, 2006a,b, 2007b,c, 2009), JECFA established specifications for steviol glycosides, based on the available analytical data, such that commercial preparations contain at least 95% steviol glycosides[2], with the remainder of the preparation being unidentified. Additionally, 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 glucuronides, and excreted. 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 for up to 16 weeks by individuals with type-2 diabetes mellitus 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 weights, expressed as steviol equivalents, using 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 evaluated at the 51st meeting (Toyoda *et al*., 1997) and a 100-fold safety factor for inter-and intra-species differences. Similar to JECFA's conclusions, other regulatory authorities have conducted their own evaluation on the safety of

[2] Not less than 95% of the following 7 steviol glycosides, on a dried weight basis: stevioside, rebaudioside A, B, and C, dulcoside A, rubusoside, and steviolbioside.

steviol glycosides (FSANZ, 2008; EFSA, 2010; Health Canada 2012) and established an ADI of 4 mg/kg body weight, expressed as steviol equivalents.

Recent evaluations of steviol glycosides have resulted in expanded specifications for steviol glycosides. JECFA revised their specifications to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria[3] (JECFA, 2010). Additionally, the European Commission includes rebaudioside E within the purity definition of steviol glycosides. Although no specific studies have been conducted with these steviol glycosides individually, their inclusion within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all glycosides are degraded to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general. Moreover, Health Canada recently expanded the purity definition of steviol glycosides to include rebaudioside M as being one 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).

In addition to the evaluations conducted by international authorities, numerous GRAS notices regarding the use of steviol glycosides in foods have been submitted and reviewed by the FDA. While in the majority of instances these GRAS Notices pertain to steviol glycoside products with rebaudioside A and/or stevioside as the principal component(s), 4 GRAS Notices have concerned preparations identifying other related steviol glycosides including rebaudioside D (GRN No. 000456 and 000548 – U.S. FDA, 2013c; 2015c) rebaudioside X (also referred to as rebaudioside M) (GRN No. 000473 – U.S. FDA, 2013f), and rebaudioside C (GRN No. 000536 – U.S. FDA, 2015b) as the principal glycoside present. As summarized in the FDA's responses to the GRAS Notice for these individual glycosides, the assessment of their safety was largely based on the shared metabolic fate of steviol glycosides, wherein steviol glycosides are hydrolyzed to steviol by colonic microflora. Additional published and unpublished toxicological studies were considered by the notifiers and it was concluded that based on the comparability of these steviol glycosides to rebaudioside A that conclusions regarding the safety of stevioside and rebaudioside A could be bridged to steviol glycosides in general. Further corroborating this point, EFSA recently concluded that there is a consistent picture of high break down by the microflora *in vitro* of steviosides, rebaudiosides and dulcosides with production of steviol which is subsequently absorbed, glucuronidated and eliminated (EFSA, 2015). Furthermore, as previously concluded, toxicological studies on steviol are considered relevant to the assessment of these compounds.

[3] Not less than 95% of the following 9 steviol glycosides, on a dried weight basis: stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside.

IV.D Additional Toxicological Studies on Steviol Glycosides

No new studies were identified on the chronic toxicity or reproductive and developmental effects of steviol glycosides; however, several additional repeated-dose rat studies (Ramanathan and Sellappan, 2010; Awney *et al*., 2011; Nikiforov *et al*., 2013; Rumelhard *et al*., 2016) have been published. While some changes in a number of toxicologically relevant parameters, including body weights, food intakes, hematological and clinical chemistry indices, and organ weights, were observed in 2 of these studies (Ramanathan and Sellappan, 2010; Awney *et al.*, 2011), these studies were considered to be of equivocal design and/or were conducted with steviol glycoside materials of unspecified purity. As such, the interpretability of these studies with regard to steviol glycoside safety is confounded. Conversely, the results of the study by Nikiforov *et al.* (2013), which was a well-designed study involving administration of rebaudioside D to rats for a period of 28 days, were consistent with the previous body of literature pertaining to the safety of steviol glycosides. Additionally, the results from a recent 90-day toxicity study in rats utilizing rebaudioside A produced *via* fermentation by a genetically engineered yeast (*Yarrowia lipolytica; Y. lipolytica*) expressing the *S. rebaudiana* metabolic pathway also corroborates the conclusions regarding the safety of steviol glycosides by scientific and regulatory authorities (Rumelhard *et al*., 2016).

Moreover, additional studies investigating the genotoxic potential of rebaudioside A (95.6% purity) and rebaudioside A produced *via* fermentation by *Y. lipolytica* expressing the *S. rebaudiana* metabolic pathway corroborated the conclusions by other expert reviews, that rebaudioside A is not genotoxic *in vitro* in the Ames reverse mutagenicity test, chromosomal aberration test, mouse lymphoma assay, and human peripheral lymphocyte micronucleus assay in the presence and absence of metabolic activation at concentrations up to 5,000 µg/mL or plate (Williams and Burdock, 2009; Rumelhard *et al*., 2016). Rebaudioside A also was found to be not mutagenic in an unscheduled DNA synthesis test in rats (given at a dose of 2,000 mg/kg body weight/day) or in a micronucleus test in mice (given at doses up to 750 mg/kg body weight/day) (Williams and Burdock, 2009).

The results of these study therefore further corroborate the safety of steviol glycosides and also further confirm that steviol glycosides are commonly metabolized to steviol. As an extension of this, collective consideration of all data available on steviol glycosides to date supports that safety data generated on any one steviol glycoside can be extended to steviol glycosides in general.

IV.E Safety of the Parental Strain

S. cerevisiae has a long history of safe-use in the production of food (*e.g.*, brewing, baking) and food ingredients (*e.g.*, food-grade enzymes, flavorings). Dried *S. cerevisiae* yeast is permitted for use in food (21 CFR §172.896) provided that the total folic acid content of the yeast does not exceed 0.04 mg/g (U.S. FDA, 2015a). Baker's yeast protein and baker's yeast glycan are

approved food additives, permitted for direct addition to food for human consumption (21 CFR §172.325 and 172.898, respectively) (U.S. FDA, 2015a). Baker's yeast extract (concentration of the solubles of mechanically ruptured cells of *S. cerevisiae*) is affirmed as GRAS for use as a flavoring agent and adjuvant at a level not to exceed 5% in food (U.S. FDA, 2015a).

IV.F Safety of the Production Strain

The production strain contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. The majority of genes used to create the production are synthetic and are based on deposited sequences from *S. rebaudiana*, *A. thaliana*, *Oryza sativa*, and *Synechococcus* sp, with additional DNA from other plant sources. The fermentation broth is subjected to heat treatment and undergoes several separation and purification steps to remove the production strain. As evidenced by the lack of protein following ion-exchange chromatography and crystallization (Section II.C.5), the lack of residual DNA (Section II.C.5) and the high steviol glycoside purity of RebDM (≥95%), the inserted DNA from these organisms is of no safety concern.

IV.G Allergenicity

In order to confirm the lack of potential for cross-reactivity among the inserted heterologous gene sequences in the production strain, the AllergenOnline database version 14 (FARRP, 2014) was used to conduct a preliminary screen for relevant matches against to known putative allergens. This database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. A FASTA 35.04 overall search of AllergenOnline was conducted using default settings (E cutoff = 1 and maximum alignments of 20). No matches to allergen sequences (E <1.0) for the geranylgeranyl pyrophosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurenoic acid hydroxylase, cytochrome P450 reductase (*S. rebaudiana*-8), UDP-glucosyl transferase 74G1, UDP-glucosyl transferase 91D2, or UDP-glucosyl transferase enzymes were identified. Although E-values ranging between 0.064 and 0.99 were identified for the sequences of kaurene oxidase, cytochrome P450 reductase (*A. thaliana*), cytochrome P450 reductase (*S. rebaudiana*-1), UDP-glucosyl transferase 85C2 and UDP-glucosyl transferase 76G1 in comparison to known allergens from wheat, fish, peanut, pear, snails, mollusks, and corn, E-values larger than 1×10^{-7} are unlikely to identify proteins that may share immunologic or allergic cross-reactivity to known allergens (Hileman *et al.*, 2002). Additionally, none of the sequences of kaurene oxidase, cytochrome P450 reductase (*A. thaliana*), cytochrome P450 reductase (*S. rebaudiana*-1), UDP-glucosyl transferase 85C2 and UDP-glucosyl transferase 76G1 shared greater than 50% identity with the identified allergens, indicating the unlikely potential for cross-reactivity to the allergens listed in the tables below (Aalberse, 2000).

Additionally, in accordance with the Codex Alimentarius Commission (2003) criterion for use in flagging proteins that might be of some concern of cross-reactivity for genetically engineered plants, an 80 amino acid sliding window (segments 1-80, 2-81, 3-82, *etc*.) was used to scan the amino acid sequence of each inserted heterologous gene remaining in the production strain against the allergen database using FASTA to search for matches of 35% identity or more. With the exception of the cytochrome P450 reductases (*S. rebaudiana*-1 and *S. rebaudiana*-8), no matches greater than 35% were identified among the 80mer sliding windows in comparison to known allergens. Amino acid sequences from olive allergens were identified as having more than 35% sequence identity to 80mer segments from the cytochrome P450 reductases (*S. rebaudiana*-1 and *S. rebaudiana*-8). Although this suggests potential cross-reactivity in accordance with the Codex criterion, no potential for cross-reactivity is indicated when the allergen is compared to the full amino acid sequence. Furthermore, as demonstrated in Section II.C.5, no protein or residual DNA was identified in the steviol glycoside product following the purification techniques used in the manufacturing process. Therefore, the likelihood of cross-reactivity in the final steviol glycoside product is low.

IV.H Expert Panel Evaluation

Cargill, Incorporated has determined that a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes, as described herein, is GRAS for the intended uses as described in Section I.D, on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain and information on steviol glycosides that were considered and reviewed by several scientific bodies and regulatory agencies, as well as the consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of steviol glycosides as a component of food. The Expert Panel consisted of the following qualified scientific experts: Joseph F. Borzelleca Ph.D. (Virginia Commonwealth University School of Medicine); Michael W. Pariza Ph.D. (University of Wisconsin-Madison), and I. Glenn Sipes, Ph.D. (University of Arizona).

The Expert Panel, convened by Cargill, independently and critically evaluated all data and information presented herein, and also concluded that a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes is GRAS for use as a general purpose sweetening agent as described in Section I.D, based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes is presented in Appendix A.

IV.I Conclusion

Based on the above data and information presented herein, Cargill has concluded that the intended food-uses of a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes, as described in Section I.D, is GRAS based on scientific procedures. General recognition of Cargill's GRAS self-affirmation is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of a purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes, who similarly concluded that the intended uses of the purified steviol glycoside mixture produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes described herein are GRAS.

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
58— Good laboratory practice for nonclinical laboratory studies	[all]	
170—Food additives	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
	172.325	Bakers yeast protein
	172.375	Sodium iodide
	172.896	Dried yeasts
	172.898	Bakers yeast glycan
173—Secondary direct food additives permitted in food for human consumption	173.25	Ion-exchange resins
	173.310	Boiler water additives
	173.340	Defoaming agent
182—Substances generally recognized as safe	182.1	Substances that are generally recognized as safe
	182.3640	Potassium sorbate
	182.8159	Biotin
	182.8997	Zinc sulfate
184—Direct food substances affirmed as generally recognized as safe	184.1139	Ammonium hydroxide
	184.1193	Calcium chloride
	184.1212	Calcium pantothenate
	184.1261	Copper sulfate
	184.1315	Ferrous sulfate
	184.1370	Inositol

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	184.1443	Magnesium sulfate
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	184.1634	Potassium iodide
	184.1676	Pyridoxine hydrochloride
	184.1733	Sodium benzoate
	184.1763	Sodium hydroxide
	184.1857	Corn sugar
	184.1875	Thiamine hydrochloride
	184.1983	Bakers yeast extract

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Expert Panel Consensus Statement:

Generally Recognized as Safe (GRAS) Determination of Steviol Glycosides from *Saccharomyces cerevisiae* Expressing Steviol Glycoside Biosynthesis Pathway Genes for Use as a General Purpose Sweetener

December 2, 2014

Introduction

Cargill, Incorporated (Cargill) intends to market a mixture of purified steviol glycosides obtained from *Saccharomyces cerevisiae* (*S. cerevisiae*) expressing steviol glycoside biosynthesis pathway genes as a general purpose sweetening agent.

At the request of Cargill, a panel of independent scientists, qualified by their scientific training and relevant national and international experience in evaluating the safety of food ingredients (the "Expert Panel"), was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether the proposed uses of the purified steviol glycoside mixture, primarily consisting of rebaudiosides D and M and produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes (herein referred to as RebDM) as a general purpose sweetening agent are safe and would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of Joseph F. Borzelleca Ph.D. (Virginia Commonwealth University School of Medicine); Michael W. Pariza Ph.D. (University of Wisconsin-Madison), and I. Glenn Sipes, Ph.D. (University of Arizona). The members of the Expert Panel are qualified by their scientific training and relevant experience to evaluate the safety of RebDM under the aforementioned food uses.

The Expert Panel, independently and collectively, critically evaluated a dossier, *Documentation Supporting the Evaluation of Steviol Glycosides from Saccharomyces cerevisiae Expressing Steviol Glycoside Biosynthesis Pathway Genes as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener*, that included a comprehensive summary of scientific information on steviol glycosides prepared from information available within the public domain and also included details pertaining to the construction of the production strain of *S. cerevisiae*, method of manufacturing and product specifications of RebDM, supporting analytical data, intended use-levels of RebDM in foods, consumption estimates for all intended uses, and an unpublished *in vitro* fecal microbial metabolism study of RebDM. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent evaluation of such data and information, the Expert Panel convened on December 2, 2014 *via* teleconference with members of Cargill and unanimously concluded that the proposed uses described herein for RebDM, meeting appropriate food-grade specifications as described in the supporting dossier[1] and manufactured consistent with current Good Manufacturing Practice (cGMP), are safe and GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

Summary and Basis for the GRAS Determination of the Proposed Uses of RebDM

The ingredient that is the subject of this GRAS evaluation is a mixture of steviol glycosides produced from the fermentation of dextrose with a *S. cerevisiae* strain engineered to express steviol glycoside biosynthesis pathway genes (Production strain). The Expert Panel reviewed information pertaining to the construction of the novel *S. cerevisiae* strain using DNA constructs inserted in non-essential locations of the yeast genome using homologous recombination. The majority of genes used to create the production are synthetic and are based on deposited sequences from *S. rebaudiana*, *A. thaliana*, *Oryza sativa*, and *Synechococcus* sp with additional DNA from other plant sources.

The Expert Panel reviewed information provided by Cargill describing the chemistry and manufacturing of RebDM. In the presence of vitamins, minerals, and nitrogen sources (*e.g.*, yeast extract), dextrose is fermented by the production strain to produce steviol glycosides. Following fermentation, the yeast is removed by heat treatment, centrifugation, and microfiltration. The resulting filtrate is then purified and concentrated using steps common to the production of steviol glycosides derived from *S. rebaudiana* leaves, to obtain the final steviol glycoside ingredient (RebDM). All raw materials, processing aids, and equipment used during the manufacture of RebDM are either food-grade ingredients permitted by regulation or previously determined to be safe and suitable for use in the production of probiotic ingredients or microbial derived enzyme preparations.

Chemical and microbiological specifications for RebDM also were reviewed by the Expert Panel. Based on the chemical and physical specifications for steviol glycosides established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Commission, RebDM, meeting or exceeding a purity of ≥95% steviol glycosides, may consist of any of the following 11 steviol glycosides included either as the primary steviol glycoside and/or in various concentrations: rebaudioside A, B, C, D, E, F, M, stevioside, steviolbioside, rubusoside, and dulcoside A. The potential presence of microbial contaminants is limited by establishing rigorous critical control points and microbiological specification parameters. Consistency in the manufacturing process is supported by batch analyses of 4 non-consecutive lots of RebDM in compliance with the set chemical and microbiological parameters. Additionally, analyses of

[1] *Documentation Supporting the Evaluation of Steviol Glycosides from Saccharomyces cerevisiae Expressing Steviol Glycoside Biosynthesis Pathway Genes as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener*

purified preparations of RebDM confirmed the absence of protein and residual DNA. The degradation of RebDM under accelerated conditions was investigated by Cargill to confirm the similarities in stability between the finished ingredient and other steviol glycosides, as reviewed by JECFA.

The Expert Panel understands that RebDM is proposed for use as a general purpose sweetener that will be added to a variety of food products, consistent with the current uses of other related high-intensity sweeteners (HIS) that are already in the market (*e.g.*, aspartame). Using published dietary exposure data for other already approved HIS in the United States (U.S.) market (*e.g.*, aspartame) and adjusting for relative sweetness intensity, the mean intake of RebDM is predicted to range from 1.3 mg/kg body weight/day (0.40 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 3.4 mg/kg body weight/day (1.03 mg/kg body weight/day as steviol equivalents) for diabetic children. Predicted intakes for heavy consumer range from 3.4 mg/kg body weight/day (1.03 mg/kg body weight/day as steviol equivalents) for non-diabetic adults to 5.0 mg/kg body weight/day (1.52 mg/kg body weight/day as steviol equivalents) for non-diabetic children. Accordingly, the highest intake estimate for RebDM of 1.52 mg/kg body weight/day, as steviol equivalents, derived for non-diabetic children under the proposed conditions of use is well below the current acceptable daily intake (ADI) for steviol glycosides of 4 mg/kg body weight, expressed as steviol, as set by JECFA (2009).

The Expert Panel reviewed the available data to support the safety of RebDM, which included a detailed discussion of the metabolic fate of steviol glycosides, a summary of the conclusions made by global scientific and regulatory authorities regarding the safety of steviol glycosides, and information related to the safety of the *S. cerevisiae* parental and production strains. In order to corroborate previous conclusions regarding the shared metabolic pathway for steviol glycosides, results of an *in vitro* study examining the microbial metabolism of RebDM in the presence of human fecal homogenates under anaerobic conditions also were reviewed.

Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Hutapea *et al*., 1997; Gardana *et al*., 2003; Geuns *et al*., 2003, 2007; Koyama *et al*., 2003a,b; Renwick and Tarka, 2008). Studies conducted with mixtures of steviol glycosides and individual steviol glycosides (*e.g.*, rebaudioside A, B, D, and M) confirmed that all steviol glycosides are hydrolyzed to steviol prior to absorption (Wingard *et al*., 1980; Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b; Nikiforov *et al*., 2013; Prakash Chaturvedula and Prakash, 2013; Purkayastha *et al*., 2014). In order to confirm the similarities in microbial metabolism of RebDM to that of other steviol glycosides, an *in vitro* study examining the metabolism of the mother liquor from the final crystallization step in the purification process of RebDM (Lot #141022-B1)[2] and rebaudioside A in the presence of human fecal homogenates under anaerobic conditions was conducted

[2] Includes a number of other steviol glycosides not previously evaluated in other steviol glycoside products.

(Bonnema and Gaspard, 2014). The results of this study corroborate the findings from previously published studies demonstrating that all steviol glycosides, including RebDM and other related isomers, sharing the same steviol backbone are degraded by fecal microbes to steviol in the gastrointestinal tract.

Studies comparing the metabolic fate of steviol glycosides (*e.g.*, rebaudioside A and stevioside) demonstrate that steviol glycosides have similar pharmacokinetics in the rat; they are metabolized in the gut to steviol prior to absorption followed by glucuronidation in the liver and excretion in the feces *via* the bile (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Roberts and Renwick, 2008). In both rats and humans, steviol was shown to be metabolized to steviol glucuronide following absorption (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). However, in humans, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, occurs *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). With the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. As such, all steviol glycosides, including those present in RebDM are expected to follow the same metabolic pathway as demonstrated for rebaudioside A and stevioside. Therefore, the results of toxicology studies on either stevioside or rebaudioside A are applicable to the safety of all steviol glycosides.

Following hydrolysis to steviol, the aglycone is absorbed systemically *via* the portal vein and primarily undergoes conjugation with glucuronic acid to steviol glucuronide in the liver (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Wang *et al*., 2004; Roberts and Renwick, 2008). The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide (*via* the bile in rats and in the urine in humans) occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da (Renwick, 2007). Although the primary routes of elimination of steviol glucuronide differ between rats and humans, the metabolism and pharmacokinetics of steviol glycosides are quite similar, which confirms the rat as an acceptable model for risk assessment in humans. The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are applicable to assess the safety of steviol glycosides in humans.

As a result of the characteristically sweet taste of steviol, extracts of the stevia plant have a long history of human consumption. The safety of steviol glycosides has been the subject of numerous reviews over the last couple of decades, and recently multiple jurisdictions including the U.S., European Union (EU), Australia and New Zealand, and Canada have concluded that

preparations containing at least 95% steviol glycosides are safe when used in accordance with cGMP (FSANZ, 2008; U.S. FDA, 2008a-b, 2009a-d, 2010a-e, 2011a-i, 2012a-e, 2013a-f, 2014a-c; EU, 2011; Health Canada, 2012). Furthermore, JECFA has conducted several safety reviews of steviol glycosides over 4 separate meetings (JECFA, 1999, 2006, 2007, 2009). Specifically, based on the similar metabolic pathway for all steviol glycosides in rats and humans, as presented above, 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). Based on the similar metabolic fate of several steviol glycosides, including those present in RebDM, it was determined that the ADI established by JECFA for steviol glycosides also would extend to RebDM.

S. cerevisiae has a long history of safe-use in the production of food (*e.g.*, brewing, baking) and food ingredients (*e.g.*, food-grade enzymes, flavorings). Preparations of *S. cerevisiae* are acceptable for use in foods in the U.S. (U.S. FDA, 2014d). The final production strain contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. A search of the amino acid sequences of the inserted heterologous gene sequences in the production strain, using the web-based database AllergenOnline (FARRP, 2014), for matches to known putative allergens did not identify relevant matches against total sequence[3]. However, amino acid sequences from olive allergens were identified as having more than 35% sequence identity to 80mer segments from CPR-8 and CPR-1, in accordance with the Codex Alimentarius Commission (2003) criterion. Given that no protein was identified in RebDM following the purification techniques used in the manufacturing process, the Expert Panel concluded that the allergenicity of the steviol glycoside biosynthesis enzymes should not be a health concern.

The scientific evidence examined by the Expert Panel demonstrates that under the conditions of intended use, RebDM would not produce any adverse health effects.

[3] E-values $<1 \times 10^{-7}$ (Hileman *et al.*, 2002) and >50% identity with the identified allergens (Aalberse, 2000).

Conclusions

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses of RebDM, meeting appropriate food-grade specifications presented in the supporting dossier, *Documentation Supporting the Evaluation of Steviol Glycosides from Saccharomyces cerevisiae Expressing Steviol Glycoside Biosynthesis Pathway Genes as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener*, and produced consistent with current Good Manufacturing Practices (cGMP), are safe and suitable and are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

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Part	**Section §**	**Section Title**
172— Food additives permitted for direct addition to food for human consumption	172.325	Bakers yeast protein
	172.896	Dried yeasts
	172.898	Bakers yeast glycan
184—Direct food substances affirmed as generally recognized as safe	184.1983	Bakers yeast extract

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SUBMISSION END