#799

July 2, 2018

Dr. Paulette Gaynor
Office of Food Additive Safety (FHS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Campus Drive
College Park, MD 20740

Re: GRAS Notice for a Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Dear Dr. Gaynor

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Sichuan Ingia Biosynthetic Co., Ltd. hereby informs the United States Food and Drug Administration of the conclusion that a Rebaudioside M-rich Steviol Glycoside Preparation (≥95% Rebaudioside M), manufactured by Sichuan Ingia Biosynthetic Co., Ltd., as defined in the enclosed documents, is Generally Recognized as Safe (GRAS) under specific conditions of use as a food ingredient, and therefore, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

I hereby certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using using Symantec Endpoint Protection 12.1.5.

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

Yours sincerely,

(b) (6)

Hua Jun
President
Sichuan Ingia Biosynthetic Co., Ltd.
huajun@scingia.com

GRAS NOTICE FOR A REBAUDIOSIDE M-RICH STEVIOL GLYCOSIDE PREPARATION (≥95% REBAUDIOSIDE M)

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

Date:
25 June 2018

GRAS Notice for a Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

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GRAS Notice for a Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Part 1. §170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Sichuan Ingia Biosynthetic Co., Ltd. (Sichuan Ingia) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that a rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), manufactured by Sichuan Ingia, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Sichuan Ingia's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Sichuan Ingia, the undersigned hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and considered all unfavorable as well as favorable information known to Sichuan Ingia and pertinent to the evaluation of the safety and GRAS status of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) as a general purpose sweetener, as described herein.

Signed, (b) (6)

2018.6.29

Hua Jun
President
Sichuan Ingia Biosynthetic Co., Ltd.
huajun@scingia.com

Date

1.1 Name and Address of Notifier

Sichuan Ingia Biosynthetic Co., Ltd.
Room 7-701#, Tongwei International Centre, No., 588
Central Tianfu Avenue, High-tech Zone
Chengdu, Sichuan Province
China

1.2 Common Name of Notified Substance

Steviol glycosides; rebaudioside M; reb M; RM95

1.3 Conditions of Use

Sichuan Ingia intends to market a rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) as a general purpose sweetener in the U.S., in accordance with current Good Manufacturing Practice (cGMP), excluding infant formulas and meat and poultry products.

The U.S. FDA has approved the use of most other high-intensity sweeteners as general purpose sweeteners without their uses being restricted to specific foods or use-levels. The foods to which high-intensity sweeteners are added and the use-levels are controlled by technological properties (*e.g.,* sweetness potency). Considering that steviol glycosides, including the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), are characterized by a sweetness profile that is, for the most part, comparable to that of other high-intensity sweeteners, the uses and use-levels of Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) are likely to primarily reflect those currently permitted for other high-intensity sweeteners in the U.S.

1.4 Basis for GRAS

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR), the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) manufactured by Sichuan Ingia has been concluded to have GRAS status on the basis of scientific procedures. The GRAS determination is based on information generally available in the public domain pertaining to the safety of steviol glycosides and the enzyme production strains, as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) as a general purpose sweetener [see Appendix A, entitled "**Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of Rebaudioside M-Rich (≥95% Rebaudioside M) Steviol Glycoside Preparation (RM95) for Use as a General Purpose Sweetener**"].

1.5 Availability of Information

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

Sichuan Ingia Biosynthetic Co., Ltd.
Room 7-701#, Tongwei International Centre, No., 588
Central Tianfu Avenue, High-tech Zone
Chengdu, Sichuan Province
China

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

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

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

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

2.1 Identity

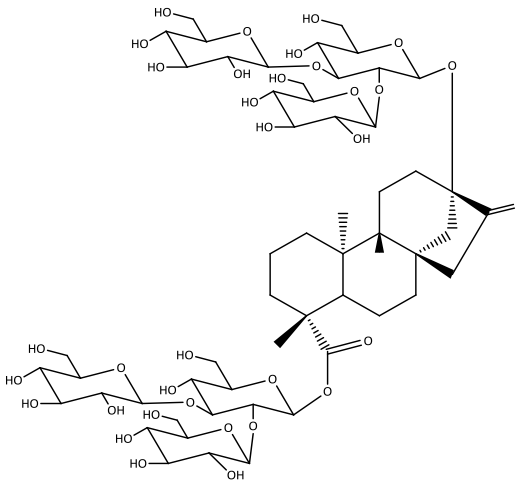
2.1.1 Common or Usual Name

Steviol glycosides; rebaudioside M; reb M; RM95

2.1.2 Chemical and Physical Characteristics

Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf is a white powder with a characteristic sweet taste and odor. The rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is 300 times sweeter than sucrose and is consistent with the sweetness profile of steviol glycosides (FAO, 2016). The chemical structure of the primary component, rebaudioside M, is presented in Figure 2.1.2-1 below. Consistent with the purity criteria for steviol glycosides as established by the Joint Expert Committee for Food Additives (JECFA) (2017a), the total steviol glycoside content of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is not less than 95% steviol glycosides. The remaining 5% may also include additional steviol glycosides as defined by JECFA as compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties of glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, and/or galactose in any orientation occurring in the leaves of *S. rebaudiana* Bertoni.

Figure 2.1.2-1 Chemical Structure of Rebaudioside M



2.2 Method of Manufacturing

The rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is produced *via* an enzymatic conversion process using a strain of *Pichia pastoris* that has been genetically modified to express the genes encoding for the UDP-glucosyltransferases (EUGT11 and 76G1) that are used in the enzymatic bioconversion reaction of high-purity rebaudioside A that is extracted from the leaves of the *S. rebaudiana* Bertoni plant. In the first stage of manufacturing, the starting material, a steviol glycoside primary extract from the leaves

of *S. rebaudiana* Bertoni containing ≥95% rebaudioside A, is produced according to the methodology outlined in the Chemical and Technical Assessment (CTA) published by the Food and Agriculture Organization (FAO)/JECFA for steviol glycosides (FAO, 2016). In the next stage, the *P. pastoris* production strains are subjected to a fermentation step to generate the UDP-glucosyltransferases (EUGT11 and 76G1) that are then utilized in the enzymatic bioconversion reaction of the steviol glycoside primary extract (≥95% rebaudioside A) to rebaudioside M. In the last stage, the crude rebaudioside M solution is purified and concentrated according to the methodology described in the CTA for steviol glycosides, yielding a final product that contains ≥95% rebaudioside M. Each manufacturing stage is discussed in more detail in the sections that follow.

2.2.1 Raw Materials and Processing Aids

All raw materials, processing aids, and equipment used in the manufacture of Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) are listed in Table 2.2.1-1 below. It should be noted that all raw materials, processing aids, and equipment are food grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses.

Table 2.2.1-1 Raw Materials, Processing Aids, and Equipment Used in the Manufacture of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Raw Material/Processing Aid/Equipment	Technical Function	Regulatory Status
Glucose	Fermentation medium (nutrient)	Permitted for use in food with no limitations apart from cGMP (21 CFR §184.1857) (U.S. FDA, 2017a)
Yeast extract	Fermentation medium (nutrient)	GRAS, 21 CFR §184.1983 (U.S. FDA, 2017a)
Peptone	Fermentation medium (nutrient)	GRAS, 21 CFR §184.1553 (U.S. FDA, 2017a)
Adenine sulfate	Fermentation medium (nutrient)	N/A
Rebaudioside A (≥95%) extracted from the leaves of *S. rebaudiana* Bertoni	Starting raw material	GRAS
UDP-glucose	Reactant; reaction medium (glucose donor)	N/A
Magnesium chloride	Reactant; reaction medium	GRAS when used in accordance with cGMP (21 CFR §184.1426) (U.S. FDA, 2017a)
Sodium citrate	Reactant; reaction medium	GRAS when used in accordance with cGMP (21 CFR §582.1751, 582.6751, 184.1751) (U.S. FDA, 201a7)
Ethanol (food-grade)	Crystallization and elution solvent	GRAS when used in accordance with cGMP (21 CFR §184.1293) (U.S. FDA, 201a7)
Macroporous resin	Purification	Used in accordance with 21 CFR §173.25 (U.S. FDA, 2017a)
Activated charcoal	Decolorizing agent/filtration aid	GRAS

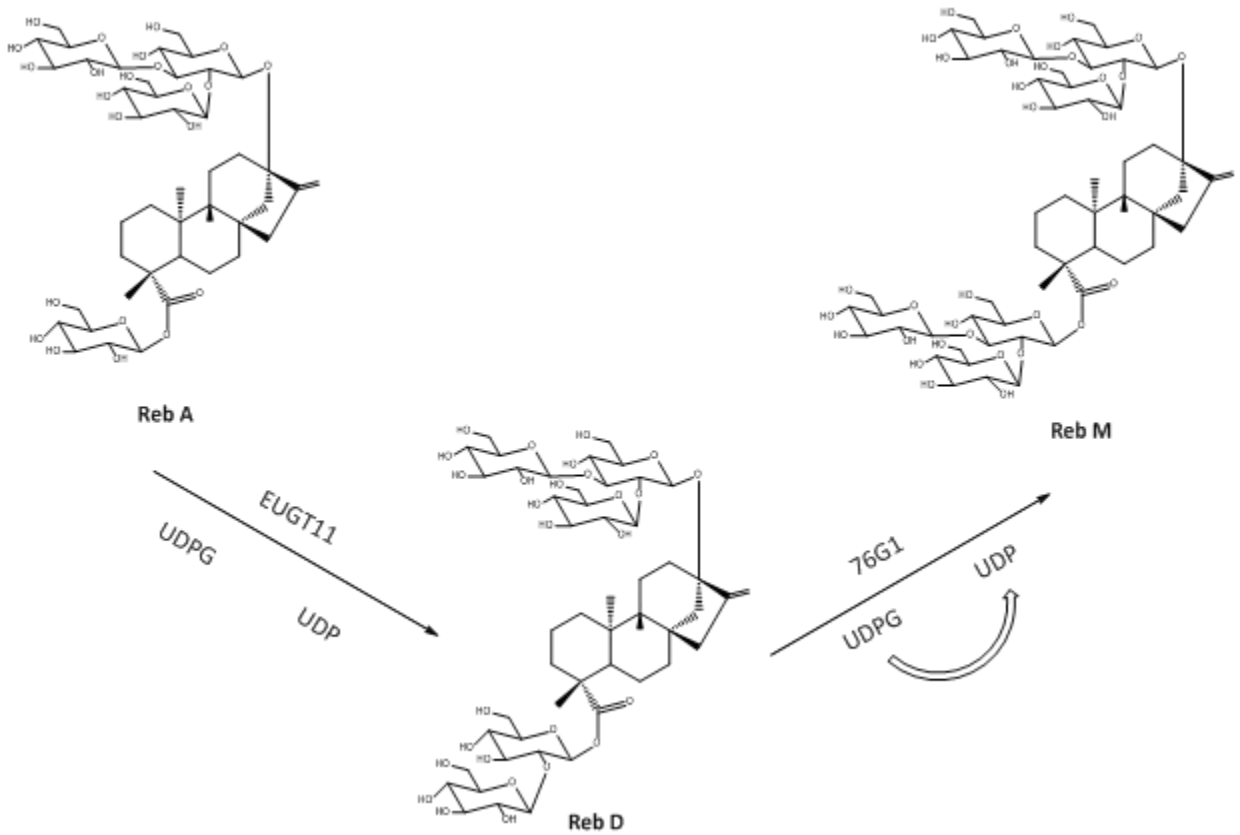
CFR = Code of Federal Regulations; cGMP = current Good Manufacturing Practice; GRAS = Generally Recognized as Safe; N/A = not available; *S. rebaudiana* Bertoni = *Stevia rebaudiana* Bertoni; U.S. FDA = United States Food and Drug Administration; UDP = uridine 5'- diphosphate.

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

2.2.2 Enzymes

The enzymatic bioconversion reaction involves the use of 2 enzymes that convert rebaudioside A to rebaudioside M, specifically, UDP-glucosyltransferase EUGT11, which is derived from a species of rice (*Oryza sativa* Japonica), and UDP-glucosyltransferase 76G1, which is derived from *S. rebaudiana* Bertoni. As shown in Figure 2.2.2-1, UDP-glucosyltransferase EUGT11 catalyzes the transfer of glucose from UDP-glucose to the 19-O-glucosyl C-2 position of rebaudioside A by 1,2-19-O-glucose glycosylation to generate rebaudioside D. Subsequently, UDP-glucosyltransferase 76G1 catalyzes the transfer of glucose from UDP-glucose to the 19-O-glucosyl C-3 position of rebaudioside D by 1,3-19-O-glucose glycosylation to generate rebaudioside M. The UDP-glucosyltransferase EUGT11 and UDP-glucosyltransferase 76G1 are produced by microbial fermentation of non-pathogenic and non-toxicogenic strains of *P. pastoris* that have been genetically modified to express the genes encoding for each respective enzyme (see Section 2.2.4 for further details). In the production of the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), the enzymes are denatured in a heating step and all residual enzymes are removed from the final product in subsequent purification processes. To demonstrate the success of the purification processes, 3 non-consecutive batches of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) were analyzed for residual protein; the results of the analysis demonstrated that protein was below the limit of detection (5 ppm) in the final product, providing evidence that subsequent purification of the final product successfully removed the enzymes and other residual proteins in the final product (see Section 2.3.5 for further details).

Figure 2.2.2-1 Enzymatic Conversion of Rebaudioside A to Rebaudioside M by UDP-Glucosyltransferases EUGT11 and 76G1



Reb = rebaudioside; UDP = uridine 5'-diphosphate; UDPG = UDP-glucose.

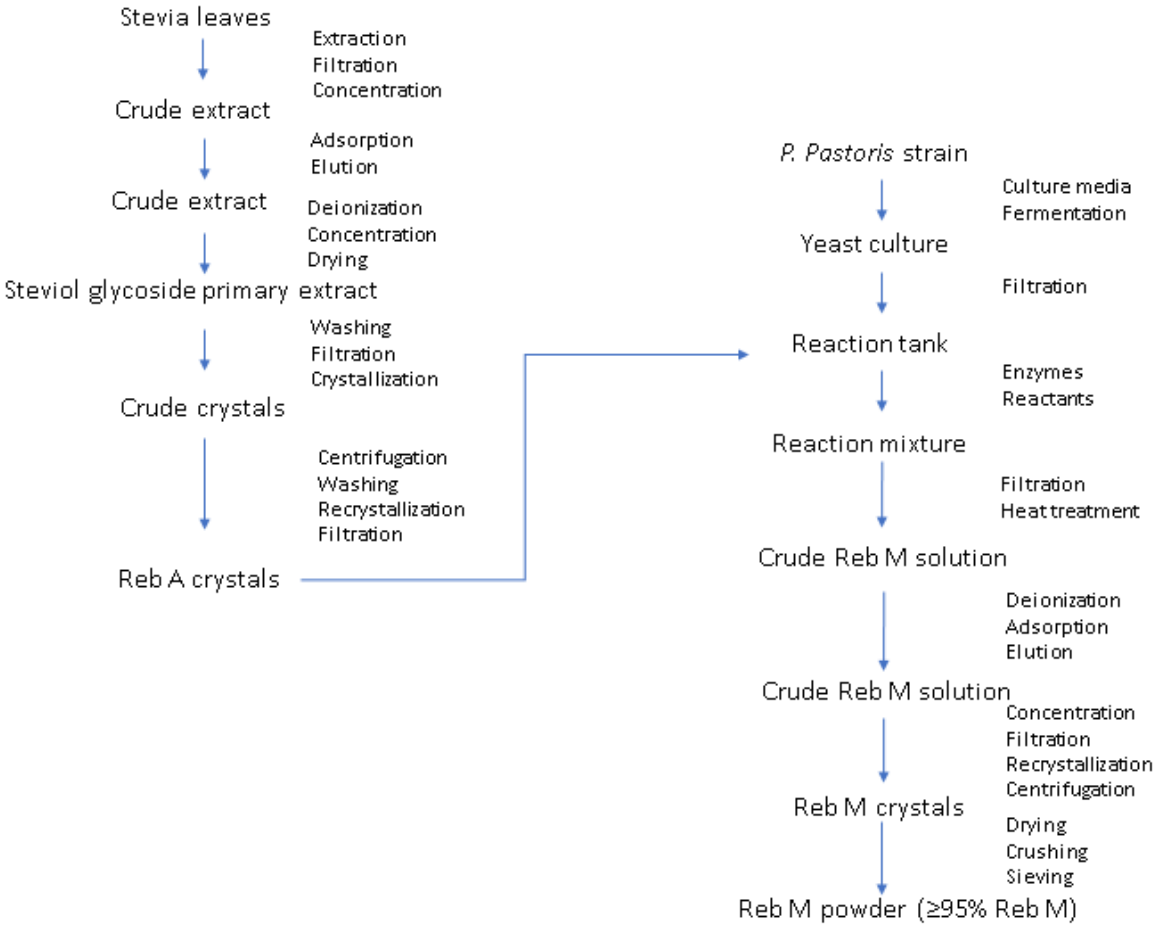
2.2.3 Manufacturing Process

A schematic overview of the manufacturing process for rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf is illustrated in Figure 2.2.3-1 and each stage of the manufacturing process is discussed in detail below.

In the first stage of manufacturing, a steviol glycoside primary extract containing ≥95% rebaudioside A is produced and purified according to the methodology outlined in the CTA for steviol glycosides (FAO, 2016). The steviol glycoside primary extract contains ≥95% rebaudioside A and therefore meets the JECFA specifications for steviol glycosides. In the next step, the enzymes required for the bioconversion process, EUGT11 and 76G1, are generated by strains of *P. pastoris* that have been genetically modified to express the genes encoding for each respective enzyme. The *P. pastoris* production strains are cultured in yeast culture seed media. Yeast cells are harvested by filtration, resuspended in a sodium phosphate buffer solution, and transferred to a reaction tank. The steviol glycoside primary extract containing ≥95% rebaudioside A and other reactants are slowly added to the reaction tank to initiate the bioconversion reaction. The reaction is allowed to proceed for 24 hours. After the reaction period, the crude rebaudioside M mixture is filtered through a 0.22 μm membrane to remove the precipitate and any remaining yeast cells. The filtered solution is heated to deactivate any residual enzymes and to kill any remaining yeast cells.

Next, the crude rebaudioside M solution is subjected to a series of purification and concentration steps that are consistent with the methodology described in the CTA for steviol glycosides (FAO, 2016). Briefly, the crude rebaudioside M solution is loaded onto a macroporous resin column and allowed to flow through by gravity. The crude rebaudioside M solution adsorbs to the column. The column is washed with food-grade ethanol to elute the adsorbed rebaudioside M solution from the column. The eluate is collected and concentrated by a scraping film evaporator to recover the ethanol. The concentrate is cooled and centrifuged to obtain a wet crystalline precipitate that is dissolved in ethanol. Activated carbon is added, the solution is filtered, recrystallized, and centrifuged to obtain wet rebaudioside M crystals. The wet crystals are then processed to generate the final high-purity rebaudioside M product (≥95% rebaudioside M). The dried crystals are subsequently packaged.

Figure 2.2.3-1 Schematic Overview of the Manufacturing Process for Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)



P. pastoris = *Pichia pastoris*; RM95 = rebaudioside M-rich (≥95% rebaudioside M) steviol glycoside preparation; Reb A = rebaudioside A; Reb M = rebaudioside M; UDP = uridine 5'- diphosphate.

2.2.4 Construction of the Production Strains

The *P. pastoris* enzyme production strains are derived from the parental strain, *P. pastoris* ATCC 20864, which is a non-pathogenic and non-toxigenic species that is ubiquitous in nature and is commonly used in the food industry. Moreover, *P. pastoris*, has been granted qualified presumption of safety (QPS) status for enzyme production by the European Food Safety Authority (EFSA) (EFSA, 2017).

The genes encoding UDP-glucosyltransferase EUGT11 and 76G1 were obtained from a species of rice and stevia leaf, respectively. The EUGT11 and 76G1 genes were introduced into the expression vector using site-directed DNA integration to produce the recombinant plasmids. The EUGT11 and 76G1 fragments and the expression vector were digested using restriction enzymes and the target fragments were ligated to produce the recombinant plasmids, which were then transformed into *P. pastoris* ATCC 20864 competent cells. The cells were grown on ampicillin-resistant lysogeny broth plates. Colonies that were successfully transformed (*i.e.,* the *P. pastoris* EUGT11 and 76G1 production strains) were obtained by ampicillin resistance screening. All plasmids and resistance genes were removed from the production strains, and therefore no residual vector sequences or antibiotic resistance genes are present in the production strains.

Stocks of the *P. pastoris* production strains were stored in glycerol at -70°C.

2.3 Product Specifications and Batch Analyses

2.3.1 Product Specifications

The product specifications for rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf are presented in Table 2.3.1-1. All analytical methods used to measure each specification parameter are internationally-recognized methods [*e.g.,* United States Pharmacopeia (USP), FDA Bacterial Analytical Manual (FDA BAM), or JECFA]. Total steviol glycoside content is measured using the high-performance liquid chromatography (HPLC) method described in the JECFA specification monograph for steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2017a,b).

Table 2.3.1-1 Product Specifications for Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Specification Parameter	Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) Specifications	JECFA Specifications for Steviol Glycosides (JECFA, 2017a)	Method of Analysis
Physical Parameters			
Appearance	White fine powder	White to light yellow powder	Visual
Odor	Characteristic	Odorless or having a slight characteristic odor	Olfactory
Taste	Characteristic	NS	Gustatory
Particle size	100% pass 80 mesh	NS	USP 34
Solubility	Soluble in water	Freely soluble in a mixture of ethanol and water (50:50)	Visual
Chemical Parameters			
Rebaudioside M	≥95% (wt/wt, on a dry basis)	NS	JECFA HPLC
Total steviol glycosides	≥95% (wt/wt, on a dry basis)	≥95% total steviol glycosides[a]	JECFA HPLC
Loss on drying	≤6% (105°, 2 h)	≤6% (105°, 2 h)	USP 34
pH	4.5 to 7 (1 in 100 solution)	4.5 to 7.0 (1 in 100 solution)	USP 34
Ash	≤1%	≤1%	USP 34
Lead	≤1 mg/kg	≤1 mg/kg	ICP-MS
Arsenic	≤1 mg/kg	≤1 mg/kg	ICP-MS
Mercury	≤1 mg/kg	NS	ICP-MS
Cadmium	≤1 mg/kg	NS	ICP-MS
Residual ethanol	≤5,000 mg/kg	≤5,000 mg/kg	USP 34
Residual methanol	≤200 mg/kg	≤200 mg/kg	USP 34
Microbiological Parameters			
Total plate count	≤1,000 CFU/g	≤1,000 CFU/g	FDA BAM
Yeast and mold	≤100 CFU/g	≤200 CFU/g	FDA BAM
Escherichia coli	Negative/g	Negative/g	FDA BAM
Salmonella	Negative/g	Negative/ 25g	FDA BAM
Staphylococcus aureus	Negative/g	N/A	FDA BAM

BAM = Bacteriological Analytical Manual; CFU = colony forming unit; FDA = Food and Drug Administration; HPLC = high-performance liquid chromatography; ICP-MS = inductively-coupled plasma mass spectrometry; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NS = not specified; ppm = parts-per-million; USP = United States Pharmacopeia.

Table 2.3.1-1 Product Specifications for Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Specification Parameter	Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) Specifications	JECFA Specifications for Steviol Glycosides (JECFA, 2017a)	Method of Analysis

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

2.3.2 Batch Analyses

Data from the analysis of 5 separate lots of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf demonstrates that the manufacturing process, as described in Section 2.2.3, produces a consistent product that meets the product specifications. A summary of the analytical data for the 5 lots of the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is presented in Table 2.3.2-1.

Table 2.3.2-1 Summary of the Analytical Data for 5 Separate Lots of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Specification Parameter	Specification	Manufacturing Lot No.				
		20180101	20180102	20180103	20180301	20180302
Physical Parameters						
Appearance	White fine powder	Complies	Complies	Complies	Complies	Complies
Odor	Characteristic	Complies	Complies	Complies	Complies	Complies
Taste	Characteristic	Complies	Complies	Complies	Complies	Complies
Particle size	100% pass 80 mesh	Complies	Complies	Complies	Complies	Complies
Chemical Parameters						
Rebaudioside M	≥95% (wt/wt, on a dry basis)	95.86%	96.06%	96.65%	96.48%	96.27%
Total steviol glycosides	≥95% (wt/wt, on a dry basis)	98.94%	98.85%	99.17%	99.64%	99.24%
Loss on drying	≤6% (105°, 2 h)	2.58%	2.53%	2.39%	2.73%	2.78%
pH	4.5 to 7 (1 in 100 solution)	5.1	5.0	5.0	5.0	5.0
Ash	≤1.0%	0.09%	0.07%	0.08%	0.07%	0.05%
Lead	≤1mg/kg	0.05	0.05	0.05	0.05	0.05
Arsenic	≤1mg/kg	0.05	0.05	0.05	0.05	0.05
Mercury	≤1mg/kg	0.05	0.05	0.05	0.05	0.05
Cadmium	≤1mg/kg	0.05	0.05	0.05	0.05	0.05
Residual Ethanol	≤5,000 mg/kg	190.71	157.56	177.85	246.28	243.75
Residual Methanol	≤200 mg/kg	30.29	36.21	37.22	47.52	28.79
Microbiological Parameters						
Total plate count	≤1,000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Yeast and mold	≤100 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Escherichia coli	Negative/g	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative/g	Negative	Negative	Negative	Negative	Negative
Staphylococcus aureus	Negative/g	Negative	Negative	Negative	Negative	Negative

CFU = colony-forming unit.

2.3.3 Pesticide Residue Analysis

Since the starting steviol glycoside material is extracted from the leaves of *S. rebaudiana* Bertoni, pesticide residue analyses were conducted on 1 lot of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) product. The results of the analysis demonstrate the absence of any residual commonly used pesticides in the final product.

2.3.4 Residual Protein Analysis

To confirm the absence of residual protein in the final product, 3 batches of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) were analyzed using the bicinchoninic acid (BCA) method. Protein was below the limit of detection (5 ppm), demonstrating that downstream processing successfully removed the enzymes and other residual proteins from the final product.

2.4 Stability Data

The stability of steviol glycosides has been evaluated by a number of authoritative and scientific bodies, including JECFA, the European Food Safety Authority (EFSA), and Food Standards Australia/New Zealand (FSANZ). JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in food (JECFA, 2007a). The Committee noted that steviol glycosides do not undergo browning or caramelization when heated and are reasonably stable under elevated temperatures used in food processing. As a result, the Committee 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% purity) are stable for at least 180 days when stored at temperatures up to 24°C in acidic conditions (pH 2 to 4). However, at higher temperatures (80°C and pH 3 and 4) 8 and 4% decomposition were observed in solutions of steviol glycosides, respectively, indicating that the stability of steviol glycosides is pH- and temperature-dependent. As expected, higher rates of decomposition were observed at greater temperatures (100°C). The U.S. FDA has also reviewed the stability of a number of steviol glycoside preparations, including high-purity rebaudioside M preparations (*e.g.,* GRN 512 and 667), the results of which demonstrate that steviol glycosides are thermally stable under normal storage conditions, consistent with JECFA's conclusions.

Sichuan Ingia conducted a series of stability tests on their rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), including short-term (10 days), accelerated (6 months), and long-term stability (up to 9 months), the results of which are summarized in Sections 2.4.1 to 2.4.3 below. The results of the stability studies conducted with rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) demonstrate that the product is stable under different storage conditions for up to 9 months, which is consistent with the stability conclusions drawn by JECFA for steviol glycosides.

2.4.1 Short-term Stability

Sichuan Ingia evaluated the short-term stability of 1 lot of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) (Lot No. 20170603) under different storage conditions, including high illumination (4,500±500 lumens), high temperature (40°C), and high humidity (90% relative humidity), when kept in commercial packaging. Physical characteristics such as the appearance, odor, and taste, were evaluated and rebaudioside M content was measured using HPLC at days 0, 5, and 10. The results of the analyses are shown in Table 2.4.1-1 below. Overall, the results demonstrate that different storage conditions for 10 days (high illumination, temperature, humidity) do not significantly impact the physical characteristics

(*i.e.*, appearance, odor, taste) or rebaudioside M content of the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M).

Table 2.4.1-1 Short-term Stability of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) (Lot No. 20170603) Under Different Storage Conditions

Parameter	Day		
	0	5	10
High illumination (4,500±500 LX)			
Rebaudioside M (%)	95.78	95.64	95.48
Physical Characteristics	White powder with sweet odor and taste		
High temperature (40°C)			
Rebaudioside M (%)	95.86	95.59	95.42
Physical Characteristics	White powder with sweet odor and taste		
High humidity (90% RH)			
Rebaudioside M (%)	95.86	95.59	95.43
Physical Characteristics	White powder with sweet odor and taste		

LX = lumens; RH = relative humidity.

2.4.2 Accelerated Stability

An accelerated stability study was conducted with 3 non-consecutive lots of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) (Lot No. 20170603, 20170605, and 20170607) under storage conditions of 40±2°C and 75±5% relative humidity for 6 months in commercial packaging. The appearance, odor, taste, moisture content, and rebaudioside M content of each lot was tested at 0, 1, 2, 3, and 6 months. The results of the analyses are shown in Table 2.4.2-1 below and demonstrate that rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is stable for up to 6 months under accelerated storage conditions.

Table 2.4.2-1 Accelerated Stability of 3 Non-Consecutive Lots of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
Lot No. 20170603			
0 months	White powder with sweet odor and taste	2.67	95.66
1 months		2.71	95.62
2 months		2.73	95.51
3 months		2.77	95.46
6 months		2.83	95.43
Lot No. 20170605			
0 months	White powder with sweet odor and taste	2.52	95.69
1 months		2.55	95.64
2 months		2.49	95.58
3 months		2.52	95.49
6 months		2.63	95.64
Lot No. 20170607			
0 months	White powder with sweet odor and taste	2.86	95.58
1 months		2.84	95.76

Table 2.4.2-1 Accelerated Stability of 3 Non-Consecutive Lots of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
2 months		2.93	95.65
3 months		2.97	95.47
6 months		2.88	95.54

2.4.3 Long-term Stability

The long-term stability of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) was investigated in 3 non-consecutive lots (Lot No. 20170603, 20170605, and 20170607) at a temperature of 25±2°C and 60±10% relative humidity. Samples are to be maintained in commercial packaging for up to 36 months. The study is currently ongoing; however, the available results indicate that rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is stable for up to 9 months when maintained at room temperature (25±2°C) and a relative humidity of 60±10% (Table 2.4.3-1).

Table 2.4.3-1 Long-Term Stability of 3 Non-Consecutive Lots of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
Lot No. 20170603			
0 months	White powder with sweet odor and taste	2.67	95.66
3 months		2.75	95.72
6 months		2.71	95.57
9 months		2.78	95.54
Lot No. 20170605			
0 months	White powder with sweet odor and taste	2.52	95.69
3 months		2.61	95.54
6 months		2.66	95.61
9 months		2.58	95.78
Lot No. 20170607			
0 months	White powder with sweet odor and taste	2.86	95.58
3 months		2.82	95.63
6 months		2.85	95.60
9 months		2.89	95.48

Part 3. §170.235 Dietary Exposure

3.1 Intended Use of Rebaudioside M-rich Steviol Glycoside Preparation (≥95% Rebaudioside M) and Levels of Use in Foods

Rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf is intended for use as a general purpose sweetening agent in accordance with cGMP and has a sweetness intensity of approximately 300 times that of sucrose. The U.S. FDA has approved the use of most other high-intensity sweeteners as general purpose sweeteners without their uses being restricted to specific foods or use-levels. The foods to which high-intensity sweeteners are added and the use-level are controlled by technological properties (*e.g.,* sweetness potency). Considering that steviol glycosides, including rebaudioside M, are characterized by a sweetness profile that is, for the most part, comparable to that of other high-intensity sweeteners, the uses and use-levels of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) are likely to primarily reflect those currently permitted for other high-intensity sweeteners in the U.S.

3.2 Estimated Consumption of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) Based Upon Intended Food Uses

3.2.1 History of Consumption of Steviol Glycosides

The *S. rebaudiana* Bertoni plant has been consumed for hundreds of years by humans in various countries, in particular South American countries, due to its sweetening properties (Geuns, 2003). To date, there have been no reports of adverse effects due to consumption of *S. rebaudiana* extracts (Lee *et al*., 1979; Ferlow, 2005). The native peoples of Brazil and Paraguay have used the leaves of *S. rebaudiana* for hundreds of years as both a food ingredient and as a tea (Blumenthal, 1995). The native Indians of the Guarani Tribe also have been documented to use stevia leaves as a sweetener since pre-Columbian times (Ferlow, 2005). Stevia became a popular herbal tea ingredient in the U.S. in the 1980s and in Japan, stevioside has been used as a sweetener for more than 30 years with no reported adverse effects (Blumenthal, 1995; Ferlow, 2005). Stevioside or *S. rebaudiana* has been used as a sweetener in South Korea and China for at least 16 and 12 years, respectively.

3.2.2 Estimated Consumption of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) from Proposed Food Uses

The daily consumption estimates of other well-established high-intensity sweeteners (*e.g.,* aspartame, saccharin, and sucralose) have been investigated in the marketplace of several countries such as the U.S., Canada, Brazil, Australia/New Zealand, and countries in the European Union. The available post-market surveillance data for other high-intensity sweeteners was used by Renwick (2008) as the basis for the assessment of dietary exposure for rebaudioside A by assuming full replacement of the approved intense sweeteners with the new sweetener. This intake assessment methodology yields conservative intake estimates as it is unlikely that the novel sweetener would entirely replace all other sweeteners in the marketplace, but they are realistic in that they reflect actual post-market intakes of high-intensity sweeteners. To estimate rebaudioside A intakes, Renwick (2008) first expressed the post-market surveillance intake estimates for intense sweeteners presently used in the global marketplace as sucrose equivalents in various population groups (for average and high-end non-diabetic and diabetic adult and child consumers). The data used in these analyses were primarily derived from studies that used specifically designed food diaries combined with actual use-levels or approved levels in different foods and beverages.

In order to predict dietary exposure to rebaudioside A, the intake estimates for the high-intensity sweeteners (expressed as sucrose equivalents) were adjusted for the sweetness intensity of rebaudioside A relative to sucrose (approximately 200).

In the case of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), the same methodology as applied by Renwick (2008) was used to estimate dietary intake. Since rebaudioside M is 300 times as sweet as sucrose, the intake values for intense sweeteners were adjusted accordingly to derive an estimated intake range for rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M). The estimated intake ranges were then converted to steviol equivalents based upon the molecular weight for rebaudioside M of 1,291 g/mol (Table 3.2.2-1).

Table 3.2.2-1 Estimated Consumption of Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) Using Renwick's (2008) Methodology of Intense Sweetener Intake Assessment Based on Post-Market Surveillance Intake Data for Currently Used Sweeteners

Population Group	Intakes of Intense Sweeteners (Expressed as Sucrose Equivalents) (mg/kg bw/day)		Consumption Estimates for:			
			Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M)[a] (mg/kg bw/day)		Rebaudioside M-Rich Steviol Glycoside Preparation (≥95% Rebaudioside M) as Steviol Equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic adults	255	675	0.85	2.25	0.21	0.55
Diabetic adults	280	897	0.93	2.99	0.23	0.74
Non-diabetic children	425	990	1.42	3.30	0.35	0.81
Diabetic children	672	908	2.24	3.03	0.55	0.74

bw = body weight.

[a] Rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is approximately 300 times as sweet as sucrose.

[b] Calculated based on the molecular weights of steviol (318.45 g/mol) and rebaudioside M of 1,291 g/mol [steviol conversion factor of 0.25].

For non-diabetic adults, average and high-end intakes of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) up to 0.21and 0.55 mg/kg body weight/day expressed as steviol equivalents, respectively, were calculated. For diabetic adults, average and high-end intakes were slightly higher at up to 0.23 and 0.74 mg/kg body weight/day. Average and high-end exposures to rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), expressed as steviol equivalents, in non-diabetic children were calculated to be up to 0.35 and 0.81 mg/kg body weight/day, respectively. Although average intakes of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), expressed as steviol equivalents, were estimated to be higher at up to 0.55 mg/kg body weight/day in diabetic children compared to values for non-diabetic children (0.35 mg/kg body weight/day), high-end values in diabetic children (0.74 mg/kg body weight/day) were lower than high-end values in non-diabetic children (0.81 mg/kg body weight/day). The predicted intakes of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), expressed as steviol equivalents, for all population groups are below the current acceptable daily intake (ADI) defined by JECFA for steviol glycosides (JECFA, 2007b) of 0 to 4 mg/kg body weight as steviol.

As part of their evaluation of the safety of steviol glycosides in 2008, JECFA considered various intake models for the estimation of dietary exposure to steviol glycosides, including the intake analysis conducted by Renwick (2008). Although higher intake estimates than those presented by Renwick (2008) were identified using other methodologies, including ones considering replacement of all sweeteners used in or

as food (up to approximately 6 mg/kg body weight/day, expressed as steviol equivalents), JECFA noted that such replacement estimates were highly conservative and that actual exposures to steviol glycosides (expressed as steviol equivalents) would be 20 to 30% of these values (1 to 2 mg/kg body weight/day, expressed as steviol equivalents). Furthermore, JECFA noted that the intake estimates based on post-market surveillance further confirmed the lower range.

Recently, JECFA re-assessed the dietary exposure to steviol glycosides using sugar/intense sweetener substitution methods as described above (FAO, 2016). In their evaluation, the Committee included mixtures of steviol glycosides and applied conversion factors ranging from 0.2 to 0.7 to account for the different molecular weights of the different individual steviol glycosides. The Committee also assumed the most conservative sucrose equivalence of 200. When substituting various sugar/intense sweetener consumption data from various global jurisdictions for steviol glycosides, such as the U.S. and Australia, the Committee determined consumption estimates ranging from 0.4 to 7.2 mg/kg body weight/day, expressed as steviol equivalents. Based on their findings, the Committee made note that the described sugar substitution methods were "*generally overestimates of dietary exposure, as not all sugar in food products would be replaced by intense sweeteners, and a number of intense sweeteners are used in the marketplace*". Thus, dietary exposure to rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is estimated to be consistent with the current consumption estimates for steviol glycosides and are within the established ADI of 0 to 4 mg/kg body weight, expressed as steviol equivalents.

Part 4. §170.240 Self-Limiting Levels of Use

The use of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is largely limited by the desired sweetness intended for a particular food or beverage product. Therefore, the use of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) as a general purpose sweetener in foods is self-limiting based on its organoleptic properties.

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

Not applicable as rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) was not used in food before 1958.

Part 6. §170.250 Narrative and Safety Information

The safety of steviol glycosides, including rebaudioside M, has been previously evaluated by the U.S. FDA through their review of several GRAS notices. With the exception of 4 GRAS notices (GRNs 759, 764, 768, and 780), which are still pending review, the Agency has raised no objections to over 50 GRAS notices describing the GRAS status of major individual steviol glycosides (including stevioside, and rebaudiosides A, C, D, and X/M), steviol glycoside mixtures, and glucosylated or enzyme-modified steviol glycosides (GRN 252, 253, 275, 278, 282, 287, 303, 304, 318, 323, 329, 337, 348, 349, 354, 365, 367, 369, 375, 380, 388, 389, 393, 395, 418, 448, 452, 456, 461, 467, 473, 493, 512, 516, 536, 548, 555, 607, 619, 626, 632, 638, 656, 662, 667, 702, 715, 733, 744, 745). In addition, several scientific bodies and regulatory agencies, including JECFA, European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada, have also evaluated the safety of steviol glycosides. An extensive database exists to support the safety of steviol glycosides, which includes an extensive evaluation of the metabolism and pharmacokinetics of steviol glycosides in rodents and humans, and a standard battery of toxicological tests, including acute toxicity,

short- and long-term toxicity and carcinogenicity, reproductive and developmental toxicity, in vitro and in vivo mutagenicity and genotoxicity, as well as several human studies.

The predominant steviol glycoside in *S. rebaudiana* leaves, stevioside, was the main subject of the earliest studies investigating the safety of steviol glycosides (Aze *et al.*, 1991; Toyoda *et al.*, 1997). Following this, additional toxicity testing was conducted on rebaudioside A and D (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). Since then, the scientific bodies and regulatory agencies described above have extended their safety opinions to include all steviol glycosides, owing to their common metabolic fate (JECFA, 2017a,b). Therefore, given that the existing safety database on steviol glycosides has been reviewed extensively by the FDA, the pertinent generally available data and information used to support the safety of steviol glycosides (including major individual steviol glycosides and other steviol glycoside mixtures/preparations) is incorporated by reference to information cited within prior GRAS notifications. Updated searches of the scientific literature were conducted through June 2018 to identify new data and information relevant to the safety of steviol glycosides that have been published since the FDA's most recent review[2]. In the following sections, the common metabolic fate of steviol glycosides is briefly discussed in Section 6.1, and a brief summary of the conclusions of the scientific and authoritative bodies is provided (Section 6.2). Following this, studies in the scientific literature that have been newly identified and published since GRN 733 are outlined (Section 6.3), and the safety of the production strains (*P. pastoris*) used to produce the enzymes required for the manufacturing process is discussed (Section 6.4) as well as the safety of the enzymes.

6.1 Absorption, Distribution, Metabolism, and Elimination

The metabolic fate (absorption, distribution, metabolism, and elimination) of steviol glycosides has been extensively studied and discussed in recent GRAS notices (*e.g.,* GRNs 619, 626, 667, and 744). Thus, the information on the metabolic fate of individual steviol glycosides, as discussed within those GRAS notices, are incorporated by reference in this notice for Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M). Owing to the presence of β-glycosidic bonds, steviol glycosides are not hydrolyzed in the upper gastrointestinal tract and therefore are not absorbed; the unchanged steviol glycosides enter the colon intact and are subject to degradation by microbes of the *Bacteriodaceae* family, resulting in the release of the aglycone steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Geuns *et al*., 2003, 2007; Renwick and Tarka, 2008; Nikiforov *et al.*, 2013; Purkayastha *et al.,* 2016). The rate at which steviol glycosides are metabolized in the colon is dependent on their structural complexity since steviol glycosides are hydrolyzed sequentially, in which one sugar moiety is removed at a time (Wingard *et al*., 1980; Koyama *et al*., 2003b). However, despite any differences in chemical structure, relatively similar hydrolysis rates are reported for different steviol glycosides to steviol, as supported by *in vitro* metabolic studies with human fecal homogenates, particularly during the first 24 hours of incubation (Purkayastha *et al.,* 2014, 2015, 2016). Following microbial degradation, systemic absorption of the steviol metabolite into the portal vein and distribution to the liver, spleen, adrenal glands, fat, and blood has been reported (Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and Renwick, 2008). In the liver, steviol glucuronide is formed by conjugation of steviol with glucuronic acid. The steviol glucuronide metabolite and any unconjugated steviol of the administered glycosides are excreted primarily in the urine, and, to a lesser extent, feces in humans (Wingard *et al*., 1980; Nakayama *et al*., 1986; Kraemer and Maurer, 1994; Sung, 2002 [unpublished]; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). The difference in the route of elimination between rats and humans occurs due to a

[2] At the time of this dossier preparation, GRN 744 was the most recent steviol glycoside GRAS notice to receive a "no questions" letter from the FDA, which summarized literature up to October 2017.

lower molecular weight threshold for biliary excretion in rats (325 kDa), in comparison to humans (500 to 600 kDa; molecular weight of steviol glucuronide is 495 kDa) (Renwick, 2007). Although the primary routes of elimination of steviol glucuronide differ between rats and humans, it 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.

In a recent study conducted by Roberts *et al.* (2016), toxicokinetic/pharmacokinetic differences of steviol and steviol glucuronide were examined in the plasma of rats and humans. A single oral dose of stevioside (40 mg/kg body weight) was administered to male and female Sprague-Dawley rats, as well as male human volunteers. Following administration, plasma samples were taken from test subjects over a period of 72 hours and analyzed for steviol and steviol glucuronide using a validated liquid chromatography-tandem mass spectrometry method. Peak plasma concentrations (C_{max}) of steviol were similar among rats and humans; however, C_{max} values of steviol and steviol glucuronide were slightly delayed in human subjects, as compared to rats. Comparing C_{max} values for steviol glucuronide in the plasma of humans and rats, human levels were approximately 25-fold higher (approximately 4,440 ng/mL *vs*. 180 ng/mL). Systemic exposure was also considered by assessing the area under the curve ($AUC_{0.75-72h}$) of steviol and a 2.8-fold greater value was observed in humans compared to rats (1,650 ng*h/mL *vs*. 590 ng*h/mL). Likewise, the steviol glucuronide AUC was 57-fold greater in humans than rats (approximately 136,000 ng*h/mL *vs.* 2,400 ng*h/mL). These data demonstrate that the extent of steviol glucuronide formation is higher in humans than in rats.

In summary, due to the common molecular structure of steviol glycosides, which consists of a steviol backbone conjugated to different numbers and types of sugar moieties (*e.g.,* glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, and/or galactose), all steviol glycosides share a common metabolic fate as described above. Therefore, the safety database that has been established for individual steviol glycosides (*e.g.,* stevioside, rebaudioside A, rebaudioside D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless of the steviol glycoside distribution of the preparation, including rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced by enzymatic bioconversion of rebaudioside A extracted from stevia leaf.

6.2 Safety Evaluations on Steviol Glycosides

Steviol glycosides and their safety have been reviewed by JECFA at their 51st, 63rd, 68th, 69th, and 82nd meetings. The safety of steviol glycosides has also been reviewed by several other scientific bodies and regulatory agencies including the U.S. FDA, FSANZ, the European Commission's SCF, EFSA, and Health Canada (SCF, 1985, 1999; FSANZ, 2008; EFSA, 2010, 2015; Health Canada, 2012b). These scientific bodies and regulatory agencies have consistently concluded that consumption of steviol glycosides is not a safety concern. Based on the available data, the aforementioned scientific bodies and regulatory agencies have established an ADI of 0 to 4 mg/kg body weight, expressed as steviol equivalents, for steviol glycosides.

Recently, EFSA concluded that safety studies conducted with individual steviol glycosides rebaudioside A and stevioside can be extended to other steviol glycosides due to their shared metabolic fate (EFSA, 2015). EFSA specifically concluded that "*extending the current specifications to include [two additional steviol glycosides], rebaudiosides D and M, as alternatives to Reb A in the predominant components of steviol glycosides would not be of safety concern*" (EFSA, 2015), whereas JECFA, FSANZ, and Health Canada recently expanded the definition of steviol glycosides to include all individual steviol glycosides present in the *S. rebaudiana* Bertoni leaf (FSANZ, 2017b; Health Canada, 2017; JECFA, 2017a,b).

In a recent evaluation in response to a proposed amendment of the specifications of steviol glycosides, EFSA concluded that the available data was not sufficient to expand the definition of steviol glycosides to include all individual steviol glycosides due to their questions regarding uncertainties in the rate and extent of the metabolism of the different steviol glycosides to steviol (EFSA, 2018a). Specifically, there were uncertainties in the evidence provided to EFSA on the rate and extent of the metabolism of different steviol glycosides to steviol that did not allow the EFSA panel to conclude that they agreed with the applicant's amendment request. Likewise, in another recent evaluation of glucosylated steviol glycosides, EFSA concluded that the data provided was not sufficient to assess the safety of glucosylated steviol glycosides due to the limited evidence provided in the dossier. EFSA's conclusions on glucosylated steviol glycosides do not apply to Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) as the material is not a glucosylated steviol glycoside; furthermore, EFSA has previously evaluated the safety of rebaudioside M and based upon their conclusion that there are no safety concerns rebaudioside M is now listed in EFSA's steviol glycosides specifications (EFSA, 2015).

The safety data and information that were reviewed in the evaluations by these scientific bodies and regulatory agencies were generally available in the published scientific literature. The results of a 2-year study in rats by Toyoda *et al.* (1997) served as the basis for the establishment of the ADI for steviol glycosides. In this study, no carcinogenicity or adverse effects in any study parameter were observed, and a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day, equivalent to 383 mg/kg body weight/day as steviol, was determined (Toyoda *et al*., 1997). Following application of a safety factor of 100, the ADI for steviol glycosides was established to be 0 to 4 mg/kg body weight, expressed as steviol equivalents (JECFA, 2006; FSANZ, 2008; EFSA, 2010; Health Canada, 2012b).

It should be noted that in addition to the described safety evaluations, the safety of over 50 different steviol glycoside preparations has also been reviewed by the U.S. FDA who have raised no objections regarding the GRAS status of the different steviol glycosides preparations.

6.3 Additional Safety Data for Steviol Glycosides

As outlined previously, the safety of steviol glycosides has been extensively reviewed in a number of GRAS notifications submitted to the U.S. FDA, which are incorporated by reference in this dossier. The safety of steviol glycosides was most recently evaluated by the U.S. FDA in its evaluation of GRN 744 for steviol glycosides consisting primarily of rebaudioside M. In order to identify new data related to the safety of steviol glycosides that have been published since the review of GRN 744, a comprehensive search of the scientific literature was conducted from March 2018 to June 2018. The search was limited to articles with full texts within peer-reviewed scientific journals. The following databases were searched: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The studies identified included 1 male reproductive toxicity study in rats and 1 human study. Upon review, the results of these studies do not raise any new safety concerns with respect to steviol glycosides and provide further support to the safety of steviol glycosides; thus, the safety of steviol glycosides, including rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), are supported by the existing safety database and the conclusions of a number of authoritative scientific bodies (*e.g.,* JECFA, EFSA, FSANZ, U.S. FDA, and Health Canada).

6.4 Safety of the Production Strains and the Enzyme

The production strains used to produce the UDP-glucosyltransferases that enzymatically convert rebaudioside A to rebaudioside M are derived from the parental strain, *P. pastoris* ATCC 20864. As described in detail in Section 2.2.2, the UDP-glucosyltransferase EUGT11 gene is derived from a species of rice (*O. sativa* Japonica) and the UDP-glucosyltransferase 76G1 gene is derived from *S. rebaudiana* Bertoni. The safety of the UDP-glucosyltransferases EUGT11 and 76G1 derived from the *P. pastoris* production strains were evaluated using the decision tree for evaluating the safety of microbially-derived food enzymes published by Pariza and Johnson (2001). The enzymes were determined to be "accepted" as per the decision tree criteria and based on the conclusion that the final product meets JECFA specifications. Furthermore, the manufacturing process includes a heating step in which residual enzymes are denatured and remaining yeast cells are killed, and subsequent filtration and purification steps that remove the production strains and enzyme from the final rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) product. Successful removal of residual enzymes and yeast cells were confirmed based on the results of analysis using the BCA assay (see Section 2.3.4 for further details).

6.4.1 History of Use and the Production Strains

P. pastoris was first introduced for commercial use in the production of proteins for use as animal feed additives over 40 years ago (Ahmad *et al.,* 2014). Currently, *P. pastoris* is used in the production of foods such as cheese and wine, and in the biopharmaceutical industry to produce recombinant proteins (De Schutter *et al.,* 2009; Weinacker *et al.,* 2013; Ahmad *et al.,* 2014). Dried *P. pastoris* is a permitted food additive for use in feed formulations of broiler chickens in the U.S. under 21 CFR §573.750 (U.S. FDA, 2017a). In addition, *P. pastoris* is used as a source organism in the production of phospholipase C enzyme preparation to which the U.S. FDA responded with a "no questions" letter concerning its GRAS status (U.S. FDA, 2006). The phospholipase C enzyme preparation also was reviewed by JECFA in which no safety concerns were expressed (JECFA, 2009).

6.4.2 Pathogenicity/Toxicogenicity of the Production Strains

P. pastoris is non-pathogenic and non-toxigenic and has not been associated with any known human or animal disease (JECFA, 2009; Chang *et al.,* 2011). *P. pastoris* has been granted QPS status by the EFSA for use in enzyme production (EFSA, 2017).

6.4.3 Potential Toxicity of the Enzymes

Although the UDP-glucosyltransferases are not present in the final rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) product, bioinformatic searches were conducted with the UDP-glucosyltransferase EUGT11 and UDP-glucosyltransferase 76G1 sequences to confirm that they do not harbor any toxic potential. The Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information was used to conduct a sequence alignment query of the UDP-glucosyltransferase FASTA protein sequences against downloaded protein sequences obtained from a curated database of venom proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot[3]). BLAST searches also were conducted against curated virulence proteins and toxins maintained by UniProt

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

(UniProtKB/Swiss-Prot/TrEMBL[4]). A sequence alignment of ≥35% identity was used as a threshold for identification as a positive alignment (Codex Alimentarius, 2003; Goodman *et al.,* 2008; Goodman and Tetteh, 2011). The searches were performed on May 3, 2018.

UDP-glucosyltransferase EUGT11 was found to have greater than 35% identity with 1 toxin, ringhalexin (39% identity), and 1 virulence factor, ESAT-6 secretion system extracellular protein B (43% identity). The query cover for all ESAT-6 secretion system extracellular protein B sequences was only 6% of the sequence length and the corresponding E-values ranged between 2.0 to 8.3. Although the query cover for ringhalexin was higher at 39%, the corresponding E-value was also much higher at 9.0. Given the low query coverage and/or high E-values for these alignments, these results were not considered to share significant homology or structural similarity with UDP-glucosyltransferase EUGT11, indicating that the enzyme does not harbor any toxic potential (Pearson, 2000; Bushey *et al.,* 2014).

UDP-glucosyltransferase 76G1 was found to have greater than or equal to 35% identity with 2 toxins, phospholipase A1 (35% identity) and trehalase (40% identity), and 1 virulence factor, Histidine protein kinase 1 (41% identity). All sequence matches had low query coverages (≤8%) paired with high E-values (0.12 to 8.8). Therefore, the assessed proteins were not considered to share homology or structural similarity with any known animal venom proteins and toxins or virulence factors (Pearson, 2000; Bushey *et al.,* 2014).

6.4.4 Potential Allergenicity of the Enzymes

The potential allergenicity of the enzymes (UDP-glucosyltransferases EUGT11 and 76G1) was investigated using an *in silico* approach in which sequence homology searches were conducted according to the approach outlined by the FAO/WHO (2001) and the Codex Alimentarius (2009) using the AllergenOnline Database version 18B (available at http://www.allergenonline.org; updated March 23, 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2018). This was done to confirm that UDP-glucosyltransferase EUGT11 and UDP-glucosyltransferase 76G1 do not contain amino acid sequences similar to other known allergens that might produce an allergenic response. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. The searches were performed for UDP-glucosyltransferase EUGT11 and UDP-glucosyltransferase 76G1 on April 17, 2018.

Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen should be considered a possibility (FAO/WHO, 2001). No matches were identified from searching with the full amino acid sequence for UDP-glucosyltransferase EUGT11.

In addition, a second homology search was conducted according to the approach outlined by the FAO/WHO (2001) and the Codex Alimentarius (2009). In accordance with this guideline, the AllergenOnline database was searched using a sliding window of 80-amino acid sequences (segments 1-80, 2-81, 3-82, *etc*.) derived from the full- length UDP-glucosyltransferase EUGT11 and 76G1 amino acid sequences. The 80-amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Using this search strategy, no matches were identified for either enzyme. A third homology search conducted using the exact 8-mer approach also did not produce any matches.

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

Based on the information provided above, no evidence exists to suggest that the UDP-glucosyltransferases used in the enzymatic conversion of rebaudioside A to rebaudioside M would be associated with an allergenic response.

6.5 Expert Panel Evaluation

Sichuan Ingia has concluded that rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf meeting appropriate food-grade specifications and manufactured consistent with cGMP is GRAS for use as an ingredient in various food products, as described in Part 1.3, on the basis of scientific procedures. Sichuan Ingia's rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) manufactured by enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf is substantially equivalent to other steviol glycoside products currently on the U.S. market, including those extracted from the leaves of *S. rebaudiana.*

The GRAS status of the rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) is based on conclusions of scientific bodies and regulatory authorities regarding steviol glycoside safety, data generally available in the public domain pertaining to the safety of steviol glycosides, and a unanimous opinion among a panel of experts ('the Expert Panel'), who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of the following qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley M. Tarka Jr., Ph.D. (The Tarka Group Inc., and The Pennsylvania State University, College of Medicine).

The Expert Panel, convened by Sichuan Ingia, independently and critically evaluated all data and information presented herein, and concluded that rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced by enzymatic conversion of a high-purity rebaudioside A (*i.e.,* RM95) is GRAS for use as a general purpose sweetener, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the Expert Panel and evaluation of such data as it pertains to the proposed GRAS uses of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M), are presented in Appendix A.

6.6 Conclusions

Based on the data and information presented herein, Sichuan Ingia has concluded that rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced by enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf, meeting appropriate food-grade specifications, and manufactured according to cGMP, is safe for use as a general purpose sweetener as presented in Section 1.3. Sichuan Ingia also has further concluded that pivotal data and information relevant to the safety of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) produced by enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf are publicly available and therefore the intended uses of rebaudioside M-rich steviol glycoside preparation (≥95% rebaudioside M) can be concluded to be GRAS on the basis of scientific procedures.

Part 7. §170.255 List of Supporting Data and Information

Ahmad M, Hirz M, Pichler H, Schwab H (2014). Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98(12):5301-5317. DOI:10.1007/s00253-014-5732-5.

Al-Dujaili EAS, Twaij H, Bataineh YA, Arshad U, Amjid F (2017). Effect of stevia consumption on blood pressure, stress hormone levels and anthropometrical parameters in healthy persons. Am J Pharmacol Toxicol 12(1):7-17. DOI:10.3844/ajptsp.2017.7.17.

Aze Y, Toyoda K, Imaida K, Hayashi S, Imazawa T, Hayashi Y, et al. (1991). [Subchronic oral toxicity study of stevioside in F344 rats]. Eisei Shikenjo Hokoku [Bull Natl Inst Hyg Sci] (109):48-54 [Japanese, English abstract].

Blumenthal M (1995). FDA lifts import alert on stevia. Herb can be imported only as dietary supplement; future use as a sweetener is still unclear. HerbalGram (35 - Fall):17-18.

Bushey DF, Bannon GA, Delaney B, Graser G, Hefford M, Jiang X, et al. (2014). Characteristics and safety assessment of intractable proteins in genetically modified crops. Regul Toxicol Pharmacol 69(2):154-170. DOI:10.1016/j.yrtph.2014.03.003.

Chang PY, Fong MY, Nissapatorn V, Lau YL (2011). Evaluation of *Pichia pastoris*-expressed recombinant rhoptry protein 2 of Toxoplasma gondii for its application in diagnosis of toxoplasmosis. Am J Trop Med Hyg 85(3):485-489 [erratum, 85(4):790]. DOI:10.4269/ajtmh.2011.11-0351.

Codex Alimentarius (2003). Appendix III, Draft Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants and Appendix IV, Annex on the assessment of possible allergenicity. In: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy 30 June-5 July, 2003 / / Report of the Third Session of the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology, Yokohama, Japan 4-8 March 2002. (Alinorm 03/34). Rome, Italy: Codex Alimentarius Commission, pp. 47-60. Available at: http://www.biosafety.be/ARGMO/Documents/codex.pdf.

Curry LL, Roberts A (2008). Subchronic toxicity of rebaudioside A. Food Chem Toxicol 46(7, Suppl.):S11-S20. DOI:10.1016/j.fct.2008.04.042.

Curry LL, Roberts A, Brown N (2008). Rebaudioside A: two-generation reproductive toxicity study in rats. Food Chem Toxicol 46(Suppl. 7):S21-S30. DOI:10.1016/j.fct.2008.05.005.

De Schutter K, Lin Y-C, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, et al. (2009) Genome sequence of the recombinant protein production host Pichia pastoris. Nat Biotechnol 27:561–566. doi:10.1038/nbt.1544.

EFSA (2010). EFSA Panel on Food Additives and Nutrient Sources scientific opinion on safety of steviol glycosides for the proposed uses as a food additive. (Question number: EFSA-Q-2007-071; EFSA-Q-2008-387; EFSA-Q-2008-401, adopted on 10 March 2010 by European Food Safety Authority). EFSA J 8(4):1537. [85 pp]. DOI:10.2903/j.efsa.2010.1537. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/1537.

EFSA (2015). Scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2014-00002, adopted on 17 November 2015 by European Food Safety Authority). EFSA J 13(12):4316 [28 pp]. DOI:10.2903/j.efsa.2015.4316. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4316.

EFSA (2017). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 5: suitability of taxonomic units notified to EFSA until September 2016 (EFSA Panel on Biological Hazards/BIOHAZ) (Question Number: EFSA-Q-2016-00018, adopted: 30 November 2016 by European Food Safety Authority, updated: 6 June 2017, corrected: 12 September 2017). EFSA J 15(3):4663. [20pp]. DOI:10.2903/j.efsa.2017.4663. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4663.

EU (2012). Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. Off J Eur Union 55(L83):1-295. Available at: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32012R0231 [Consolidated Version: 18/08/2017].

EU (2016). Commission Regulation (EU) 2016/1814 of 13 October 2016 amending the Annex to Regulation (EU) No 231/2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as regards specifications for steviol glycosides (E 960). Off J Eur Union 59(L278):37-41. Available at: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=uriserv:OJ.L_.2016.278.01.0037.01.ENG.

FAO (2016). *Steviol Glycosides: Chemical and Technical Assessment*. (*82nd JECFA - Chemical and Technical Assessment/CTA)*. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/3/a-br566e.pdf [Last accessed Oct. 4, 2017].

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en/.

FARRP (2017). *AllergenOnline Version 17: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org/ [Released: January 18, 2017].

Ferlow K (2005). Stevia - The sweetest substance on Earth. NutraCos 4(2, Suppl.):10-11.

FSANZ (2008). *Final Assessment Report: Application A540 Steviol Glycosides as Intense Sweeteners*. Canberra, Australia: Food Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/applications/documents/FAR_A540_Steviol_glycosides.pdf

FSANZ (2017). Schedule 3 — Identity and purity (F2017C00737). In: *Australia New Zealand Food Standard Code. (Food Standards Australia New Zealand)*. (Federal Register of Legislation). Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: https://www.legislation.gov.au/Details/F2017C00737 [As of Sep. 14, 2017].

Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P (2003). Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. J Agric Food Chem 51(22):6618-6622. DOI:10.1021/jf0303619.

Geuns JMC (2003). Stevioside. Phytochemistry 64(5):913-921. DOI:10.1016/S0031-9422(03)00426-6.

Geuns JMC, Pietta, P (2004) [unpublished]. *Stevioside Mtabolism by Human Volunteers*. Report from Segrate (MI), Italy: Laboratory Functional Biology, Kuleuven, Leuven Belgium and ITB-CNR. Submitted to WHO by Belgium: Federal Ministry of Social Affairs, Public Health and the Environment. Cited In: JECFA, 2006a.

Geuns JM, Augustijns P, Mols R, Buyse JG, Driessen B (2003). Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem Toxicol 41(11):1599-1607. DOI:10.1016/S0278-6915(03)00191-1.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM, Compernolle F, Toppet S (2006). Identification of steviol glucuronide in human urine. J Agric Food Chem 54(7):2794-2798. DOI:10.1021/jf052693e.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM (2007). Metabolism of stevioside by healthy subjects. Exp Biol Med 232(1):164-173.

Goodman RE, Tetteh AO (2011). Suggested improvements for the allergenicity assessment of genetically modified plants used in foods. Curr Allergy Asthma Rep 11(4):317-324. DOI:10.1007/s11882-011-0195-6.

Goodman RE, Vieths S, Sampson HA, Hill D, Ebisawa M, Taylor SL, et al. (2008). Allergenicity assessment of genetically modified crops--what makes sense? [Includes letter to the editor from Rob C Aalberse and response from Richard E Goodman et al.]. Nat Biotechnol 26(1):73-81 [erratum, 26(2):241]. DOI:10.1038/nbt1343.

Health Canada (2012). *Information and Consultation Document on Health Canada's Proposal to Allow the Use of the Food Additive Steviol Glycosides as a Table-Top Sweetener and as a Sweetener in Certain Food Categories.* Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: http://www.hc-sc.gc.ca/fn-an/consult/steviol/document-consultation-eng.php [Date modified: 2012-11-30].

Health Canada (2016). *Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Rebaudioside M as a Sweetener in Various Unstandardized Foods*. (Reference Number: NOM/ADM-0065). Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: http://www.hc-sc.gc.ca/fn-an/consult/nom-adm-0065/index-eng.php [Date Modified: 2016-01-15].

Health Canada (2017). *Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Steviol Glycosides from Stevia rebaudiana Bertoni as a Sweetener*. (Reference Number: NOM/ADM-0102). Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/acts-regulations/modification-list-permitted-sweeteners-steviol-glycosides.html [Date Modified: 2017-08-29].

Hutapea AM, Toskulkao C, Buddhasukh D, Wilairat P, Glinsukon T (1997). Digestion of stevioside, a natural sweetener, by various digestive enzymes. J Clin Biochem Nutr 23(3):177-186. DOI:10.3164/jcbn.23.177.

JECFA (1999). Stevioside. In: *Safety Evaluation of Certain Food Additives.* 51st Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 9-18, 1998. (WHO Food Additives Series, no 42). Geneva, Switz.: World Health Organization (WHO) / International Programme on Chemical Safety (IPCS), pp. 119-143. Available at: http://www.inchem.org/documents/jecfa/jecmono/v042je07.htm.

JECFA (2006a). Steviol glycosides. In: *Safety Evaluation of Certain Food Additives*. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives, June 8-17, 2004, Geneva, Switz. (WHO Food Additives Series, no 54). Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS), pp. 117-144, 638. Available at: http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

JECFA (2006b). Steviol glycosides (Tentative) [New tentative specifications prepared at the 63rd JECFA (2004), published in FNP 52 Add 12 (2004)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 65th JECFA Meetings, 1956-2005. (FAO JECFA Monographs 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/.

JECFA (2007a). Steviol glycosides. In: *68th JECFA - Chemical and Technical Assessment (CTA)*. [Sixty-eighth meeting held June 17-26, 2008]. (Revised by Paul M. Kuznesof, Ph.D., Original prepared by Harriet Wallin for the 63rd JECFA). Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/68/Steviol_glycosides.pdf.

JECFA (2007b). Steviol glycosides [Prepared at the 68th JECFA (2007) and published in FAO JECFA Monographs 4 (2007), superseding tentative specifications prepared at the 63rd JECFA (2004)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 68th Meeting, June 19-28, 2007, Geneva, Switz. (FAO JECFA Monographs 4). Rome, Italy: Food and Agriculture Organization of the United Nations. Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA), pp. 61-64. Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/monograph4/additive-442-m4.pdf.

JECFA (2009). Steviol glycosides (addendum). In: *Safety Evaluation of Certain Food Additives and Contaminants*. Sixty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 17-26-29, 2008, Geneva, Switz. (WHO Food Additives Series, vol 60). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 183-220. Available at: http://apps.who.int/iris/bitstream/10665/44063/1/9789241660600_eng.pdf.

JECFA (2010). Steviol glycosides [Prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4*. (FAO JECFA Monographs 10). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/monograph10/additive-442-m10.pdf.

JECFA (2016a). Steviol glycosides from Stevia *rebaudiana Bertoni* (tentative) [New specifications prepared at the 82nd JECFA, 2016), Superseding specifications prepared at the 73rd JECFA (2010)]. In: *Compendium of Food Additive Specifications*. 82nd Meeting, June 7-16, Geneva, Switz. (FAO JECFA Monographs 19). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 106-111. Available at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/detail/en/c/467/.

JECFA (2016b). Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica* [Prepared at the 82nd JECFA, 2016). In: *Combined Compendium of Food Additive Specifications*. 82nd Meeting, June 7-16, Geneva, Switz. (FAO JECFA Monographs 19). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 91-95. Available at: http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/detail/en/c/548/.

JECFA (2017a). 3.2. Revision of specifications and analytical methods. 3.2.4 Steviol glycosides. In: *Evaluation of Certain Food Additives*. Eighty-fourth Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 5-16, 2017, Rome, Italy. (WHO Technical Report Series no 1007). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 67, 90. Available at: http://www.who.int/foodsafety/publications/jecfa-reports/en/; http://apps.who.int/iris/bitstream/10665/259483/1/9789241210164-eng.pdf?ua=1.

JECFA (2017b). Steviol glycosides. In: *Joint FAO/WHO Expert Committee on Food Additives 84th Meeting: Summary and Conclusions*, June 7-15, 2017, Geneva. (JECFA/84/SC). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/JECFA_84_Summary_Report.pdf?ua=1.

Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, et al. (2003a). Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem Toxicol 41(6):875-883. DOI:10.1016/S0278-6915(03)00039-5.

Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, et al. (2003b). In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem Toxicol 41(3):359-374. DOI:10.1016/S0278-6915(02)00235-1.

Kraemer T, Maurer HH (1994). On the metabolism of the sweetener stevioside in humans. Eur J Pharm Sci 2(1/2):103 [abstract FC12]. DOI:10.1016/0928-0987(94)90121-X.

Lee SK, Lee KR, Park JR, Kim KS, Tchai BS (1979). [A study on the safety of stevioside as a new sweetening source]. Hanguk Sikpum Kwahakhoe Chi [Kor J Food Sci Technol] 11(4):224-231 [Korean, English abstract].

Maki KC, Curry LL, Reeves MS, Toth PD, McKenney JM, Farmer MV et al. (2008a). Chronic consumption of rebaudioside A, a steviol glycoside, in men and women with type 2 diabetes mellitus. Food Chem Toxicol 46(Suppl. 7):S47-S53. DOI:10.1016/j.fct.2008.05.007.

Maki KC, Curry LL, Carakostas MC, Tarka SM, Reeves MS, Farmer MV et al. (2008b). The hemodynamic effects of rebaudioside A in healthy adults with normal and low-normal blood pressure. Food Chem Toxicol 46(Suppl. 7):S40-S46. DOI:10.1016/j.fct.2008.04.040.

Nakayama K, Kasahara D, Yamamoto F (1986). Absorption, distribution, metabolism and excretion of stevioside in rats. Shokuhin Eiseigaku Zasshi 27(1):1-8. DOI:10.3358/shokueishi.27.1.

Nikiforov AI, Eapen AK (2008). A 90-day oral (dietary) toxicity study of rebaudioside A in Sprague-Dawley rats. Int J Toxicol 27(1):65-80.

Noosud J, Lailerd N, Kayan A, Boonkaewwan C (2017). *In vitro* and *in vivo* assessment of inhibitory effect of stevioside on pro-inflammatory cytokines. Avicenna J Phytomed 7(2):101-106. DOI:10.22038/AJP.2016.6599.

Oehme A, Wüst M, Wölwer-Rieck U (2017). Steviol glycosides are not altered during commercial extraction and purification processes. Int J Food Sci Technol 52(10):2156-2162. DOI:10.1111/ijfs.13494.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Pearson WR (2000). Flexible sequence similarity searching with the FASTA3 program package. In: Misener S, Krawetz SA, editors. *Bioinformatics Methods and Protocols*. (Methods in Molecular Biology, vol 132). Totowa (NJ) / New York (NY): Humana Press / Springer, pp. 185-219.

Philippaert K, Pironet A, Mesuere M, Sones W, Vermeiren L, Kerselaers S, et al. (2017). Steviol glycosides enhance pancreatic beta-cell function and taste sensation by potentiation of TRPM5 channel activity. Nat Commun 8:14733 [16pp]. DOI:10.1038/ncomms14733.

Purkayastha S, Pugh G Jr, Lynch B, Roberts A, Kwok D, Tarka SM (2014). *In vitro* metabolism of rebaudioside B, D, and M under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol 68(2):259-268. DOI:10.1016/j.yrtph.2013.12.004.

Purkayastha S, Bhusari S, Pugh G Jr, Teng X, Kwok D, Tarka SM (2015). In vitro metabolism of rebaudioside E under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol 72(3):646-657. DOI:10.1016/j.yrtph.2015.05.019.

Purkayastha S, Markosayan A, Prakash I, Bhusari S, Pugh G Jr, Lynch B, et al. (2016). Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. Regul Toxicol Pharmacol 77:125-133. DOI:10.1016/j.yrtph.2016.02.015.

Renwick AG (2007). Toxicokinetics [section on elimination: excretion via the gut]. In: Hayes W, editor. *Principles and Methods of Toxicology, 5th edition*. Philadelphia (PA): Taylor and Francis/CRC Press, p. 188.

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

Renwick AG, Tarka SM (2008). Microbial hydrolysis of steviol glycosides. Food Chem Toxicol 46(7, Suppl.):S70-S74. DOI:10.1016/j.fct.2008.05.008.

Roberts A, Renwick AG (2008). Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. Food Chem Toxicol 46(Suppl. 7):S31-S39. DOI:10.1016/j.fct.2008.05.006.

Roberts A, Lynch B, Rogerson R, Renwick A, Kern H, Coffee M, et al. (2016). Chemical-specific adjustment factors (inter-species toxicokinetics) to establish the ADI for steviol glycosides. Regul Toxicol Pharmacol 79:91-102. DOI:10.1016/j.yrtph.2016.05.017.

SCF (1985). Reports of the Scientific Committee for Food concerning sweeteners (Opinion expressed by the SCF on 14 September 1984) [Stevioside]. In: *Food Science and Techniques*. (Reports of the Scientific Committee for Food, 16th series). Brussels, Belgium: Commission of the European Communities (EEC), Scientific Committee for Food (SCF), 2, 4, 7, 15, 20. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_reports_16.pdf.

SCF (1999). *Opinion on Stevioside as a Sweetener (Adopted on 17 June 1999)*. (CS/ADD/EDUL/167 Final). Brussels, Belgium: European Commission, Health & Consumer Protection Directorate-General, Scientific Committee on Food (SCF). Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_out34_en.pdf.

Sharif R, Chan KM, Ooi TC, Mohammad NF (2017). Cytotoxicity and genotoxicity evaluation of stevioside on CCD18Co and HCT 116 cell lines. Int Food Res J 24(1):341-345.

Simonetti P, Gardana C, Bramati L, Pietta PG (2004). Bioavailability of stevioside from Stevia rebaudiana in humans: preliminary report. In: Geuns JMC, Buyse J, editors. *Safety of Stevioside:* Proceedings of the First Symposium Sponsored by KULeuven, April 16, 2004, Leuven, Belgium. Heverlee, Belgium: Euprint ed., pp. 51-62.

Sung LH (2002) [unpublished]. *Report on Pharmacokinetic (PK) studies of T100 Sunstevia 95% Stevioside in Rats*. Report from Singapore: Sunlabel Pte Ltd. Submitted to WHO by the Ministry of Health and Welfare, Japan. Cited In: JECFA, 2006a.

Toyoda K, Matsui H, Shoda T, Uneyama C, Takada K, Takahashi M (1997). Assessment of the carcinogenicity of stevioside in F344 rats. Food Chem Toxicol 35(6):597-603. DOI:10.1016/S0278-6915(97)00023-9.

U.S. FDA (2006). *Agency Response Letter GRAS Notice No. GRN 000204 [Phospholipase C enzyme preparation from Pichia pastoris expressing a heterologous phospholipase C gene, San Diego (CA): Diversa Corporation].* College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=204 [Date of filing: Jun 9, 2006; Date of closure: Dec 5, 2006].

U.S. FDA (2017a). *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR.

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

Part	Section §	Section Title
173—Secondary Direct Food Additives Permitted in Food for Human Consumption	173.25	Ion-exchange resins
184—Direct Food Substances Affirmed as Generally Recognized as Safe	184.1293	Ethyl alcohol
	184.1426	Magnesium chloride
	184.1553	Peptones
	184.1751	Sodium citrate
	184.1857	Corn sugar
	184.1983	Bakers yeast extract
573—Food additives permitted in feed and drinking water of animals	573.750	Pichia pastoris dried yeast
582—Substances Generally Recognized as Safe	582.1751	General purpose food additives—Sodium citrate
	582.6751	Sequestrants—Sodium citrate

U.S. FDA. (2017b). *Agency Response Letter GRAS Notice No. GRN 000715 [Rebaudioside D, Rancho Santa Margarita (CA): Blue California]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=715 [Oct. 24, 2017].

Uçar A, Yilmaz S, Yilmaz S, Kiliç MS (2018). A research on the genotoxicity of stevia in human lymphocytes. Drug Chem Toxicol 41(4):221-224. DOI:10.1080/01480545.2017.1349135.

Wang LZ, Goh BC, Fan L, Lee HS (2004). Sensitive high- performance liquid chromatography/mass spectrometry method for determination of steviol in rat plasma. Rapid Commun Mass Spectrom 18(1):83-86. DOI:10.1002/rcm.1285.

Weinacker D, Rabert C, Zepeda AB, Figueroa CA, Pessoa A, Farías JG (2013). Applications of recombinant *Pichia pastoris* in the healthcare industry. Braz J Microbiol 44(4):1043-1048. DOI:10.1590/S1517-83822013000400004.

Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X, et al. (2008). Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem Toxicol 46(Suppl. 7):S54-S60. DOI:10.1016/j.fct.2008.04.041.

WHO/FAO (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. Geneva, Switz.: World Health Organization (WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Williams LD, Burdock GA (2009). Genotoxicity studies on a high-purity rebaudioside A preparation. Food Chem Toxicol 47(8):1831-1836.

Wingard RE, Brown JP, Enderlin FE, Dale JA, Hale RL, Seitz CT (1980). Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. Experientia 36(5):519-520. DOI:10.1007/BF01965774.

Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of a Rebaudioside M-Rich (≥95% Rebaudioside M) Steviol Glycoside Preparation (RM95) for Use as a General Purpose Sweetener

1 June 2018

INTRODUCTION

Sichuan Ingia Biosynthetic Co., Ltd. (Sichuan Ingia) intends to market a rebaudioside M-rich (≥95% rebaudioside M) steviol glycoside preparation (RM95), produced *via* enzymatic bioconversion of high-purity rebaudioside A (≥95% rebaudioside A) extracted from *Stevia rebaudiana* (*S. rebaudiana*) Bertoni to rebaudioside M using enzymes derived from genetically modified strains of yeast, as a general purpose sweetener in the United States (U.S.). Rebaudioside M is a minor steviol glycoside that is naturally present in the leaves of *S. rebaudiana* Bertoni. Rebaudioside M is typically obtained from hot water extraction of the leaves of *S. rebaudiana* Bertoni, however, Sichuan Ingia has developed an alternative manufacturing process to produce high-purity rebaudioside M. The alternative manufacturing process utilizes two uridine-5'-diphosphate (UDP)-glucosyltransferase enzymes derived from genetically modified strains of *Pichia pastoris* (*P. pastoris*) that converts high-purity rebaudioside A that is extracted and purified from the leaves of *S. rebaudiana* Bertoni to rebaudioside M. The manufacturing process used by Sichuan Ingia to produce RM95 is similar to that of other enzymatic bioconversion processes to produce steviol glycosides, specifically rebaudioside M and rebaudioside D (GRN 667 and 715, respectively), which have received "no questions" by the U.S. Food and Drug Administration (FDA) with respect to their Generally Recognized as Safe (GRAS) status. When manufactured as described, the final rebaudioside M-rich (≥95% rebaudioside M) steviol glycoside preparation meets or exceeds the ≥95% steviol glycoside purity criteria established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC).

At the request of Sichuan Ingia, an Expert Panel of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether, under the conditions of intended use as a sweetening agent, RM95 would be GRAS, based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley M. Tarka Jr., Ph.D. (The Tarka Group Inc., and The Pennsylvania State University, College of Medicine). For purposes of the Expert Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined by the U.S. Food and Drug Administration (FDA) in 21 CFR 170.3(i) (U.S. FDA, 2017a).

The Expert Panel independently and collectively evaluated a dossier entitled "*Documentation Supporting the Evaluation of a Rebaudioside M-Rich (≥95% Rebaudioside M) Steviol Glycoside Preparation (RM95) as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener*" that included a comprehensive summary of scientific information on RM95. This dossier was prepared with information available in the public domain and also included details pertaining to the manufacturing method, product specifications and supporting batch analyses, intended uses and use-levels in food and beverages, consumption estimates for all intended uses,

and a summary of the scientific literature pertaining to the safety of steviol glycosides. The Expert Panel also evaluated other information deemed appropriate or necessary.

Following their independent and critical evaluation of such data and information, the Expert Panel convened on 1 June 2018 *via* teleconference and unanimously concluded that the intended use described herein for RM95, meeting appropriate food-grade specifications as described in the supporting dossier and manufactured according to current Good Manufacturing Practice (cGMP), is safe, suitable, and GRAS based on scientific procedures. A summary of the basis of the Expert Panel's conclusion is presented below.

CHEMISTRY AND MANUFACTURING

The ingredient that is the subject of this GRAS evaluation is a rebaudioside M-rich steviol glycoside preparation that is comprised of ≥95% rebaudioside M, which is consistent with the purity criteria for steviol glycosides as established by JECFA (JECFA, 2017a). The remaining 5% of the preparation may include additional steviol glycosides containing sugar moieties of glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, and/or galactose conjugated to the steviol backbone in any combination or orientation.

All raw materials, processing aids, and equipment used in the manufacture of RM95 are food-grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses. Sichuan Ingia's RM95 is produced *via* an enzymatic bioconversion process using strains of *P. pastoris* that have been genetically modified to express the UDP-glucosyltransferases, EUGT11 and 76G1. In the first stage of manufacturing, the starting material, a steviol glycoside primary extract containing ≥95% rebaudioside A, is produced and purified according to the methodology outlined in the Chemical and Technical Assessment (CTA) published by FAO/JECFA for steviol glycosides (FAO, 2016). In the next stage, the *P. pastoris* production strains carrying the expression vector encoding for the enzymes required for the enzymatic bioconversion reaction (EUGT11 and 76G1) are subjected to a fermentation step to express the UDP-glucosyltransferases. The production strains are mixed with the steviol glycoside primary extract in order for the enzymatic bioconversion reaction to proceed to generate a crude rebaudioside M solution. The EUGT11 and 76G1 enzymes catalyze the reaction in which the high-purity rebaudioside A is converted to rebaudioside M. In the last stage of manufacturing, the crude rebaudioside M solution that was generated in the previous stage is purified and concentrated according to the methodology described in the CTA for steviol glycosides, yielding a final product that contains ≥95% rebaudioside M.

The Expert Panel also reviewed information pertinent to the construction of the production strains used to generate the UDP-glucosyltransferases and noted that the inserted gene sequences encoding for EUGT11 and 76G1 were obtained from plant sources (*i.e., Oryza sativa* Japonica and *S. rebaudiana* Bertoni, respectively). These plant sources are not associated with any known allergens or toxins. In addition, the Expert Panel noted that the parental strain, *Pichia pastoris* ATCC 20864, is non-pathogenic and non-toxigenic and is commonly used in the food industry.

Sichuan Ingia has established product specifications (physical, chemical, and microbiological) for RM95 based on those established by JECFA for steviol glycosides from *S. rebaudiana* Bertoni. Total steviol glycoside content

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

is measured using the high-performance liquid chromatography (HPLC) method described in the JECFA specification monograph for steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2017a).

The Expert Panel reviewed data for 5 separate lots of RM95 and confirmed that the final product complies with the established product parameters. Pesticide residue analysis was available for 1 lot of RM95, the results of which demonstrate the absence of commonly used pesticides in the final product. Residual protein was not detected in 3 non-consecutive batches of RM95 using the bicinchoninic acid (BCA) assay, demonstrating successful removal of any enzyme or production strains in the final product.

Sichuan Ingia conducted a series of stability tests on RM95, including short-term (10 days), accelerated (6 months), and long-term stability (36 months with results available through 9 months). The results of these stability tests demonstrate that RM95 is stable under different storage conditions (*i.e.,* different temperature, humidity, and illumination conditions) for up to 9 months when kept in commercial packaging. These conclusions are consistent with those of JECFA in which it was determined that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions (JECFA, 2007).

INTENDED FOOD USES AND ESTIMATED INTAKE

Sichuan Ingia's RM95 is intended for use as a general purpose sweetener that will be added to a variety of food and beverage products, consistent with the current uses of other related high-intensity sweeteners that are currently on the U.S. market. The estimated intakes of RM95 were calculated for adults and children based on post-market surveillance data for other high-intensity sweeteners and by adjusting these data for the relative sweetness intensity of RM95 (*i.e.,* approximately 300 times sweeter than sucrose). The results are shown in Table 1. For the average consumer, the mean intake of RM95 across all groups was predicted to range from 0.85 mg/kg body weight/day for non-diabetic adults to 2.24 mg/kg body weight/day for diabetic children, equivalent to 0.21 to 0.55 mg/kg body weight/day as steviol equivalents, respectively. For high consumers, the mean intake of RM95 across all groups was predicted to range from 2.25 mg/kg body weight for non-diabetic adults to 3.30 mg/kg body weight/day for non-diabetic children. These intake values are equivalent to 0.55 and 0.81 mg steviol equivalents/kg body weight/day for non-diabetic adults and children, respectively. It should be noted that the highest intake estimate for RM95 of 3.30 mg/kg body weight/day, equivalent to 0.81 mg/kg body weight/day as steviol equivalents, under the proposed conditions of use, is below the current Acceptable Daily Intake (ADI) for steviol glycosides of 0 to 4 mg/kg body weight, expressed as steviol, as established by JECFA (2010). JECFA recently re-assessed the dietary exposure to steviol glycosides using the sugar/intense sweetener substitution method and determined consumption estimates ranging from 0.4 to 7.2 mg/kg body weight/day, expressed as steviol equivalents, and made note that this method overestimates dietary exposure (FAO, 2016).

Table 1 **Estimated Consumption of RM95 Using Renwick's (2008) Methodology of Intense Sweetener Intake Assessment Based on Post-Market Surveillance Intake Data for Currently Used Sweeteners**

Population Group	Intakes of intense sweeteners (expressed as sucrose equivalents) (mg/kg bw/day)		Consumption estimates for:			
			RM95[a] (mg/kg bw/day)		RM95 as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	0.85	2.25	0.21	0.55
Diabetic Adults	280	897	0.93	2.99	0.23	0.74
Non-diabetic Children	425	990	1.42	3.30	0.35	0.81
Diabetic Children	672	908	2.24	3.03	0.55	0.74

bw = body weight; RM95 = rebaudioside M-rich (≥95% rebaudioside M) steviol glycoside preparation.

[a] RM95 is approximately 300 times as sweet as sucrose.

[b] Calculated based on the molecular weights of steviol (318.45 g/mol) and rebaudioside M of 1,291 g/mol [steviol conversion factor of 0.25].

INFORMATION TO ESTABLISH SAFETY

The Expert Panel reviewed the available data supporting the safety of steviol glycosides in order to evaluate the safety of RM95. The available data included a detailed discussion on the metabolic fate of steviol glycosides, a summary of the conclusions of several global scientific and regulatory authorities/bodies regarding the safety of steviol glycosides, and other data deemed pivotal in determining the safety. The Expert Panel also reviewed information regarding the safety of the production strains used to produce the enzymes required for the enzymatic conversion process, including an *in silico* assessment of the potential allergenicity, toxigenicity, and virulence of the EUGT11 and 76G1 enzymes.

Due to the presence of β-glycosidic bonds, steviol glycosides are not hydrolyzed in the upper gastrointestinal tract and therefore are not absorbed; the unchanged steviol glycosides enter the colon intact and are subject to degradation by microbes of the *Bacteriodaceae* family, resulting in the release of the aglycone steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Geuns *et al*., 2003, 2007; Renwick and Tarka, 2008; Nikiforov *et al*., 2013; Purkayastha *et al*., 2016). The rate at which steviol glycosides are metabolized in the colon is dependent on their structural complexity since steviol glycosides are hydrolyzed sequentially, in which one sugar moiety is removed at a time (Wingard *et al*., 1980; Koyama *et al*., 2003b). However, despite any differences in chemical structure, relatively similar hydrolysis rates are reported for different steviol glycosides to steviol, as supported by *in vitro* metabolic studies with human fecal homogenates, particularly during the first 24 hours of incubation (Purkayastha *et al.,* 2014, 2015, 2016). Following microbial degradation, systemic absorption of the steviol metabolite into the portal vein and distribution to the liver, spleen, adrenal glands, fat, and blood has been reported (Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and Renwick, 2008). In the liver, steviol glucuronide is formed by conjugation of steviol with glucuronic acid. The steviol glucuronide metabolite and any unconjugated steviol of the administered glycosides are excreted primarily in the urine, and, to a lesser extent, feces in humans (Wingard *et al*., 1980; Nakayama *et al*., 1986; Kraemer and Maurer, 1994; Sung, 2002 [unpublished]; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). Therefore, due to the similar metabolic fate of steviol glycosides, the safety database that has been established for individual steviol glycosides (*e.g.,* stevioside, rebaudioside A, rebaudioside D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless

of the steviol glycoside distribution of the preparation, including RM95 produced by enzymatic bioconversion of steviol glycosides.

Steviol glycosides and their safety have been extensively reviewed by various scientific and regulatory authorities/bodies, such as JECFA, U.S. FDA, Food Standards Australia New Zealand (FSANZ), the European Commission's Scientific Committee on Food (SCF), the European Food Safety Authority (EFSA), and Health Canada (SCF, 1985, 1999; FSANZ, 2008, 2017; EFSA, 2010, 2015, 2018a,b; Health Canada, 2012, 2017; JECFA, 2006, 2017a,b). These scientific bodies and regulatory agencies have consistently concluded that consumption of steviol glycosides is not a safety concern. Based on the available data, the aforementioned scientific bodies and regulatory agencies have established an ADI of 0 to 4 mg/kg body weight, expressed as steviol equivalents, for steviol glycosides based on 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 the results of the carcinogenicity study in rats by Toyoda *et al.* (1997), and following application of a 100-fold safety factor for inter- and intra-species differences (JECFA, 2009; FSANZ, 2008; EFSA, 2010; Health Canada, 2012). Recently, EFSA concluded that safety studies conducted with individual steviol glycosides rebaudioside A and stevioside can be extended to other steviol glycosides due to their shared metabolic fate (EFSA, 2015). EFSA specifically concluded that "*extending the current specifications to include [two additional steviol glycosides], rebaudiosides D and M, as alternatives to Reb A in the predominant components of steviol glycosides would not be of safety concern*" (EFSA, 2015). JECFA, FSANZ, and Health Canada recently expanded the definition of steviol glycosides to include all individual steviol glycosides present in the *S. rebaudiana* Bertoni leaf (FSANZ, 2017; Health Canada, 2017; JECFA, 2017a,b). In a recent evaluation in response to a proposed amendment of the specifications of steviol glycosides, EFSA concluded that the available data were not sufficient to expand the definition of steviol glycosides to include all individual steviol glycosides due to their questions regarding uncertainties in the rate and extent of the metabolism of the different steviol glycosides to steviol (EFSA, 2018a). Specifically, there were uncertainties in the evidence provided to EFSA on the rate and extent of the metabolism of different steviol glycosides to steviol that did not allow the EFSA panel to conclude that they agreed with the applicant's amendment request. Likewise, in another recent evaluation of glucosylated steviol glycosides, EFSA concluded that the data provided were not sufficient to assess the safety of glucosylated steviol glycosides due to the limited evidence provided. Specifically, the lack of availability of data to support the complete hydrolysis of glucosylated steviol glycosides under what the Panel considered extreme hydrolysis conditions resulted in their determination that the metabolic fate data for steviol glycosides cannot be used in a read-across approach to apply to glucosylated steviol glycosides (EFSA, 2018b). Also, the EFSA panel noted that no toxicological studies on glucosylated steviol glycoside preparations that were under evaluation were provided for its assessment. EFSA's conclusions on glucosylated steviol glycosides do not apply to Sichuan Ingia's RM95 as the material is not a glucosylated steviol glycoside; furthermore, EFSA has previously evaluated the safety of rebaudioside M and based upon their conclusion that there are no safety concerns rebaudioside M is now listed in EFSA's steviol glycosides specifications (EFSA, 2015).

It should also be noted that in addition to the described safety evaluations, the U.S. FDA has reviewed the safety of over 50 different steviol glycoside preparations (including stevioside, and rebaudiosides A, C, D, and X/M) and have consistently raised no objections regarding the GRAS status of steviol glycosides for use as general purpose sweeteners in food and beverage products. Of note, GRNs 667 and 715, which pertain to rebaudioside M and rebaudioside D, respectively, received "no questions" from the U.S. FDA regarding the GRAS status of rebaudioside M and rebaudioside D produced by enzymatic bioconversion for use as a sweetener in foods (U.S. FDA, 2017b,c). Similar to Sichuan Ingia's RM95, the steviol glycosides, specifically rebaudioside M and rebaudioside D, described in GRNs 667 and 715 are also produced *via* enzymatic conversion of purified stevia leaf extract using UDP-glucosyltransferases that are derived from genetically modified *P. pastoris*.

A comprehensive search of the scientific literature was conducted through May 2018 to identify publications relevant to the safety of steviol glycosides that became available following the U.S. FDA review of GRN 744[2]. The studies identified included 1 reproductive toxicity study (Ahmad *et al.,* 2018) and 1 human study (Ghaheri *et al.,* 2018). The results of these recent studies provide further support for the safety of steviol glycosides, and therefore, the safety of RM95 is supported by the existing safety database on steviol glycosides and the conclusions of a number of authoritative scientific bodies (*e.g.,* JECFA, EFSA, FSANZ, U.S. FDA, and Health Canada).

The production strain, *P. pastoris*, is derived from *P. pastoris* ATCC 20864, which has an extensive history of use in food processing and in the biopharmaceutical industry to produce recombinant proteins. Dried *P. pastoris* is a permitted food additive in feed formulations for broiler chickens under 21 CFR §573.750 (U.S. FDA, 2017d), and a phospholipase C enzyme preparation derived from *P. pastoris* has GRAS status for use in foods in the U.S. (U.S. FDA, 2006). *P. pastoris* is non-pathogenic and non-toxigenic, and is not associated with any known human or animal disease. EFSA has granted *P. pastoris* qualified presumption of safety (QPS) status for use in enzyme production in the European Union (EFSA, 2017).

The Expert Panel reviewed potential allergenicity, toxigenicity and virulence of the EUGT11 and 76G1 enzymes, using an *in silico* approach. Using the Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information, a sequence alignment query of the EUGT11 FASTA protein sequence was conducted against protein sequences obtained from a curated database of venom proteins and toxins and virulence factors. EUGT11 was found to have greater than 35% identity with 1 toxin, ringhalexin (39% identity) and 1 virulence factor, ESAT-6 secretion system extracellular protein B (43% identity), however, given the low query coverage and/or high E-values for these alignments, these results were not considered to share significant homology or structural similarity with EUGT11, indicating that the enzyme does not harbor any toxic potential (Pearson, 2000; Bushey *et al.,* 2014). 76G1 was found to have greater than 35% identity with 2 toxins, phospholipase A1 (35% identity) and trehalase (40% identity), and 1 virulence factor, Histidine protein kinase 1 (41% identity); however, due to the low query coverage paired with high E-values for these alignments, these results were not considered to share significant homology or structural similarity with 76G1, indicating that the enzyme does not harbor any toxic potential (Pearson, 2000; Bushey *et al.,* 2014). The potential for allergenic cross-reactivity also was investigated in accordance with the FAO/WHO protocol for allergenicity assessment (FAO/WHO, 2001) and Codex Alimentarius (2009) using the AllergenOnline Database Version 18B (FARRP, 2018). The database contains a comprehensive list of putative allergenic proteins developed *via* a peer-reviewed process for the purpose of evaluating food safety. No structural similarity greater than 35% to known allergen sequences was identified for either EUGT11 or 76G1, indicating the unlikely potential for cross-reactivity to any known allergens. The safety of the glucosyltransferases derived from the *P. pastoris* production strains were evaluated using the Pariza and Johnson (2001) decision tree for evaluating the safety of microbially-derived food enzymes. It is noted that the RM95 produced by enzymatic bioconversion of rebaudioside A meets the established JECFA specifications, and that the enzymes are absent in the final product. Furthermore, given that the manufacturing process includes a heat-kill treatment step and filtration steps to produce a high-purity final product, and analytical data demonstrate the absence of residual protein that could carry over from the enzymatic bioconversion step, the Expert Panel concluded that the potential allergenicity of the inserted gene sequence would not be a health concern.

[2] At the time of the Expert Panel's evaluation, GRN 744 was the most recent GRAS notice reviewed by the U.S. FDA to receive a "no questions" letter.

The scientific evidence reviewed by the Expert Panel demonstrates that under the conditions of intended use, RM95 would not produce any adverse health effects.

CONCLUSION

We, the members of the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that RM95, meeting appropriate food grade specifications and produced in according with current good manufacturing practice (cGMP), is safe for use as a general purpose sweetener in foods and beverages.

We further unanimously conclude that the proposed use of Sichuan Ingia's RM95 meeting appropriate food grade specifications, as presented in the supporting dossier and produced consistent with current Good Manufacturing Practices (cGMP) is Generally Recognized as Safe (GRAS) under its intended conditions of use as a general purpose sweetener in food and beverages based on scientific procedures.

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

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REFERENCES

Bushey DF, Bannon GA, Delaney B, Graser G, Hefford M, Jiang X, et al. (2014). Characteristics and safety assessment of intractable proteins in genetically modified crops. Regul Toxicol Pharmacol 69(2):154-170. DOI:10.1016/j.yrtph.2014.03.003.

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. Geneva, Switz.: World Health Organization (WHO), / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

EFSA (2010). EFSA Panel on Food Additives and Nutrient Sources scientific opinion on safety of steviol glycosides for the proposed uses as a food additive. (Question number: EFSA-Q-2007-071; EFSA-Q-2008-387; EFSA-Q-2008-401, adopted on 10 March 2010 by European Food Safety Authority). EFSA J 8(4):1537. [85 pp]. DOI:10.2903/j.efsa.2010.1537. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/1537.

EFSA (2015). Scientific opinion on the safety of the proposed amendment of the specifications for steviol glycosides (E 960) as a food additive. (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2014-00002, adopted on 17 November 2015 by European Food Safety Authority). EFSA J 13(12):4316 [28 pp]. DOI:10.2903/j.efsa.2015.4316. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4316.

EFSA (2017). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 6: suitability of taxonomic units notified to EFSA until March 2017 (EFSA Panel on Biological Hazards/BIOHAZ) (Question no EFSA-Q-2016-00827, adopted: 7 June 2017 by European Food Safety Authority) EFSA J 15(7):4884 [32pp]. DOI:10.2903/j.efsa.2017.4884. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4884.

EFSA (2018a). Scientific Opinion on the safety of the proposed amendment of the specifications of the food additive steviol glycosides (E 960) (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2017-00036, adopted: 15 March 2018 by European Food Safety Authority). EFSA J 16(3):5236 [11 pp.]. DOI:10.2903/j.efsa.2018.5236. Available at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5236.

EFSA (2018b). Scientific opinion on the safety in use of glucosylated steviol glycosides as a food additive in different food categories (EFSA Panel on Food Additives and Nutrient Sources Added to Food/ANS) (Question no EFSA-Q-2017-00247, adopted: dd Month 2018 by European Food Safety Authority). EFSA J 16(2):5181 [11 pp.]. DOI:10.2903/j.efsa.2018.5181. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5181.

FAO (2016). Steviol glycosides. In: *82nd JECFA - Chemical and Technical Assessment (CTA)* [82nd meeting held June 7-16, 2016]. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives Meeting (JECFA). Available at: http://www.fao.org/3/a-br566e.pdf.

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en/.

FARRP (2018). *AllergenOnline Version 18: Home of the FARRP Allergen Protein Database*. (Version 18B). Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org/ [Released: March 23, 2018].

FSANZ (2008). *Final Assessment Report: Application A540 Steviol Glycosides as Intense Sweeteners*. Canberra, Australia: Food Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/applications/documents/FAR_A540_Steviol_glycosides.pdf

FSANZ (2017). *A1132 – Broaden Definition of Steviol Glycosides (Intense Sweetener) [Approval Report]*. (20 February 2017). Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/applications/Pages/A1132Definition-of-Steviol-Glycosides.aspx.

Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P (2003). Metabolism of stevioside and rebaudioside A from *Stevia rebaudiana* extracts by human microflora. J Agric Food Chem 51(22):6618-6622. DOI:10.1021/jf0303619.

Geuns JM, Augustijns P, Mols R, Buyse JG, Driessen B (2003). Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem Toxicol 41(11):1599-1607. DOI:10.1016/S0278-6915(03)00191-1.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM, Compernolle F, Toppet S (2006). Identification of steviol glucuronide in human urine. J Agric Food Chem 54(7):2794-2798. DOI:10.1021/jf052693e.

Geuns JMC, Buyse J, Vankeirsbilck A, Temme EHM (2007). Metabolism of stevioside by healthy subjects. Exp Biol Med 232(1):164-173.

Geuns JMC, Pietta, P (2004) [unpublished]. *Stevioside Metabolism by Human Volunteers*. Report from Segrate (MI), Italy: Laboratory Functional Biology, Kuleuven, Leuven Belgium and ITB-CNR. Submitted to WHO by Belgium: Federal Ministry of Social Affairs, Public Health and the Environment. Cited In: JECFA, 2006.

Health Canada (2012). *Notice of Modification to the Lists of Permitted Food Additives to Enable the Use of Steviol Glycosides as a Table-Top Sweetener and as a Sweetener in Certain Food Categories*. Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: http://www.hc-sc.gc.ca/fn-an/consult/nom-adm-0002/index-eng.php [Date modified: 2012-11-30].

Health Canada (2017). *Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Steviol Glycosides from Stevia rebaudiana Bertoni as a Sweetener*. (Reference Number: NOM/ADM-0102). Ottawa (ON): Health Canada, Bureau of Chemical Safety, Food Directorate, Health Products and Food Branch. Available at: https://www.canada.ca/en/health-canada/services/food-nutrition/legislation-guidelines/acts-regulations/modification-list-permitted-sweeteners-steviol-glycosides.html [Date Modified: 2017-08-29].

Hutapea AM, Toskulkao C, Buddhasukh D, Wilairat P, Glinsukon T (1997). Digestion of stevioside, a natural sweetener, by various digestive enzymes. J Clin Biochem Nutr 23(3):177-186. DOI:10.3164/jcbn.23.177.

JECFA (2006). Steviol glycosides. In: *Safety Evaluation of Certain Food Additives*. Sixty-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives, June 8-17, 2004, Geneva, Switz. (WHO Food Additives Series, no 54). Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS), pp. 117-144, 638. Available at: http://whqlibdoc.who.int/publications/2006/9241660546_eng.pdf.

JECFA (2007). Steviol glycosides. In: *68th JECFA - Chemical and Technical Assessment (CTA)*. [Sixty-eighth meeting held June 17-26, 2008]. (Revised by Paul M. Kuznesof, Ph.D., Original prepared by Harriet Wallin for the 63rd JECFA). Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/fileadmin/templates/agns/pdf/jecfa/cta/68/Steviol_glycosides.pdf.

JECFA (2009). Steviol glycosides (addendum). In: *Safety Evaluation of Certain Food Additives and Contaminants*. Sixty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 17-26-29, 2008, Geneva, Switz. (WHO Food Additives Series, vol 60). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 183-220. Available at: http://apps.who.int/iris/bitstream/10665/44063/1/9789241660600_eng.pdf.

JECFA (2010). Steviol glycosides [Prepared at the 73rd JECFA (2010) and published in FAO JECFA Monographs 10 (2010)]. In: *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4*. (FAO JECFA Monographs 10). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: http://www.fao.org/ag/agn/jecfa-additives/specs/monograph10/additive-442-m10.pdf.

JECFA (2017a). Steviol glycosides from Stevia *rebaudiana Bertoni* [New specifications prepared at the 84th JECFA, 2017), Superseding tentative specifications prepared at the 82nd JECFA (2016)]. In: *Compendium of Food Additive Specifications*. 84th Meeting, Rome, 6-15 June 2017 (FAO JECFA Monographs 20). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 50-69. Available at: http://www.fao.org/documents/card/en/c/4b06cdda-3e70-4c80-b7e5-56034601836b/.

JECFA (2017b). 3.2. Revision of specifications and analytical methods. 3.2.4 Steviol glycosides. In: *Evaluation of Certain Food Additives*. Eighty-fourth Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 5-16, 2017, Rome, Italy. (WHO Technical Report Series no 1007). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 67, 90. Available at: http://www.who.int/foodsafety/publications/jecfa-reports/en/; http://apps.who.int/iris/bitstream/10665/259483/1/9789241210164-eng.pdf?ua=1.

Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, et al. (2003a). In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem Toxicol 41(3):359-374. DOI:10.1016/S0278-6915(02)00235-1.

Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, et al. (2003b). Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. Food Chem Toxicol 41(6):875-883. DOI:10.1016/S0278-6915(03)00039-5.

Kraemer T, Maurer HH (1994). On the metabolism of the sweetener stevioside in humans. Eur J Pharm Sci 2(1/2):103 [abstract FC12]. DOI:10.1016/0928-0987(94)90121-X.

Nakayama K, Kasahara D, Yamamoto F (1986). Absorption, distribution, metabolism and excretion of stevioside in rats. Shokuhin Eiseigaku Zasshi 27(1):1-8. DOI:10.3358/shokueishi.27.1.

Nikiforov AI, Rihner MO, Eapen AK, Thomas JA (2013). Metabolism and toxicity studies supporting the safety of rebaudioside D. Int J Toxicol 32(4):261-273. DOI:10.1177/1091581813492828.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Pearson WR (2000). Flexible sequence similarity searching with the FASTA3 program package. In: Misener S, Krawetz SA, editors. *Bioinformatics Methods and Protocols*. (Methods in Molecular Biology, vol 132). Totowa (NJ) / New York (NY): Humana Press / Springer, pp. 185-219.

Purkayastha S, Pugh G Jr, Lynch B, Roberts A, Kwok D, Tarka SM (2014). *In vitro* metabolism of rebaudioside B, D, and M under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol 68(2):259-268. DOI:10.1016/j.yrtph.2013.12.004.

Purkayastha S, Bhusari S, Pugh G Jr, Teng X, Kwok D, Tarka SM (2015). In vitro metabolism of rebaudioside E under anaerobic conditions: comparison with rebaudioside A. Regul Toxicol Pharmacol 72(3):646-657. DOI:10.1016/j.yrtph.2015.05.019.

Purkayastha S, Markosayan A, Prakash I, Bhusari S, Pugh G Jr, Lynch B, et al. (2016). Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. Regul Toxicol Pharmacol 77:125-133. DOI:10.1016/j.yrtph.2016.02.015.

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

Renwick AG, Tarka SM (2008). Microbial hydrolysis of steviol glycosides. Food Chem Toxicol 46(Suppl. 7):S70-S74. DOI:10.1016/j.fct.2008.05.008.

Roberts A, Renwick AG (2008). Comparative toxicokinetics and metabolism of rebaudioside A, stevioside, and steviol in rats. Food Chem Toxicol 46(Suppl. 7):S31-S39. DOI:10.1016/j.fct.2008.05.006.

SCF (1985). Reports of the Scientific Committee for Food concerning sweeteners (Opinion expressed by the SCF on 14 September 1984) [Stevioside]. In: *Food Science and Techniques*. (Reports of the Scientific Committee for Food, 16th series). Brussels, Belgium: Commission of the European Communities (EEC), Scientific Committee for Food (SCF), 2, 4, 7, 15, 20. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_reports_16.pdf.

SCF (1999). *Opinion on Stevioside as a Sweetener (Adopted on 17 June 1999)*. (CS/ADD/EDUL/167 Final). Brussels, Belgium: European Commission, Health & Consumer Protection Directorate-General, Scientific Committee on Food (SCF). Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_out34_en.pdf.

Simonetti P, Gardana C, Bramati L, Pietta PG (2004). Bioavailability of stevioside from Stevia rebaudiana in humans: preliminary report. In: Geuns JMC, Buyse J, editors. *Safety of Stevioside:* Proceedings of the First Symposium Sponsored by KULeuven, April 16, 2004, Leuven, Belgium. Heverlee, Belgium: Euprint ed., pp. 51-62.

Sung LH (2002) [unpublished]. *Report on Pharmacokinetic (PK) studies of T100 Sunstevia 95% Stevioside in Rats*. Report from Singapore: Sunlabel Pte Ltd. Submitted to WHO by the Ministry of Health and Welfare, Japan. Cited In: JECFA, 2006.

Toyoda K, Matsui H, Shoda T, Uneyama C, Takada K, Takahashi M (1997). Assessment of the carcinogenicity of stevioside in F344 rats. Food Chem Toxicol 35(6):597-603. DOI:10.1016/S0278-6915(97)00023-9.

U.S. FDA (2006). *Agency Response Letter GRAS Notice No. GRN 000204 [Phospholipase C enzyme preparation from Pichia pastoris expressing a heterologous phospholipase C gene, San Diego (CA): Diversa Corporation].* College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=204 [Date of filing: Jun 9, 2006; Date of closure: Dec 5, 2006].

U.S. FDA (2017a). Part 170—Food additives. §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR.

U.S. FDA (2017b). *Agency Response Letter GRAS Notice No. GRN 000667 [Rebaudioside M, Rancho Santa Margarita (CA): Blue California]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=667 [Sep. 17, 2017].

U.S. FDA (2017c). *Agency Response Letter GRAS Notice No. GRN 000715 [Rebaudioside D, Rancho Santa Margarita (CA): Blue California]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=715 [Oct. 24, 2017].

U.S. FDA (2017d). Part 573—Food additives permitted in feed and drinking water of animals. §573.750—Pichia pastoris dried yeast. In: *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR.

Wang LZ, Goh BC, Fan L, Lee HS (2004). Sensitive high- performance liquid chromatography/mass spectrometry method for determination of steviol in rat plasma. Rapid Commun Mass Spectrom 18(1):83-86. DOI:10.1002/rcm.1285.

Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X, et al. (2008). Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem Toxicol 46(Suppl. 7):S54-S60. DOI:10.1016/j.fct.2008.04.041.

Wingard RE, Brown JP, Enderlin FE, Dale JA, Hale RL, Seitz CT (1980). Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. Experientia 36(5):519-520. DOI:10.1007/BF01965774.