764

February 26, 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D), manufactured by Sichuan Ingia Biosynthetic Co., Ltd., as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, and therefore, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

I hereby certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using McAfee VirusScan 8.8.

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

Yours sincerely,

(b) (6)

Hua Jun
President
Sichuan Ingia Biosynthetic Co., Ltd.

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FEB 27 2018
OFFICE OF
FOOD ADDITIVE SAFETY

GRN # 764

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

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:
13 February 2018

RECEIVED
FEB 27 2018
OFFICE OF
FOOD ADDITIVE SAFETY

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

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

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 D-rich steviol glycoside preparation (≥95% rebaudioside D), 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) as a general purpose sweetener, as described herein.

Signed,

[redacted]

2018. 2. 15

Name [redacted]
Date

Title President
Sichuan Ingia Biosynthetic Co., Ltd.
Email [redacted]

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 D; reb D; RD95; D Plus

1.3 Conditions of Use

Sichuan Ingia intends to market a rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D), 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 strain, 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) as a general purpose sweetener [see Appendix A, entitled "**Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of Rebaudioside D-rich (≥95% Rebaudioside D) Steviol Glycoside Preparation (RD95) 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

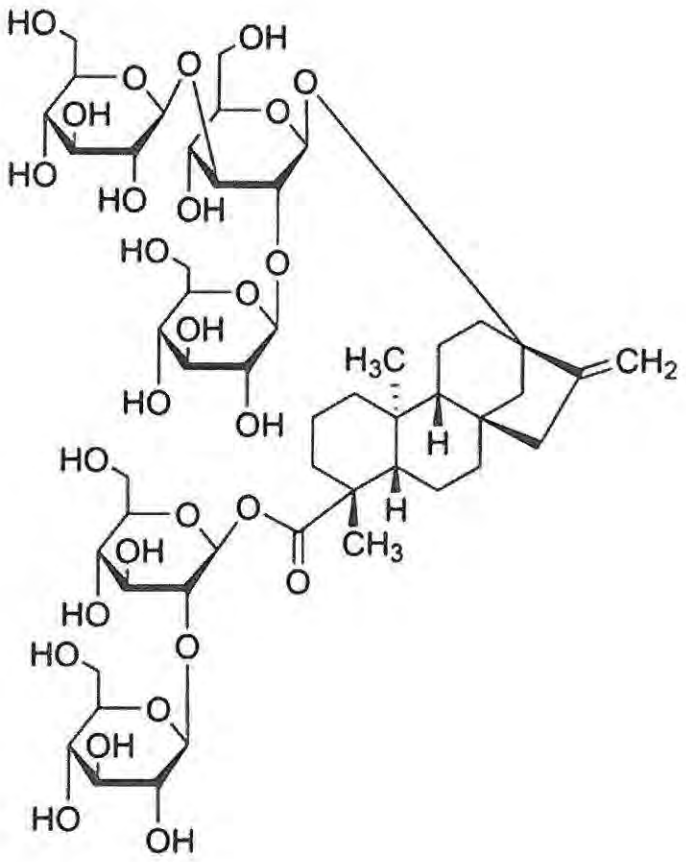
2.1.1 Common or Usual Name

Steviol glycosides; rebaudioside D; reb D; RD95; D Plus.

2.1.2 Chemical and Physical Characteristics

Sichuan Ingia's rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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. Rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is 250 times sweeter than sucrose and is consistent with the sweetness profile of steviol glycosides (FAO, 2016). The chemical structure of the primary component, rebaudioside D, 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 galactose in any orientation occurring in the leaves of *S. rebaudiana* Bertoni.

Figure 2.1.2-1 Chemical Structure of Rebaudioside D



2.2 Method of Manufacturing

The rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is produced *via* an enzymatic conversion process using a strain of *Pichia pastoris* that has been genetically modified to express UDP-glucosyltransferase EUGT11. In the first stage of manufacturing, a steviol glycoside primary extract from the leaves of *S. rebaudiana* Bertoni containing 55±5% of 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 step, the steviol glycoside primary extract is further purified to a purity of ≥95% rebaudioside A through crystallization, also consistent with the CTA methodology. In the third stage, the *P. pastoris* production strain is subjected to a fermentation step to express the UGT-glucosyltransferase EUGT11, which is used to catalyze the conversion of the high-purity rebaudioside A extracted from *S. rebaudiana* Bertoni to rebaudioside D. In the last stage, the rebaudioside D solution is purified and concentrated according to the methodology described in the steviol glycoside CTA, yielding a final product that contains ≥95% rebaudioside D.

2.2.1 Raw Materials and Processing Aids

All raw materials, processing aids, and equipment used in the manufacture of Sichuan Ingia's rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

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	Reaction medium (glucose donor)	N/A
Magnesium chloride	Reaction medium	GRAS when used in accordance with cGMP (21 CFR §184.1426) (U.S. FDA, 2017a)
Sodium citrate	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)
Methanol	Processing aid	21 CFR §173.250 (U.S. FDA, 2017a)
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.

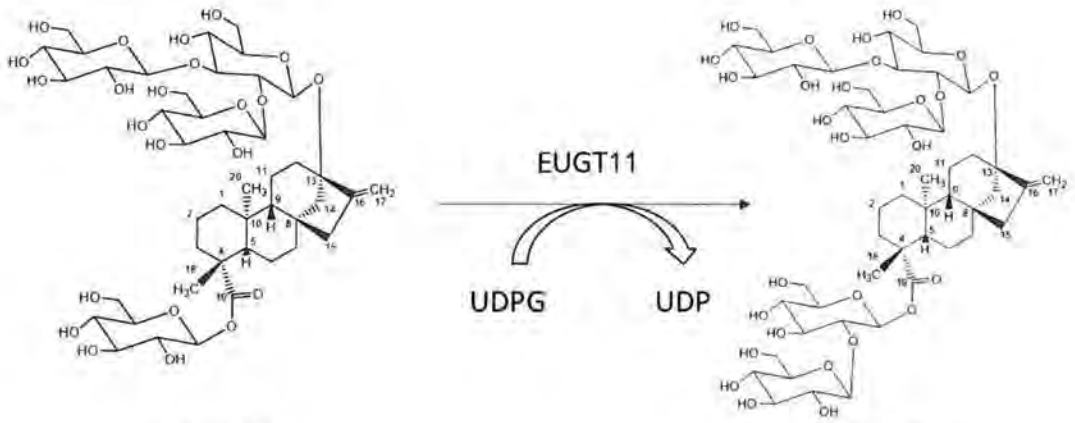
2.2.2 Enzyme

The enzyme utilized in the manufacturing process that converts rebaudioside A to rebaudioside D, UDP-glucosyltransferase EUGT11, is derived from a species of rice (*Oryza sativa* Japonica). 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. The UDP-glucosyltransferase EUGT11 is produced by microbial fermentation of a non-pathogenic and non-toxicogenic strain of *P. pastoris* that has been genetically modified to produce the enzyme (see Section 2.2.4 for further details). The manufacturing process includes a heating step in which the enzymes are denatured, and subsequent purification steps that remove all residual enzymes from the final product. To demonstrate the success of the purification processes, Sichuan Ingia assessed 3 batches of rebaudioside

[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]).

D-rich steviol glycoside preparation (≥95% rebaudioside D) for residual protein and the results of the analysis indicated that protein was not present in the final product (see Section 2.3.5 for further details).

Figure 2.2.2-1 Enzymatic Conversion of Rebaudioside A to Rebaudioside D by UDP-glucosyltransferase EUGT11



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

2.2.3 Manufacturing Process

A schematic overview of the manufacturing process for rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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.

Stage 1 – Steviol Glycoside Extraction from *S. rebaudiana* Bertoni

A steviol glycoside primary extract containing 55±5% of rebaudioside A is produced according to the methodology outlined in the CTA for steviol glycosides (FAO, 2016). Briefly, dried/crushed leaves of the *S. rebaudiana* Bertoni plant are extracted with hot water, filtered, and concentrated. The crude extract is adsorbed with a polar resin, followed by elution with methanol. The crude extract is deionized using an ion exchange resin, concentrated, and dried by either spray or vacuum to yield a steviol glycoside primary extract containing 55±5% rebaudioside A.

Stage 2 – Purification of Crude Rebaudioside A Extract

The steviol glycoside primary extract containing 55±5% rebaudioside A is further purified in accordance with the methodologies outlined in the CTA for steviol glycosides (FAO, 2016). Briefly, the steviol glycoside primary extract is dissolved in ethanol and water, filtered, and crystallized. The crude crystals containing 85±5% rebaudioside A are separated by centrifugation, rinsed with ethanol, recrystallized, and filtered. The resulting rebaudioside A crystals of ≥95% purity are spray dried and sifted through 80 to 150 mesh screens and packaged.

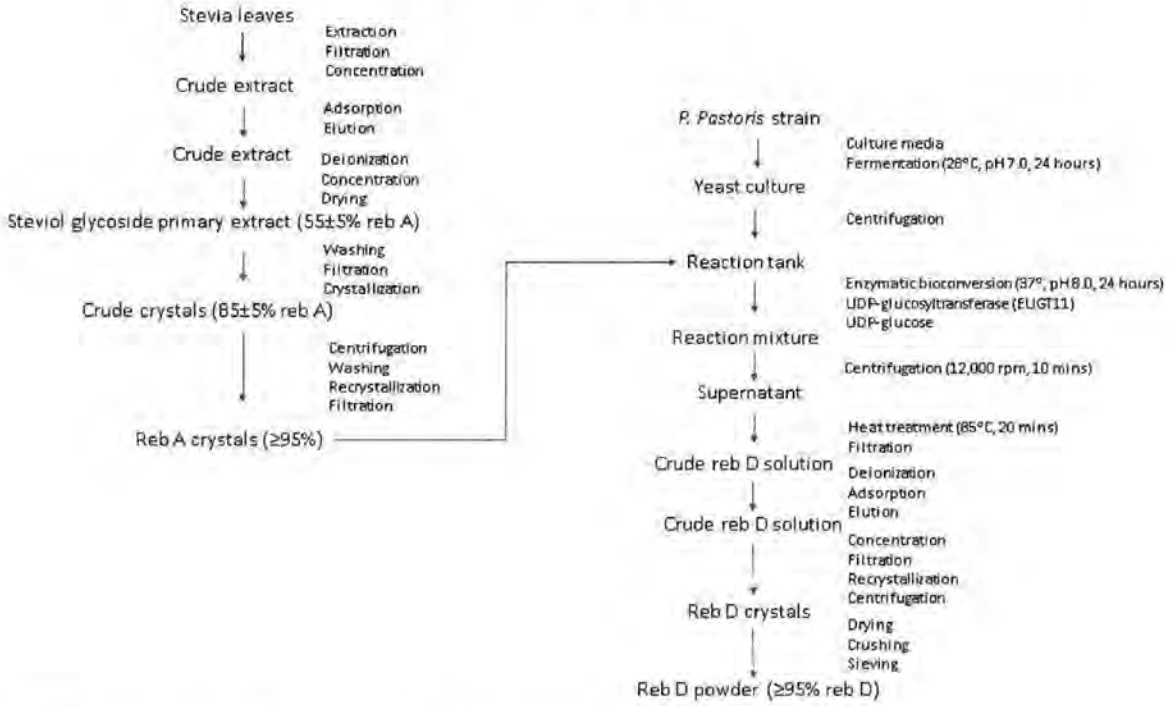
Stage 3 – Enzymatic Conversion of Rebaudioside A to Rebaudioside D

The enzyme required for the conversion process, UDP-glucosyltransferase EUGT11, is generated by a strain of *P. pastoris* that has been genetically modified. A glycerol stock of the *P. pastoris* enzyme production strain is removed from the -70°C freezer, thawed to room temperature, and cultured in 2 L of yeast culture seed media (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone, 0.075 g/L adenine sulfate, pH 7.0) for 12 hours. The cell culture is transferred to a 500 L fermenter and cultured until the OD_{600} is equal to 0.5, after which point the cells are transferred to a 10-ton production fermenter. Following the 24-hour incubation period, cells are harvested by centrifugation and then transferred to a reaction tank. The purified rebaudioside A (≥95%) extracted from *S. rebaudiana* Bertoni (*i.e.*, the product of Stage 2) is slowly added to the reaction tank of *P. pastoris* enzyme production strain containing the expressed UDP-glucosyltransferase EUGT11 enzyme and mixed to initiate the enzymatic conversion process. After the reaction period, the mixture is centrifuged to remove the precipitate and the supernatant is heated to 85°C for 20 minutes to deactivate any residual enzymes and to kill any remaining yeast cells. The heat-killed supernatant containing rebaudioside D is then filtered through a 0.22 µm membrane.

Stage 4 – Purification of Rebaudioside D

The crude rebaudioside D solution from Stage 3 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 D solution is loaded onto a macroporous resin column and allowed to flow through by gravity. The column is washed with food-grade ethanol to elute the adsorbed rebaudioside D solution, the eluate is collected and concentrated by a scraping film evaporator. The concentrate is cooled and centrifuged to obtain a wet crystalline precipitate. Activated carbon is added to remove any remaining impurities, and the solution is filtered and recrystallized to obtain wet rebaudioside D crystals. The wet crystals are then processed to generate the final high-purity rebaudioside D product (≥95% rebaudioside D). The dried crystals are subsequently packaged.

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



P. pastoris = *Pichia pastoris*; Reb = rebaudioside; UDP = uridine 5'- diphosphate.

2.2.4 Construction of the Production Strain

The *P. pastoris* enzyme production strain is 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 gene encoding UDP-glucosyltransferase EUGT11 was obtained from a species of rice. The EUGT11 gene was introduced into the expression vector using site-directed DNA integration to produce the recombinant plasmid. The EUGT11 fragment and the expression vector were digested using restriction enzymes and the target fragment was ligated to produce the recombinant plasmid, which was 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 production strain) were obtained by ampicillin resistance screening. All plasmids and resistance genes were removed from the production strain, and therefore no residual vector sequences or antibiotic resistance genes are present in the production strain. Stocks of the *P. pastoris* production strain were stored in glycerol at -70°C.

2.3 Product Specifications and Batch Analyses

2.3.1 Physical and Chemical Specifications

The physical and chemical specifications for rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf are presented in Table 2.3.1-1.

Table 2.3.1-1 Physical and Chemical Specifications for Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Specification Parameter	Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) Specifications	JECFA Specifications for Steviol Glycosides (JECFA, 2017a)	Method of Analysis
Physical Tests			
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
Chemical Tests			
Rebaudioside D	≥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	≤6.0%	≤6% (105°, 2 h)	USP 34
pH	5 to 7 (1 in 100 solution)	4.5 to 7.0 (1 in 100 solution)	USP 34
Ash	≤1.0%	≤1%	USP 34
Total heavy metals	≤10 ppm	NS	USP 34
Lead	≤0.05 ppm	≤1 mg/kg	ICP-MS
Arsenic	≤0.05 ppm	≤1 mg/kg	ICP-MS
Mercury	≤0.05 ppm	NS	ICP-MS
Cadmium	≤0.05 ppm	NS	ICP-MS
Residual ethanol	<1,000 ppm	≤5,000 mg/kg	USP 34
Residual methanol	<200 ppm	≤200 mg/kg	USP 34

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.
[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 Microbiological Specifications

The microbiological specifications for rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf are presented in Table 2.3.2-1.

Table 2.3.2-1 Microbiological Specifications for Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Specification Parameter	Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) Specifications	JECFA Specifications for Steviol Glycosides (JECFA, 2017a)	Method of Analysis
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/ 25 g	FDA BAM
Staphylococcus aureus	Negative/g	N/A	FDA BAM

BAM = Bacteriological Analytical Manual; CFU = colony forming unit; FDA = Food and Drug Administration; JECFA = Joint FAO/WHO Expert Committee on Food Additives.

2.3.3 Batch Analyses

2.3.3.1 Physical and Chemical Analysis

Data from the analysis of 5 non-consecutive lots of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf (Lot No. 20170704RD-95HT, 20170705RD-95HT, 20170706RD-95HT, 20170801RD-95HT, 20170802RD-95HT) 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 physical and chemical analyses for the 5 lots of the rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is presented in Table 2.3.3.1-1.

Table 2.3.3.1-1 Summary of the Physical and Chemical Product Analysis for 5 Non-Consecutive Lots of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Specification Parameter	Specification	Manufacturing Lot No.				
		20170704RD-95HT	20170705RD-95HT	20170706RD-95HT	20170801RD-95HT	20170802RD-95HT
Physical Tests						
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 Tests						
Rebaudioside D	≥95% (wt/wt, on a dry basis)	95.31%	95.76%	96.46%	96.34%	97.09%
Total steviol glycosides	≥95% (wt/wt, on a dry basis)	97.45%	97.92%	98.24%	97.99%	98.58%
Loss on drying	≤6.0%	2.1%	1.8%	1.7%	2.2%	1.6%
pH	5 to 7 (1 in 100 solution)	6.3	6.1	6.0	5.8	5.9
Ash	≤1.0%	0.09%	0.11%	0.09%	0.12%	0.09%
Total heavy metals	≤10 ppm	Complies	Complies	Complies	Complies	Complies
Lead	≤0.05 ppm	0.013 ppm	0.012 ppm	0.013 ppm	0.010 ppm	0.010 ppm
Arsenic	≤0.05 ppm	0.011 ppm	0.010 ppm	0.010 ppm	0.009 ppm	0.011 ppm
Mercury	≤0.05 ppm	0.009 ppm	0.009 ppm	0.009 ppm	0.009 ppm	0.008 ppm
Cadmium	≤0.05 ppm	0.011 ppm	0.011 ppm	0.008 ppm	0.011 ppm	0.009 ppm

Table 2.3.3.1-1 Summary of the Physical and Chemical Product Analysis for 5 Non-Consecutive Lots of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Specification Parameter	Specification	Manufacturing Lot No.				
		20170704RD-95HT	20170705RD-95HT	20170706RD-95HT	20170801RD-95HT	20170802RD-95HT
Residual ethanol	<1,000 ppm	<200 ppm	<200 ppm	<200 ppm	<200 ppm	<200 ppm
Residual methanol	<200 ppm	<100 ppm	<100 ppm	<100 ppm	<100 ppm	<100 ppm

ppm = parts-per-million.

2.3.3.2 Microbiological Analysis

Analysis of 5 non-consecutive lots of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf (Lot No. 20170704RD-95HT, 20170705RD-95HT, 20170706RD-95HT, 20170801RD-95HT, 20170802RD-95HT) demonstrates that the product meets the microbiological specifications outlined in Section 2.3.2. A summary of the microbiological analyses for the 5 lots of the rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is presented in Table 2.3.3.2-1.

Table 2.3.3.2-1 Summary of the Microbiological Analysis for 5 Non-Consecutive Lots of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Specification Parameter	Specification	Manufacturing Lot No.				
		20170704RD-95HT	20170705RD-95HT	20170706RD-95HT	20170801RD-95HT	20170802RD-95HT
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.

2.3.4 Pesticide Residue Analysis

Pesticide residue analysis was conducted on 1 lot of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) (Lot No. 20160402RD-95) as the starting steviol glycoside material is extracted from the leaves of *S. rebaudiana* Bertoni. The results of the analysis demonstrate the absence of any residual commonly used pesticides in the final product.

2.3.5 Residual Protein Analysis

To confirm the absence of residual protein in the final product, 3 batches of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) (Lot No. 20171205RD95, 20171208RD95, 20171209RD95) were analyzed using the bicinchoninic acid (BCA) method. The limit of detection was 5 µg/mL (5 ppm). No protein was detected, demonstrating that downstream processing successfully removed the enzymes and other residual proteins from the final product.

2.4 Stability Data

At their 68th meeting, 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). Recently, the structural stability of 3 commercial batches each of the dried stevia leaves, the first aqueous infusion of the ground stevia, and a high-purity stevia leaf extract (≥95% steviol glycosides), was evaluated to determine whether the manufacturing process adversely impacts steviol glycoside composition (Oehme *et al.*, 2017). Changes in steviol glycosides were analyzed by HPLC-UV and HPLC-ESI-MS/MS and the authors reported that the 9 steviol glycosides defined in the JECFA (2010) specifications were detected in all samples. Based on the results of this study the authors made note that processing does not chemically alter or modify the steviol glycoside content.

Sichuan Ingia conducted a series of stability tests on their rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), including short-term (10 days), accelerated (6 months), and long-term stability (up to 18 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) demonstrate that the product is stable under different storage conditions for up to 18 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) (Lot No. 20160101) under different storage conditions, including high illumination (4,500±500 lumens), high temperature (40°C), and high humidity (90% relative humidity), kept in commercial packaging. Physical characteristics such as the appearance, odor, and taste, were evaluated and rebaudioside D 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 appearance, odor, taste, or rebaudioside D content of the rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D).

Table 2.4.1-1 Short-term Stability of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) (Lot No. 20160101) Under Different Storage Conditions

Parameter	Day		
	0	5	10
High illumination (4,500±500 LX)			
Rebaudioside D (%)	95.76	95.61	95.68
Appearance, odor, taste	White granular with sweet odor and taste		
High temperature (40°C)			
Rebaudioside D (%)	95.26	95.83	95.15
Appearance, odor, taste	White granular with sweet odor and taste		

Table 2.4.1-1 Short-term Stability of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) (Lot No. 20160101) Under Different Storage Conditions

Parameter	Day		
	0	5	10
High humidity (90% RH)			
Rebaudioside D (%)	95.26	95.69	95.88
Appearance, odor, taste	White granular 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) (Lot No. 20160101, 20160102, and 20160103) 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 D 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside D Content (%)
Lot No. 20160101			
0 months	White granular with sweet odor and taste	3.52	95.76
1 months		3.64	95.68
2 months		3.68	95.54
3 months		3.70	95.78
6 months		3.69	95.58
Lot No. 20160102			
0 months	White granular with sweet odor and taste	3.46	95.50
1 months		3.51	95.66
2 months		3.52	95.48
3 months		3.55	95.32
6 months		3.53	95.44
Lot No. 20160103			
0 months	White granular with sweet odor and taste	3.86	95.56
1 months		3.88	95.76
2 months		3.91	95.65
3 months		3.96	95.46
6 months		3.95	95.53

2.4.3 Long-term Stability

The long-term stability of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) was investigated in 3 non-consecutive lots (Lot No. 20160101, 20160102, 20160103) at a temperature of 25±2°C and 60±10% relative humidity maintained in commercial packaging for up to 18 months. The results indicate that rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is stable for up to 18 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside D Content (%)
Lot No. 20160101			
0 months	White granular with sweet odor and taste	3.52	95.76
3 months		3.54	95.47
6 months		3.57	95.56
9 months		3.60	95.47
12 months		3.58	95.72
18 months		3.62	95.34
Lot No. 20160102			
0 months	White granular with sweet odor and taste	3.46	95.50
3 months		3.51	95.62
6 months		3.49	95.53
9 months		3.52	95.44
12 months		3.48	95.28
18 months		3.58	95.36
Lot No. 20160103			
0 months	White granular with sweet odor and taste	3.86	95.56
3 months		3.81	95.43
6 months		3.84	95.48
9 months		3.82	95.61
12 months		3.84	95.46
18 months		3.82	95.42

Part 3. §170.235 Dietary Exposure

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

Rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 250 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 D, 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) are likely to primarily reflect those currently permitted for other high-intensity sweeteners in the U.S.

3.2 Estimated Consumption of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) from Proposed Food Uses

The daily consumption estimates of other well-established high-intensity sweeteners (*e.g.,* aspartame, cyclamate, 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 D-rich steviol glycoside preparation (≥95% rebaudioside D), the same methodology as applied by Renwick (2008) was used to estimate dietary intake. Since rebaudioside D is 250 times as sweet as sucrose, the intake values for intense sweeteners were adjusted accordingly to derive an estimated intake range for rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D). The estimated intake ranges were then converted to steviol equivalents based upon the molecular weight for rebaudioside D of 1,129 g/mol (Table 3.2.2-1).

Table 3.3.2-1 Estimated Consumption of Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) 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 D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D)[a] (mg/kg bw/day)		Rebaudioside D-rich Steviol Glycoside Preparation (≥95% Rebaudioside D) as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic adults	255	675	1.02	2.70	0.26	0.69
Diabetic adults	280	897	1.12	3.59	0.29	0.91
Non-diabetic children	425	990	1.70	3.96	0.43	1.01
Diabetic children	672	908	2.69	3.63	0.68	0.92

bw = body weight

[a] Rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) is approximately 250 times as sweet as sucrose.

[b] Calculated based on the molecular weight of rebaudioside D of 1,129 g/mol [steviol conversion factor of 0.28].

For non-diabetic adults, average and high-end intakes of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) up to 0.26 and 0.69 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 0.29 and 0.91 mg/kg body weight/day. Average and high-end exposures to rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), expressed as steviol equivalents, in non-diabetic children were calculated to be up to 0.43 and 1.01 mg/kg body weight/day, respectively. Although average intakes of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), expressed as steviol equivalents, were estimated to be higher at up to 0.68 mg/kg body weight/day in diabetic children compared to values for non-diabetic children, high-end values in diabetic children (0.92 mg/kg body weight/day) were lower than high-end values in non-diabetic children. The predicted intakes of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) is largely limited by the desired sweetness intended for a particular food or beverage product. Therefore, the use of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) was not used in food before 1958.

Part 6. §170.250 Narrative and Safety Information

The safety of steviol glycosides, including rebaudioside D, has been previously evaluated by the U.S. FDA through their review of numerous GRAS notices for the use of steviol glycosides as general purpose sweeteners, to which the U.S. FDA has consistently responded with "no questions". In addition, the safety of steviol glycosides has been evaluated by several scientific bodies and regulatory agencies, including JECFA, EFSA, Food Standards Australia New Zealand (FSANZ), and Health Canada. It has been demonstrated that all steviol glycosides share the same metabolic fate of microbial hydrolysis to steviol and therefore, safety data available for specific individual steviol glycosides can be extended to support the safety of other steviol glycosides. A large safety database exists for steviol glycosides, consisting of comparative metabolism and pharmacokinetic studies of steviol glycosides in animals and humans, studies evaluating the acute toxicity, short- and long-term toxicity, and carcinogenicity, reproductive/developmental toxicity, and *in vitro* and *in vivo* genotoxicity and mutagenicity, and human safety (Aze *et al.*, 1991; Toyoda *et al.*, 1997; Curry and Roberts, 2008; Curry *et al.*, 2008; Maki *et al.*, 2008a,b; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). In addition to the extensive database that exists to support steviol glycoside safety, a comprehensive and detailed search of the newly published scientific literature was conducted through December 2017 to identify scientific publications on steviol glycosides published since the latest FDA review

of the related GRAS notice (GRN) GRN 715 (U.S. FDA, 2017b). Given the shared metabolic fate of steviol glycosides, the safety of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) produced *via* enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf can be established based on the safety conclusions for steviol glycosides drawn by JECFA and other scientific bodies and regulatory agencies, and is supported by the safety of the *P. pastoris* production strain and UDP-glucosyltransferase used in the manufacturing process.

6.1 Absorption, Distribution, Metabolism, and Elimination

In vitro and *ex vivo* studies have demonstrated that steviol glycosides contain β-glycosidic bonds that are not hydrolyzed by human digestive enzymes of the upper gastrointestinal tract (Hutapea *et al.*, 1997; Geuns *et al.*, 2003, 2007; Koyama *et al.*, 2003a). Steviol glycosides are therefore not absorbed through the upper gastrointestinal tract, but rather enter the colon intact and are degraded by microbes of the *Bacteriodaceae* family that release the aglycone steviol (Renwick and Tarka, 2008). Many *in vitro* studies mimicking the conditions of the anaerobic colon have demonstrated that the gut microflora of mice, rats, hamsters, and humans completely hydrolyze steviol glycosides to steviol (Wingard *et al.*, 1980; Hutapea *et al.*, 1997; Gardana *et al.*, 2003; Koyama *et al.*, 2003b; Purkayastha *et al.*, 2014, 2015, 2016). The rate at which steviol glycosides are metabolized in the colon is dependent on the complexity of the steviol glycoside structure (Wingard *et al.*, 1980; Koyama *et al.*, 2003b). During hydrolysis of rebaudioside A to steviol, the presence of the extra glucose moiety slows down the rate of metabolism, as compared to the hydrolysis of stevioside to steviol. This is indicative of the process by which microbes hydrolyze steviol glycosides by removing 1 glucose molecule at a time. For example, when stevioside is degraded a glucose molecule is released with each sequential hydrolysis reaction to yield steviolbioside, steviolmonoside, and finally steviol. In comparison, rebaudioside A is initially converted to either stevioside (major pathway) or rebaudioside B (minor pathway), before undergoing degradation to steviol (Nakayama *et al.*, 1986; Gardana *et al.*, 2003; Koyama *et al.*, 2003b). Despite these differences in structure, several recent *in vitro* studies have demonstrated that the degradation rates of individual steviol glycosides (*e.g.*, rebaudioside A, B, C, D, E, F, M, steviolbioside, and dulcoside A) to steviol in the presence of human fecal homogenates do not in fact differ significantly (Purkayastha *et al.*, 2014, 2015, 2016). The authors utilized rebaudioside A as a control in each experimental evaluation to allow for comparison between experiments with different individual glycosides and reported that the microbial hydrolysis rates were all generally similar. As such, the authors concluded that "*there is no concern that any of the steviol glycosides would result in rapid absorption of steviol in humans*" (Purkayastha *et al.*, 2016).

The degradation product, steviol, is systemically absorbed *via* the portal vein and distributed to various organs and tissues, such as the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al.*, 1986; Sung, 2002 [unpublished]; Koyama *et al.*, 2003a,b; Wang *et al.*, 2004; Roberts and Renwick, 2008; Roberts *et al.*, 2016). For instance, peak concentrations of steviol were detected in the plasma of Sprague-Dawley rats orally administered steviol within 15 to 30 minutes following administration, (Nakayama *et al.*, 1986; Koyama *et al.*, 2003a; Roberts and Renwick, 2008; Roberts *et al.*, 2016). When rebaudioside A or stevioside were orally administered, the following compounds were observed in the plasma of rats within 8 hours: free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) (Roberts and Renwick, 2008). Human studies showed similar results following ingestion of stevioside or rebaudioside A, where maximal concentrations of steviol glucuronide were identified in the plasma within 8 and 12 hours, respectively (Geuns and Pietta, 2004 [unpublished]; Simonetti *et al.*, 2004; Geuns *et al.*, 2007; Wheeler *et al.*, 2008).

Systemically absorbed steviol is conjugated to glucuronic acid and is excreted in the bile or urine, in rats and humans, respectively. In rats, free and conjugated steviol, as well as any unhydrolyzed fraction of the administered glycosides, are excreted primarily in the feces *via* the bile (generally within 48 hours), with smaller amounts appearing in the urine (less than 3%) (Wingard *et al.*, 1980; Nakayama *et al.*, 1986; Sung, 2002; Roberts and Renwick, 2008). Specifically, in Wistar rats, 2 steviol conjugates were identified in the bile, 1 of which was hydrolyzed by a weak acid and another which was hydrolyzed by a weak acid and β-glucuronidase (Nakayama *et al.*, 1986). Thus, during the elimination of steviol glucuronide in the bile of rats, steviol may be released from its conjugated form by the microflora, and enter enterohepatic circulation. In contrast, humans eliminate steviol glycosides, mainly as steviol glucuronide, with small amounts of unchanged glycoside or steviol, in the urine (Kramer and Maurer, 1994; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al.*, 2004; Geuns *et al.*, 2006, 2007; Wheeler *et al.*, 2008). Unabsorbed steviol, released from steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile, are also eliminated in the feces in similar amounts recovered in the urine (Geuns and Pietta, 2004 [unpublished]; Simonetti *et al.*, 2004; Geuns *et al.*, 2007; Wheeler *et al.*, 2008). The difference in the route of elimination between rats and humans occurs due to a 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 ($AUC_{0.75\text{-}72h}$) 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 much higher in humans than in rats.

Overall, with the exception of having different numbers and types of sugar moieties, all steviol glycosides share the same structural backbone (*i.e.,* steviol), and, as a result, have a similar metabolic fate. Steviol glycosides pass through the upper portion of the gastrointestinal tract undigested and enter the colon intact. In the colon, steviol glycosides are subjected to microbial degradation by members of the *Bacteroidaceae* family, releasing the aglycone steviol. This common metabolite is absorbed systemically, conjugated to glucuronic acid, and eliminated primarily in the urine in humans. *In vitro* studies have demonstrated that steviol glycosides have similar rates of microbial hydrolysis in the gastrointestinal tract despite the differences in the number of sugar moieties attached to the steviol backbone. Thus, the safety database that has been established for individual steviol glycosides can be extended to support the safety of purified steviol glycosides in general, including rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), regardless of the steviol glycoside distribution of the preparation.

6.2 Safety Evaluations on Steviol Glycosides

Steviol glycosides and their safety have been evaluated by several scientific bodies and regulatory agencies including the U.S. FDA, EFSA, FSANZ, Health Canada, and JECFA.

The U.S. FDA has raised no objections to 47 GRAS notices submitted since 2008 for major individual steviol glycosides, including stevioside, rebaudiosides A, C, D, and X/M, mixtures of steviol glycosides, and glucosylated/enzyme-modified steviol glycosides for use as a general purpose sweetener in food and beverage products. Of particular relevance, GRN 715 describing the use of rebaudioside D produced by enzymatic bioconversion as a sweetener in foods received a "no questions" letter from the U.S. FDA. Similar to Sichuan Ingia's rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D), the rebaudioside D described in GRN 715 is also produced *via* the enzymatic conversion of stevia leaf extract using UDP-glucosyltransferases that are derived from a genetically modified *P. pastoris* (U.S. FDA, 2017b).

JECFA evaluated the safety of steviol glycosides during their 51st, 63rd, 68th, 69th and 82nd meetings (JECFA, 1999, 2006a, 2007a, 2008, 2016a). During their 51st meeting, stevioside was reported to be hydrolyzed to the aglycone steviol before it was absorbed from the gut in rats, followed by conjugation and excretion in the bile and feces. However, limited data were available to adequately assess the safety of stevioside and to establish an ADI. JECFA re-evaluated the safety of steviol glycosides to establish an ADI in subsequent meetings. JECFA established initial specifications for steviol glycosides based on the available analytical data assessed during their 63rd, 68th, and 69th meetings, such that commercial preparations contained at least 95% steviol glycosides[2], with the remainder of the preparation being unidentified (JECFA, 2006a,b, 2007a,b, 2008, 2009). The Committee concluded that humans and rats metabolize steviol glycosides to steviol in the same manner by the removal of glucose moieties by gut microflora and that following absorption of steviol from the colon, it is rapidly conjugated to steviol glucuronide and excreted. The Committee also concluded that steviol glycosides are not mutagenic, and steviol does not have mutagenic activity *in vivo*. Human studies in which steviol glycosides, meeting the established purity specifications, were administered to individuals with type-2 diabetes mellitus for up to 16 weeks, and individuals with normal or low-normal blood pressure for 4 weeks, did not result in any adverse effects (Maki *et al.*, 2008a,b). Based on these results, JECFA calculated an ADI of 0 to 4 mg/kg body weight for steviol glycosides, expressed as steviol equivalents, using the no-observed-adverse-effect level (NOAEL) of 970 mg stevioside/kg body weight/day (equivalent to 383 mg steviol equivalents/kg body weight/day) from a carcinogenicity study in rats that was evaluated at the 51st meeting (Toyoda *et al.*, 1997), and applying a 100-fold safety factor for inter- and intra-species differences. Other regulatory authorities have conducted their own evaluations on the safety of steviol glycosides and have also established an ADI of up to 4 mg/kg body weight, expressed as steviol equivalents.

Additional evaluations of steviol glycosides by JECFA have resulted in expanded specifications for steviol glycosides. JECFA first revised their specifications to include 2 additional steviol glycosides, rebaudioside D and rebaudioside F, within the purity criteria[3] (JECFA, 2010). Although no specific studies have been conducted with these steviol glycosides individually, their inclusion within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all glycosides are degraded to the aglycone steviol and that the safety demonstrated for 1 glycoside is relevant to all glycosides in general. At the 82nd meeting, the Committee reviewed data related to the safety of steviol glycosides that had become available since the 69th meeting and confirmed the ADI of 0 to 4 mg/kg body

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

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

weight, expressed as steviol (FAO, 2016). A new specifications monograph was prepared for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (the Committee also confirmed its inclusion in the ADI) (JECFA, 2016b) and new specifications were established for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). The new specifications for steviol glycosides from *S. rebaudiana* recognize commercial products that "*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).

In the European Union, steviol glycosides must comply with the specifications for steviol glycosides (E 960) adopted by the European Commission in 2012 and recently updated in 2016 (EU, 2012, 2016). Presently, steviol glycoside products must contain no less than 95% of 11 named steviol glycosides: stevioside, rebaudiosides A, B, C, D, E, F, and M, steviolbioside, rubusoside, and dulcoside. The European Commission's Scientific Committee on Food (SCF) evaluated stevioside as a sweetener and concluded that its use was not toxicologically acceptable due to the limited data on metabolism, mutagenicity, long-term, and reproductive and developmental toxicity (SCF, 1985). In a subsequent evaluation, the SCF evaluated newly available metabolism, genotoxicity, and long-term toxicity data. However, the Committee concluded again that the data were inadequate to assess the safety of stevioside, and raised specific concerns on the potential reproductive safety of steviol glycosides (SCF, 1999). The SCF recommended additional testing in a different rat strain than F344 that was previously used as it was not possible to evaluate any potential effects on the testicular system of F344 rats as this strain normally develops testicular changes. In addition, the SCF also questioned the relevance of other studies as the test materials were not clearly defined and also made note of potential mutagenicity of steviol (SCF, 1999). As a result of their evaluation, the European Commission rejected *Stevia* and stevioside for use as a sweetener (Geuns, 2003). In a subsequent review of the data, EFSA corroborated the conclusions of JECFA regarding the safety of steviol glycosides, and agreed with the previously established ADI of 0 to 4 mg/kg body weight/day, expressed as steviol equivalents (EFSA, 2010). Recently, EFSA concluded that safety studies conducted with individual steviol glycosides rebaudioside A and stevioside can be extended to other steviol glycosides due to the shared metabolic fate (EFSA, 2015). Specifically, the EFSA Panel concluded that "*extending the current specifications to include* [two additional steviol glycosides], *rebaudiosides D and M, as alternatives to rebaudioside A in the predominant components of steviol glycosides would not be of safety concern*". Furthermore, EFSA considered the ADI of 4 mg/kg body weight/day can be applied where total steviol glycosides comprise more than 95% of the material.

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

Health Canada conducted an independent review of the available safety data for steviol glycosides and further corroborated the conclusions by JECFA, FSANZ, and EFSA, in which an ADI of 4 mg/kg body weight was established for steviol glycosides, expressed as steviol, based on the NOAEL from the 2-year carcinogenicity study conducted by Toyoda *et al.* (1997) and an uncertainty factor of 100 (Health Canada, 2012). Health Canada expanded the purity definition of steviol glycosides in 2016 to include rebaudioside M as being 1 of the 10 steviol glycosides that may be present alone or in combination in finished preparations to reach the total steviol glycoside content of at least 95% purity (Health Canada, 2016), and in their most recent safety review in 2017, further expanded the definition to include all steviol glycosides in the *S. rebaudiana* Bertoni plant (Health Canada, 2017).

6.3 Additional Safety Data for Steviol Glycosides

A comprehensive search of the scientific literature was conducted on 7 December 2017, to identify new data related to the safety of steviol glycosides since the U.S. FDA review of GRN 715 for rebaudioside D produced by enzymatic bioconversion of stevia leaf extract using enzymes derived from genetically modified strains of *P. pastoris* (U.S. FDA, 2017b). The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. The studies identified included 2 genotoxicity studies, 2 studies in animals evaluating the antidiabetic and immune effects of steviol glycosides, and 1 human study. In general, the results of these recent studies provide further support for the safety of steviol glycosides.

6.3.1 Genotoxicity

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

Uçar *et al.* (2018) investigated the *in vitro* genotoxic potential of stevia in micronucleus and chromosomal aberration tests in human lymphocytes. Human lymphocytes were collected from healthy non-smoking males and females and cell cultures were incubated at 37°C for 72 hours in the chromosome aberration and micronucleus tests. In both tests, after 24 and 48 hours of incubation, stevia (steviol glycoside purity of 99%) was tested in duplicate at concentrations of 0 (negative control), 1, 2, 4, 8, and 16 µg/mL. A positive control, 0.2 µg/mL mitomycin C, was included in both assays. In the chromosome aberration test, 0.06 µg/mL colchicine was added at 70 hours, and at 72 hours cells were collected and prepared for

analysis. A total of 400 metaphases/concentration were analyzed for chromosome aberrations. In the micronucleus test, cells were harvested at 72 hours and prepared for analysis. A total of 4,000 binucleated cells/concentration were analyzed. The authors did not observe any significant increase in the number of chromosomal aberrations or micronuclei at any tested concentration of stevia compared to the negative control. As expected, the positive control caused a significant increase in the number of chromosomal aberrations and micronuclei in each respective assay. Based on the results of this study, the authors concluded that stevia does not have genotoxic potential in the *in vitro* chromosome aberration and micronucleus tests in human lymphocytes, consistent with the results of other similarly conducted studies.

6.3.2 Antidiabetic Effects

Philippaert *et al.* (2017) studied the effects of steviol and steviol glycosides on pancreatic β-cell function and taste preferences of mice in many *in vitro* and *in vivo* studies. In particular, the authors were interested in the relationship between steviol glycosides and TRPM5, an ion channel present in pancreatic β-cells and type II taste receptors that is associated with sweet, bitter, and umami taste perception. The *in vitro* and *in vivo* studies conducted using *Trpm5-/-* mice demonstrate that: a) stevioside, reb A, and steviol potentiate the activity of TRPM5; b) TRPM5 facilitates insulin release from the islet cells; c) potentiation of TRPM5 activity by steviol glycosides modulates and intensifies bitter, sweet, and umami taste responses, and d) the glucose lowering effect of stevioside is dependent on TRPM5 expression in pancreatic islets. In addition, the effect of chronic stevioside treatment (25 mg/kg, 0.1% solution in drinking water) on the development of diabetes induced by a high-fat diet (HFD) on male mice (C57Bl6/J wildtype or *Trpm5-/-*, n=8 per group) was examined. The control group was provided a HFD while the treatment group was provided a HFD plus stevioside. Following consumption of the HFD for 20 weeks, a time-dependent development of glucose intolerance was observed in the wildtype control group using an intraperitoneal glucose tolerance test, whereas wildtype mice treated with stevioside (HFD plus stevioside) had normal glycemic profiles after 20 weeks. *Trpm5-/-* mice showed no differences in control (HFD) and treatment (HFD plus stevioside) groups. The authors also considered reversal of glucose homeostasis by stevioside withdrawal in male mice (C57Bl6/J, n=8 to 10 per group). The mice were divided into the following groups: a 15-week HFD with stevioside treatment (124 μM stevioside in drinking water; mg/kg dose not stated), a 10-week HFD with stevioside followed by a 5-week HFD without stevioside, and a control group on a 15-week HFD. Results demonstrated an improved glucose tolerance in the 15-week HFD plus stevioside group. However, deteriorated glucose tolerance was observed in mice on a HFD treated with stevioside for 10 weeks, followed by removal of stevioside for 5 weeks, with levels similar to that of untreated HFD mice. The authors concluded that targeting TRPM5 may have the potential to prevent and treat type 2 diabetes. It was also suggested that other modulators of TRPM5 including, stevioside, rebaudioside A, and steviol may play a role in the future development of TRPM5-targetted antidiabetic drugs.

6.3.3 Other Physiological Effects

Noosud *et al.* (2017) investigated the effects of stevioside (>95% purity) *in vivo* and *in vitro*. Rat plasma levels of TNF-α and IL-1β, and the release of these pro-inflammatory cytokines from isolated rat peripheral blood mononuclear cells (PBMCs) exposed to stevioside were used to study these effects. Male Wistar rats (170 to 220 g; n=6/group) were administered stevioside at the following doses: 0, 500, and 1,000 mg/kg body weight/day *via* gavage for 6 weeks. Blood samples were obtained following the exposure period and plasma and PBMCs were isolated. Cytokine production was induced through stimulation of PBMCs with and without lipopolysaccharide (LPS) *in vitro* for 24 hours, followed by the collection of supernatant fluids. Rat enzyme-linked immunosorbent assay kits were used to measure the release of TNF-α and IL-1β from PBMCs as well as their concentrations in the plasma. Viability of cells obtained from the stevioside-treated and control groups were similar, indicating that stevioside was not toxic to these cells following oral intake. The

presence of TNF-α and IL-1β were not detected in the plasma of control and treatment groups. During LPS stimulation of PBMCs *in vitro*, TNF-α and IL-1β were released from stevioside exposed cells (both doses) and were significantly decreased compared to the control group, which is indicative of the inhibitory effect on cytokine release. It was suggested that stevioside may have the potential to inhibit the release of pro-inflammatory cytokines, TNF-α and IL-1β *in vivo* and further studies should be conducted. It should be noted that the doses provided in this study greatly exceed the current ADI for steviol glycosides.

6.3.4 Studies in Humans

The potential effects of a natural stevia preparation (obtained from Boots Ltd.) on blood pressure, stress hormone levels, and anthropometric parameters were evaluated in a randomized, crossover placebo-controlled study in healthy humans (Al-Dujaili *et al.*, 2017). Healthy males and females (8/group; mean age 27.75±13.75 years; body mass index [BMI] 26.33±5.26 kg/m^2) were subjected to a 3-day washout period before and after each treatment period. Individuals consumed either the placebo (5 g sucrose) or stevia dissolved preferably in a hot drink (0.2 g; ~2.7 mg stevia/kg body weight) 3 times per day for 7 days. Subjects were instructed to refrain from consuming other sweeteners or sugars throughout the study period. A 24-hour urine sample and saliva samples (in the morning, afternoon, and evening) were collected at baseline and after each treatment period and analyzed for cortisol and cortisone concentrations. Anthropometric parameters, including blood pressure, weight, height, and BMI were also measured at baseline and after each treatment. No significant effect on weight or BMI was observed following stevia consumption. Conversely, the authors noted a significant increase in systolic and diastolic blood pressure compared to baseline following stevia consumption, yet the values were still within the expected reference range. Salivary cortisol levels in the stevia group were reported to increase slightly yet significantly in the morning *vs.* baseline, but this elevation was not sustained through the midday or afternoon time points. Levels of free urinary cortisol and cortisone were reported to significantly increase and decrease, respectively, compared to baseline following stevia consumption. No significant changes in any parameter compared to baseline were observed following consumption of the sucrose placebo. Based on the results of this study, the authors concluded that consumption of stevia for a short period caused a small but significant increase in blood pressure and that the increase in blood pressure may have been due to the increase in cortisol levels. The authors did note the limitations in the size of their study, and indicated that further research is needed to determine the significance of their findings.

6.4 Safety of the Production Strain and the Enzyme

The production strain used to produce the UDP-glucosyltransferase that enzymatically converts rebaudioside A to rebaudioside D is derived from wildtype *P. pastoris* ATCC 20864. As described in detail in Section 2.2, the UDP-glucosyltransferase EUGT11 gene introduced into the parental strain *P. pastoris* ATCC 20864 is derived from a species of rice (*Oryza sativa* Japonica). The safety of the UDP-glucosyltransferase EUGT11 derived from the *P. pastoris* production strain was evaluated using the decision tree for evaluating the safety of microbially-derived food enzymes published by Pariza and Johnson (2001). The enzyme was 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 strain and enzyme from the final product. No protein was detected in 3 non-consecutive batches of the final product, demonstrating that the enzymes and other residual proteins were successfully removed.

6.4.1 History of Use and the Production Strain

P. pastoris was first introduced for commercial use in the production of proteins 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 Strain

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 Enzyme

Although the UDP-glucosyltransferase EUGT11 is not present in the final product, bioinformatic searches were conducted with the UDP-glucosyltransferase EUGT11 sequence to confirm that it does 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 EUGT11 FASTA protein sequence against downloaded protein sequences obtained from a curated database of venom proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot[4]). BLAST searches also were conducted against curated virulence proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot/TrEMBL[5]). A sequence alignment of greater than 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 7 December 2017.

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).

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

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

6.4.4 Potential Allergenicity of the Enzyme

Although the UDP-glucosyltransferase EUGT11 is not present in the final product, a sequence homology search was conducted according to the approach outlined by the FAO/World Health Organization (WHO) (2001) and the WHO/FAO (2009) using the AllergenOnline Database version 17 (available at http://www.allergenonline.org; updated 18 January 2017) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2017). This was done to confirm that UDP-glucosyltransferase EUGT11 does 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 search was performed on 7 December 2017. No matches were identified from searching with the full amino acid sequence for UDP-glucosyltransferase EUGT11. 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).

A second homology search was conducted according to the approach outlined by the FAO/WHO (2001) and the WHO/FAO (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 amino acid sequence. The 80-amino acid alignment search was conducted using default settings (*E* value cutoff = 1 and maximum alignments of 20). Using this search strategy, again no matches were identified. A third homology search conducted using the exact 8-mer approach also did not produce any matches.

Based on the information provided above, no evidence exists to suggest that the UDP-glucosyltransferase EUGT11 used in the enzymatic conversion of rebaudioside A to rebaudioside D would be associated with an allergenic response.

6.5 Expert Panel Evaluation

Sichuan Ingia has concluded that rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) produced by enzymatic conversion of a high-purity rebaudioside A (*i.e.*, RD95) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D), are presented in Appendix A.

6.6 Conclusions

Based on the data and information presented herein, Sichuan Ingia has concluded that rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) 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 D-rich steviol glycoside preparation (≥95% rebaudioside D) produced by enzymatic conversion of a high-purity rebaudioside A extracted from stevia leaf are publicly available and therefore the intended uses of rebaudioside D-rich steviol glycoside preparation (≥95% rebaudioside D) can be concluded to be GRAS on the basis of scientific procedures.

Part 7. §170.255 List of Supporting Data and Information

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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

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Expert Panel Report Concerning the Generally Recognized as Safe (GRAS) Status of a Rebaudioside D-rich (≥95% Rebaudioside D) Steviol Glycoside Preparation (RD95) for Use as a General Purpose Sweetener

January 30th, 2018

INTRODUCTION

Sichuan Ingia Biosynthetic Co., Ltd. ("Sichuan Ingia") intends to market a rebaudioside D-rich (≥95% rebaudioside D) steviol glycoside preparation (RD95), produced *via* a manufacturing process that utilizes a glucosyltransferase derived from a strain of *Pichia pastoris* (*P. pastoris*) to convert high-purity rebaudioside A extracted from *Stevia rebaudiana* (*S. rebaudiana*) Bertoni to rebaudioside D, as a general purpose sweetener in the United States (U.S.). Steviol glycosides are typically obtained by extraction from the leaves of *S. rebaudiana* Bertoni using hot water followed by solvent purification of the water-soluble extract, and rebaudioside D is 1 of approximately 40 steviol glycosides that are naturally present in the leaves of *S. rebaudiana* Bertoni. Sichuan Ingia has developed an alternative manufacturing process to produce high-purity rebaudioside D that utilizes a uridine 5'- diphosphate (UDP)-glucosyltransferase enzyme derived from *P. pastoris* that converts high-purity rebaudioside A (extracted and purified from the leaves of *S. rebaudiana* Bertoni) to rebaudioside D. The steviol glycoside preparation under consideration herein is a rebaudioside D-rich product consisting of ≥95% rebaudioside D. When manufactured as described, the final 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.

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, RD95 would be Generally Recognized as Safe (GRAS), based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin-Madison), I. Glenn Sipes, Ph.D. (University of Arizona), and Stanley M. Tarka Jr., Ph.D. (The Tarka Group Inc., and The Pennsylvania State University, College of Medicine). For purposes of the Expert Panel's evaluation, "safe" or "safety" means there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use, as defined by the U.S. Food and Drug Administration (FDA) in 21 CFR 170.3(i) (U.S. FDA, 2017a).

The Expert Panel independently and collectively evaluated as dossier entitled "Documentation Supporting the Evaluation of a Rebaudioside D-rich (≥95% Rebaudioside D Steviol Glycoside Preparation (RD95) as Generally Recognized as Safe (GRAS) for Use as a General Purpose Sweetener" that included a comprehensive summary of scientific information on RD95. 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 January 25th, 2018 *via* teleconference and unanimously concluded that the intended use described herein for RD95, 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 steviol glycoside preparation ("RD95") that is comprised of ≥95% rebaudioside D, which is consistent with the purity criteria for steviol glycosides as established by JECFA (2016a). 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 steviol glycosides share a common metabolic fate of hydrolysis to steviol in the lower gastrointestinal tract, where steviol is absorbed, conjugated with glucuronic acid, and eliminated through the urine in humans.

All raw materials, processing aids, and equipment used in the manufacture of RD95 are food-grade ingredients[1] permitted by U.S. regulation or have GRAS status for their respective uses. Sichuan Ingia's RD95 is produced *via* an enzymatic bioconversion process using a strain of *P. pastoris* that has been genetically modified to express the UDP-glucosyltransferase EUGT11. In the first stage of manufacturing, a steviol glycoside primary extract from the leaves of *S. rebaudiana* Bertoni containing 55±5% of rebaudioside A is produced according to the methodology outlined in the Chemical and Technical Assessment (CTA) published by FAO/JECFA for steviol glycosides (FAO, 2016). In the next step, the steviol glycoside primary extract is further purified to a purity of ≥95% rebaudioside A, also consistent with the CTA methodology, using crystallization techniques. In the third stage, the *P. pastoris* production strain is subjected to a fermentation step to express the UGT-glucosyltransferase EUGT11, which catalyzes the bioconversion of the high-purity rebaudioside A extracted from *S. rebaudiana* Bertoni to rebaudioside D. In the last stage, the crude rebaudioside D solution is purified and concentrated according to the methodology described in the steviol glycoside CTA, yielding a final product that contains ≥95% rebaudioside D.

The Expert Panel also reviewed information pertinent to the construction of the production strain used to produce the UDP-glucosyltransferase and noted that the inserted gene sequence was obtained from a plant source (*i.e., Oryza sativa* Japonica) that is 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 physical and chemical specifications for RD95 based on those established by JECFA for steviol glycosides from *S. rebaudiana* Bertoni. In addition, microbiological specifications have also been established to ensure safe use in foods, similar to those for other food ingredients and other steviol glycoside preparations. 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, 2016a).

[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]).

The Expert Panel reviewed data provided for 5 non-consecutive lots of RD95 and confirmed that the final product complies with the established physical and chemical and microbiological parameters. Pesticide residue analysis was available for 1 lot of RD95, the results of which demonstrate the absence of commonly used pesticides in the final product. The absence of residual protein that may be carried over from the enzymatic bioconversion step of the manufacturing process was confirmed in 3 batches of RD95 using the bicinchoninic acid assay (BCA) method .

Sichuan Ingia conducted a series of stability tests on RD95, including short-term (10 days), accelerated (6 months), and long-term stability (up to 18 months), and concluded that RD95 is stable under different storage conditions (*i.e.,* different temperature, humidity, and illumination conditions) for up to 18 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 RD95 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 RD95 were calculated for adults and children based on post-market surveillance data for other high-intensity sweeteners and by adjusting this data for the relative sweetness intensity of RD95 (*i.e.,* approximately 250 times sweeter than sucrose). The results are shown in Table 1. For the average consumer, the mean intake of RD95 across all groups was predicted to range from1.02 mg/kg body weight/day for non-diabetic adults to 2.69 mg/kg body weight/day for diabetic children, equivalent to 0.26 to 0.68 mg/kg body weight/day as steviol equivalents, respectively. For high consumers, the mean intake of RD95 across all groups was predicted to range from 2.70 mg/kg body weight for non-diabetic adults to 3.96 mg/kg body weight/day for non-diabetic children. These intake values are equivalent to 0.69 and 1.01 mg steviol equivalents/kg body weight/day for non-diabetic adults and children, respectively. It should be noted that the highest intake estimate for RD95 of 3.96 mg/kg body weight/day, equivalent to 1.01 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 RD95 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:			
			RD95[a] (mg/kg bw/day)		RD95 as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer
Non-diabetic Adults	255	675	1.02	2.70	0.26	0.69
Diabetic Adults	280	897	1.12	3.59	0.29	0.91
Non-diabetic Children	425	990	1.70	3.96	0.43	1.01
Diabetic Children	672	908	2.69	3.63	0.68	0.92

Table 1 **Estimated Consumption of RD95 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: RD95[a] (mg/kg bw/day)		RD95 as steviol equivalents[b] (mg/kg bw/day)	
	Average Consumer	High Consumer	Average Consumer	High Consumer	Average Consumer	High Consumer

bw = body weight; RD95 = rebaudioside D-rich (≥95% rebaudioside D) steviol glycoside preparation.

[a] RD95 is approximately 250 times as sweet as sucrose.

[b] Calculated based on the molecular weight of rebaudioside D of 1,129 g/mol [steviol conversion factor of 0.28].

INFORMATION TO ESTABLISH SAFETY

The Expert Panel reviewed the available data supporting the safety of steviol glycosides in order to evaluate the safety of RD95. 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. New studies related to the safety of steviol glycosides published in the scientific literature also were reviewed. In addition, the Expert Panel reviewed information regarding the safety of the production strain used to produce the enzyme required for the enzymatic conversion process, including an *in silico* assessment of the potential allergenicity of the inserted gene sequence.

Studies conducted *in vitro* and *ex vivo* have demonstrated that steviol glycosides contain β-glycosidic bonds that are not hydrolyzed by human digestive enzymes of the upper gastrointestinal tract (Hutapea *et al.*, 1997; Geuns *et al.*, 2003, 2007; Koyama *et al.*, 2003a). Thus, steviol glycosides are not absorbed through the upper gastrointestinal tract, but rather enter the colon intact and are degraded by microbes of the *Bacteriodaceae* family that release the aglycone steviol (Renwick and Tarka, 2008). Many *in vitro* studies mimicking the conditions of the anaerobic colon have demonstrated that the gut microflora of mice, rats, hamsters, and humans completely hydrolyze steviol glycosides to steviol (Wingard *et al.*, 1980; Hutapea *et al.*, 1997; Gardana *et al.*, 2003; Koyama *et al.*, 2003b; Purkayastha *et al.*, 2014, 2015, 2016). The rate at which steviol glycosides are metabolized in the colon is dependent on the complexity of the steviol glycoside structure (Wingard *et al.*, 1980; Koyama *et al.*, 2003b). During hydrolysis of rebaudioside A to steviol, the presence of the extra glucose moiety slows down the rate of metabolism, as compared to the hydrolysis of stevioside to steviol. This is indicative of the process by which microbes hydrolyze steviol glycosides by removing 1 glucose molecule at a time. For example, when stevioside is degraded a glucose molecule is released with each sequential hydrolysis reaction to yield steviolbioside, steviolmonoside, and finally steviol. In comparison, rebaudioside A is initially converted to either stevioside (major pathway) or rebaudioside B (minor pathway), before undergoing degradation to steviol (Nakayama *et al.*, 1986; Gardana *et al.*, 2003; Koyama *et al.*, 2003b). Despite these differences in structure, several recent *in vitro* studies have demonstrated that the degradation rates of individual steviol glycosides (*e.g.*, rebaudioside A, B, C, D, E, F, M, steviolbioside, and dulcoside A) to steviol in the presence of human fecal homogenates do not in fact differ significantly (Purkayastha *et al.*, 2014, 2015, 2016). The degradation product, steviol, is systemically absorbed *via* the portal vein and distributed to various organs and tissues, such as the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al.*, 1986; Sung, 2002 [unpublished]; Koyama *et al.*, 2003a,b; Wang *et al.*, 2004; Roberts and Renwick, 2008; Roberts *et al.*, 2016). Systemically absorbed steviol is conjugated to glucuronic acid in the liver and steviol glucuronide, the

metabolite, is excreted in the urine in humans (Kramer and Maurer, 1994; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al.*, 2004; Geuns *et al.*, 2006, 2007; Wheeler *et al.*, 2008). Small amounts of unchanged glycoside or steviol are also excreted in the urine. Due to the shared metabolic fate of steviol glycosides, the existing safety database for individual steviol glycosides such as stevioside, rebaudioside A, and rebaudioside D, can be extrapolated to support the safety of purified steviol glycosides, regardless of the steviol glycoside distribution of the preparation, including RD95.

JECFA evaluated the safety of steviol glycosides during their 51st, 63rd, 68th, 69th and 82nd meetings and established an ADI of 0 to 4 mg/kg body weight for steviol glycosides, expressed as steviol equivalents, using the no-observed-adverse-effect level (NOAEL) of 970 mg stevioside/kg body weight/day (equivalent to 383 mg steviol equivalents/kg body weight/day) from a carcinogenicity study in rats that was evaluated at the 51st meeting (Toyoda *et al.*, 1997), and applying a 100-fold safety factor for inter- and intra-species differences.

Most recently, JECFA prepared a new specification monograph for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (JECFA, 2016b) and new specifications were established for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). The new specifications for steviol glycosides from *S. rebaudiana* recognize commercial products that contain "not less than 95% of total steviol glycosides (on a dried basis) determined as the sum of all compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni, including glucose, rhamnose, xylose, fructose and deoxyglucose". At the 84th meeting, the Committee also added the sugar moieties arabinose and galactose to the definition (JECFA, 2017b).

The safety of steviol glycosides has been extensively reviewed by JECFA and other scientific and regulatory authorities/bodies, including the FDA, European Commission Scientific Committee on Food (SCF), European Food Safety Authority (EFSA), Food Standards Australia New Zealand (FSANZ), and Health Canada, all of which have concluded that steviol glycoside preparations containing no less than 95% steviol glycosides are safe when used in accordance with cGMP. In addition, these authoritative bodies have corroborated the JECFA-established ADI of 0 to 4 mg/kg body weight as steviol equivalents (SCF, 1985, 1999; FSANZ, 2008, 2017; EFSA, 2010, 2015; Health Canada, 2012, 2017).

Steviol glycosides are approved for use in food and beverages in a number of jurisdictions around the world. Notably, the U.S. FDA has reviewed over 45 GRAS Notifications for high-purity steviol glycoside preparations to date and has raised no objections regarding the GRAS status of steviol glycosides for use as general purpose sweeteners in food and beverage products. Of note, GRN 715 received a "no questions" letter from the FDA regarding the GRAS status of rebaudioside D produced by enzymatic bioconversion for use as a sweetener in foods (U.S. FDA, 2017b). Similar to Sichuan Ingia's RD95, the rebaudioside D described in GRN 715 is also produced *via* the enzymatic conversion of a purified stevia leaf extract using UDP-glucosyltransferases that are derived from genetically modified *P. pastoris*.

The production strain, *P. pastoris*, is derived from wildtype *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, 2017c), 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.

The potential allergenicity, toxigenicity and virulence of the inserted gene sequence, EUGT11, was investigated *via in silico* methods. 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). The potential for allergenic cross-reactivity also was investigated in accordance with the FAO/WHO protocol for allergenicity assessment (FAO/WHO, 2001) and WHO/FAO (2009) using the AllergenOnline Database Version 17 (FARRP, 2017). The database contains a comprehensive list of putative allergenic proteins developed *via* a peer-reviewed process for the purpose of evaluating food safety. No structural similarity greater than 35% to known allergen sequences was identified, indicating the unlikely potential for cross-reactivity to any known allergens. The safety of the UDP-glucosyltransferase EUGT11 derived from the *P. pastoris* production strain was evaluated using the decision tree for evaluating the safety of microbially derived food enzymes published by Pariza and Johnson (2001) and the enzyme was determined to be "accepted" as per the decision tree criteria and based on the conclusion that the final product meets JECFA specifications. 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 demonstrates 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 should not be a health concern.

The scientific evidence reviewed by the Expert Panel demonstrates that under the conditions of intended use, RD95 would not produce any adverse health effects.

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CONCLUSION

We, the members of the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that RD95, meeting appropriate food grade specifications and produced in according with current good manufacturing practice, 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 RD95 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.

(b) (6)

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin-Madison

5 February 2018
Date

(b) (6)

I. Glenn Sipes, Ph.D.
Fellow of AAAS and ATS
Professor Emeritus Pharmacology
University of Arizona

6 February 2018
Date

(b) (6)

Stanley M. Tarka, Jr., Ph.D.
Fellow of ATS
The Tarka Group Inc.
The Pennsylvania State University, College of Medicine

31 January 2018
Date

25 April 2018

Judith D. Perrier, Ph.D., R.D.
Biologist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Dear Dr. Perrier,

Re: GRAS Notice for Rebaudioside D (GRN No. 000764)

As requested, please find following our response to your questions raised in your email sent on April 18, 2018, regarding the referenced GRAS Notice (GRN 764). In addition to addressing the questions raised in the Agency's email, Sichuan Ingia Biosynthetic has included a revised GRAS Notice which incorporates all changes to the manufacturing process, as appropriate, based on the Agency's questions. Specifically, the Agency raised several questions regarding the manufacturing process on page 10 of the GRAS Notice:

> "Following the 24-hour incubation period, cells are harvested by centrifugation and then transferred to a reaction tank. The purified rebaudioside A (≥95%) extracted from *S. rebaudiana* Bertoni (*i.e.,* the product of Stage 2) is slowly added to the reaction tank of *P. pastoris* enzyme production strain containing the expressed UDP-glucosyltransferase EUGT11 enzyme and mixed to initiate the enzymatic conversion process. After the reaction period, the mixture is centrifuged to remove the precipitate and the supernatant is heated to 85°C for 20 minutes to deactivate any residual enzymes and to kill any remaining yeast cells. The heat-killed supernatant containing rebaudioside D is then filtered through a 0.22 µm membrane."

FDA Question 1: What is transferred to the reaction tank, the centrifuged cells or the supernatant?

- If it is the cells, are they lysed prior the addition of the rebaudioside A solution?
- If only the supernatant is added, state whether the enzyme is expressed into the supernatant or discuss why a non-native enzyme would be transported out of the cell.

Notifier Response: The cells are harvested by filtration and then the cells are transferred to the reaction tank. The GRAS notice has been updated to indicate that the cells are harvested by filtration rather than by centrifugation. The cells are not lysed prior to transferring to the reaction tank.

FDA Question 2: What is in the reaction tank? Specifically, if the cells alone are transferred, what are they resuspended in?

Notifier Response: The reaction tank is comprised of the following: rebaudioside A, UDP-glucose, magnesium chloride, and sodium citrate.

FDA Question 3: What are the conditions in the reaction tank, for example, temperature and pH?

Notifier Response: The conditions in the reaction tank are: 37°C and pH 8.0 for 24 hours.

I hope that the answers outlined above address the concerns raised by the Agency. If I can be of any further assistance or provide further clarification, please do not hesitate to contact me.

Sincerely,

(b) (6)

Hua Jun
President
Sichuan Ingia Biosynthetic Co., Ltd.

From:	Huajun
To:	Perrier, Judith
Cc:	*何舟*
Subject:	Re:GRN 764 - Rebaudioside D - Additional FDA Question for Follow up
Date:	Sunday, May 06, 2018 10:47:11 PM
Attachments:	ATT00001

Dear Mr.Judith D. Perrier:

This is our answer regarding the GRAS GRN 764 from Sichuan Ingia Biosynthetic. Thank you very much. Jolin He is charge of our GRAS work.

Best Regards,

Hua Jun

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