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September 14, 2020

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

Attention: Dr. Susan Carlson
Re: GRAS Notification – *High Purity Glucosylated Steviol Glycosides*

Dear Dr. Carlson:

GRAS Associates, LLC, acting as the Agent for Shandong Shengxiangyuan Biotechnology Co., Ltd. ("Shandong", The People's Republic of China), is submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS Notification for *High Purity Glucosylated Steviol Glycosides.* Along with Shandong's determination of safety, an Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into food in general, other than infant formulas and meat and poultry products. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

William J. Rowe, President
Agent for Shandong
GRAS Associates, LLC
11810 Grand Park Ave
Suite 500
North Bethesda, MD 20852
wrowe@nutrasource.ca
Enclosure: GRAS Notification for Shandong – *High Purity Glucosylated Steviol Glycosides*

GRAS Notification

of

High Purity Glucosylated Steviol Glycosides

Food Usage Conditions for General Recognition of Safety

on behalf of

Shandong Shengxiangyuan Biotechnology Co., Ltd.

Shandong
People's Republic of China

9/14/20

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FOREWORD

Shandong Shengxiangyuan Biotechnology Co., Ltd. ("Shandong") based our Generally Recognized as Safe (GRAS) assessment of Prostevia high purity glucosylated steviol glycosides primarily on the composite safety information, i.e., scientific procedures with corroboration from history of use. The safety/toxicity of steviol glycosides, history of use of steviol glycosides, and compositional details, specifications, and method of preparation of the subject ingredient were reviewed. In addition, a search of the scientific and regulatory literature was conducted through August 6, 2020, with particular attention paid to adverse reports, as well as those that supported conclusions of safety. Those references that were deemed pertinent to this review are listed in Part 7. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

At Shandong's request, GRAS Associates, LLC ("GA") convened an Expert Panel to complete an independent safety evaluation of Shandong's Prostevia high purity enzyme glucosylated steviol glycosides preparation. The purpose of the evaluation is to ascertain whether Shandong's Prostevia high purity glucosylated steviol glycosides preparation as described in Part 3 is generally recognized as safe, i.e., GRAS, under the intended conditions of use. In addition, Shandong has asked GA to act as Agent for the submission of this GRAS notice.

PART 1. SIGNED STATEMENTS AND CERTIFICATION

A. Claim of Exclusion from the Requirement for Premarket Approval Pursuant to 21 CFR 170 Subpart E[1]

Shandong has concluded that our high purity glucosylated steviol glycosides preparations that are a blend of glucosylated steviol glycosides, unreacted steviol glycosides, and maltodextrin, referred to as "Prostevia", and which meet the specifications described below, are GRAS in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). This determination was made in concert with an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the intended conditions of food use for the designated high purity glucosylated steviol glycosides preparations.

Signed:

Agent for Shandong

[1] See 81 FR 54960, 17 August 2016. Accessible at: https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf (Accessed 5/16/20).

William J. Rowe
President
GRAS Associates, LLC
11810 Grand Park Ave.
Suite 500
North Bethesda, MD
20852

Date: 9/11/2020

B. Name and Address of Responsible Parties

Shandong Shengxiangyuan Biotechnology Co., Ltd.
No. 58 East Haiguan Rd.
Qufu
Jining
Shandong Province
The People's Republic of China

As the Responsible Party, Shandong accepts responsibility for the GRAS conclusion that has been made for our high purity glucosylated steviol glycosides preparations as described in the subject safety evaluation; consequently, the purified steviol glycosides preparations having acceptable steviol glycosides compositions which meet the conditions described herein, are not subject to premarket approval requirements for food ingredients.

C. Common Name and Identity of Notified Substance

The common name of the ingredient to be used on food labels is "glucosylated steviol glycosides" or "high purity glucosylated steviol glycosides." Shandong also plans to market our high purity glucosylated steviol glycosides preparations under the trade name "Prostevia."

D. Conditions of Intended Use in Food

Shandong's Prostevia (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) high purity glycosylated steviol glycosides preparations are intended for use as general purpose sweeteners in foods, excluding meat and poultry products and infant formulas, at levels determined by current good manufacturing practices (CGMP).

E. Basis for GRAS Conclusion

Pursuant to 21 CFR 170.30(a) and (b)[2], Shandong's Prostevia (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) high purity glucosylated steviol glycosides preparations have been concluded to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

Shandong's Prostevia high purity glucosylated steviol glycosides are not subject to premarket approval requirements of the FD&C Act based on Shandong's conclusion that the substances are GRAS under the conditions of its intended food use.

Shandong certifies, to the best of our knowledge, that this GRAS notice is a complete, representative, and balanced assessment that includes all relevant information, both favorable and unfavorable, available and pertinent to the evaluation of the safety and GRAS status of Shandong's Prostevia high purity glucosylated steviol glycosides.

F. Availability of Information

The data and information that serve as the bases for this GRAS Notice will be maintained at the offices of Shandong Shengxiangyuan Biotechnology Co., Ltd. (The People's Republic of China) and will be made available during customary business hours.

Shandong certifies that no data or information contained herein are exempt from disclosure under the Freedom of Information Act (FOIA). No non-public, safety-related data were used by the Expert Panel to reach a GRAS conclusion.

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

A. Chemical Identity of Ingredient

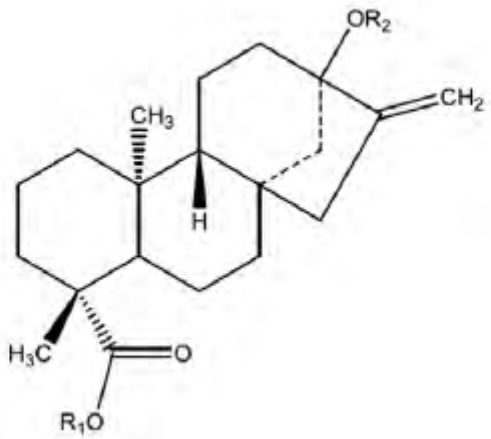
"Enzyme modified steviol glycosides" and "glucosylated steviol glycosides" are the common or usual names of the non-nutritive sweetener derived from the enzymatic glycosylation of a high purity extract of *Stevia rebaudiana* Bertoni. The compositional features of Shandong's high purity glucosylated steviol glycosides preparations are described in more detail in this section. The preparation is also marketed as Prostevia.

The general chemistry of steviol glycosides and enzyme modified steviol glycosides has previously been reviewed in a number of GRAS Notices (GRNs) including GRN 337 (NOW Foods, 2010), GRN 667 (Blue California, 2016), and GRN 715 (Blue California, 2017). Representative chemical structures of steviol glycosides that have been identified to date are presented in Figure 1.

[2] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=170.30 (Accessed 5/16/20).

No known toxins have been identified in stevia or stevia-derived products.

Figure 1. Chemical Structures of Various Steviol Glycosides[a]



Compound	R1	R2
Steviol	H-	H-
Stevioside	Glcß1-	Glcß(1-2)Glcß1-
Rebaudioside A	Glcß1-	Glcß(1-2)[Glcß(1-3)]Glcß1-
Rebaudioside B	H-	Glcß(1-2)[Glcß(1-3)]Glcß1-
Rebaudioside C	Glcß1-	Rhaα(1-2)[Glcβ(1-3)]Glcβ-
Rebaudioside D	Glcβ(1-2)Glcβ1-	Glcß(1-2)[Glcß(1-3)]Glcß1-
Rebaudioside E	Glcβ(1-2)Glcβ1-	Glcβ(1-2)Glcβ1-
Rebaudioside F	Glcβ1-	Xylβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M	Glcß(1-2)[Glcß(1-3)]Glcß1-	Glcß(1-2)[Glcß(1-3)]Glcß1-
Steviolbioside	H-	Glcβ(1-2)Glcβ1-
Dulcoside A	Glcβ1-	Rhaα(1-2)Glcβ1-
Rubusoside	Glcß1-	Glcß1-
Glc, Rha, and Xyl represent glucose, rhamnose, and xylose sugar moieties, respectively		

[a] From Perrier et al. (2018)

Enzyme modified steviol glycosides are produced when additional glucose moieties are bonded to the original steviol glycoside structure via α(1→4) linkages, resulting in α-glucosylated steviol glycosides. The product α-glucosylated steviol glycosides consists of a mixture of both α-D-glucosylated steviol glycosides and steviol glycosides, including rebaudioside A, rebaudioside C, dulcoside A, steviolbioside, rubusoside, and rebaudioside B. The enzyme attaches the additional glucose residues by sterio- and regio-specific 1,4-α-D-glycosidic bonds, whereas the glucose is attached by β-glycosidic bonds in naturally occurring steviol glycosides. The primary constituents of enzymatically modified stevia have been identified (Koyama et al., 2003a) and are described in Table 1. The chemical structures are shown in Figure 2.

Table 1. Components Expected to be Present in Glucosylated Steviol Glycosides[a]

COMPOUND	MOLECULAR WEIGHT	EMPIRICAL FORMULA	LEVEL OF ENZYME GLYCOSYLATION[B]
Steviolbioside	642	$C_{32}H_{50}O_{13}$	--
Dulcoside A	788	$C_{38}H_{60}O_{17}$	--
Stevioside	804	$C_{38}H_{60}O_{18}$	--
Rebaudioside C	950	$C_{44}H_{70}O_{22}$	--
Rebaudioside A	966	$C_{44}H_{70}O_{23}$	--
Monoglucosyl rebaudioside B	966	$C_{44}H_{70}O_{23}$	+1
Monoglucosyl stevioside	966	$C_{44}H_{70}O_{23}$	+1
Monoglucosyl rebaudioside C	1112	$C_{50}H_{80}O_{27}$	+1
Monoglucosyl rebaudioside A	1128	$C_{50}H_{80}O_{28}$	+1
Diglucosyl rebaudioside B	1128	$C_{50}H_{80}O_{28}$	+2
Diglucosylstevioside	1128	$C_{50}H_{80}O_{28}$	+2
Diglucosyl rebaudioside C	1274	$C_{56}H_{90}O_{32}$	+2
Diglucosyl rebaudioside A	1290	$C_{56}H_{90}O_{33}$	+2
Triglucosyl rebaudioside B	1290	$C_{56}H_{90}O_{33}$	+3
Triglucosyl rebaudioside A	1452	$C_{62}H_{100}O_{38}$	+3

[a] Data from Koyama et al. (2003a)
[b] The level of enzymatic glycosylation indicates the number of glucose units that have been added via enzyme modification.

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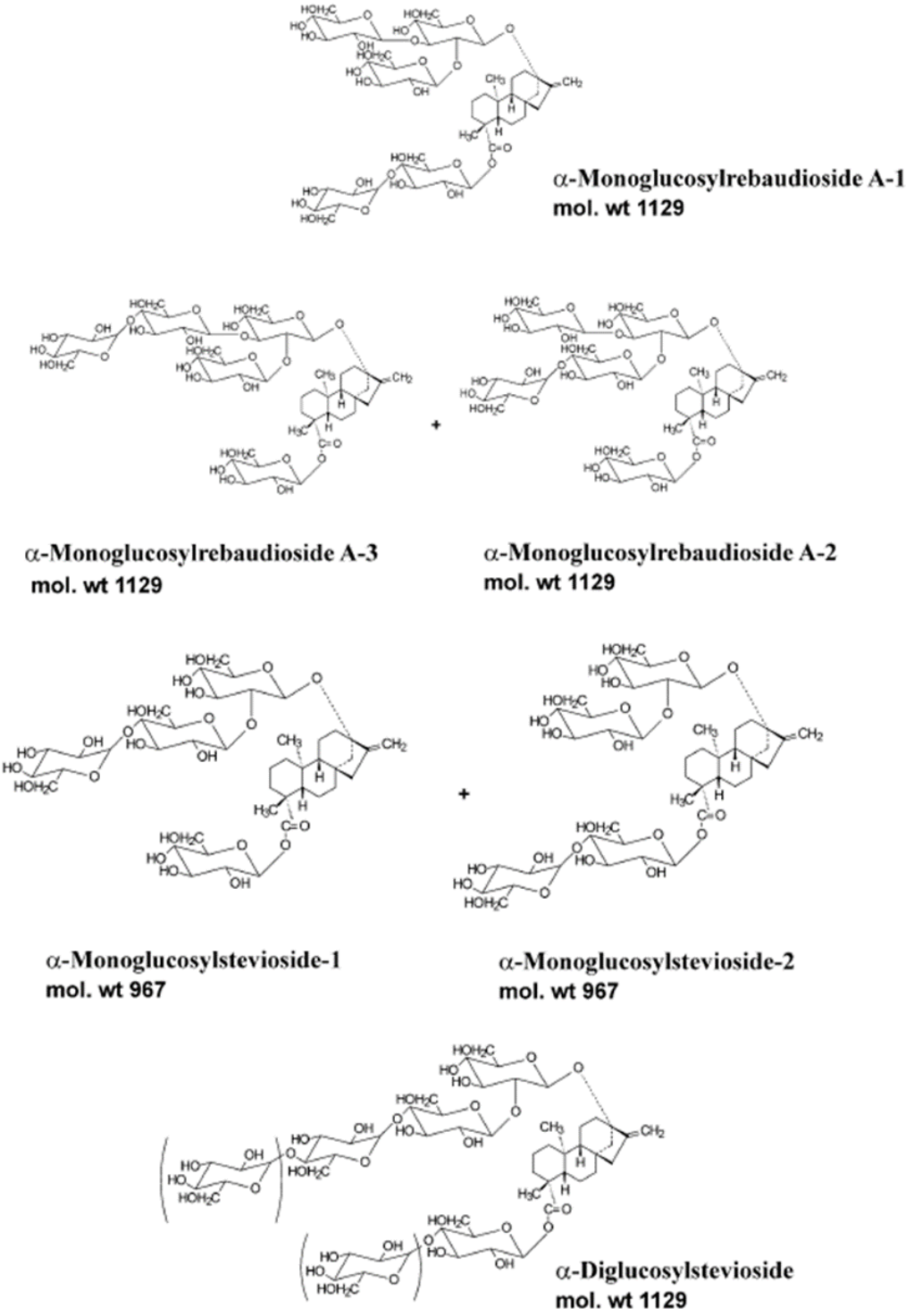
Figure 2. Chemical Structures of Various Glucosylated Steviol Glycosides[a]



[a] From Koyama et al. (2003a)

B. Manufacturing Processes

Shandong's Prostevia glucosylated steviol glycosides preparations are manufactured via an enzymatic reaction with *Stevia rebaudiana* Bertoni extract [>95% total steviol glycosides, which meets Joint FAO/WHO Expert Committee on Food Additives (JECFA) specifications] using cyclomaltodextrin glucanotransferase (CGTase). The resulting preparation is high purity enzyme modified steviol glycosides: Prostevia (≥80% glucosylated steviol glycosides and <15% unreacted steviol glycosides). The remaining ≤5% of the finished product is unreacted maltodextrin.

1. Steviol Glycosides Raw Material

For the manufacturing of the starting steviol glycosides, Shandong employs a fairly typical aqueous extraction process that is used in the industry for the production of stevia extracts. In short, dried *Stevia rebaudiana* Bertoni leaves are soaked in cold water, the extract is flocculated with calcium hydroxide and ferrous sulfate, and the steviol glycosides are purified through filtration, adsorption and elution, and decolorization processes. The resulting extract is concentrated using membranes and evaporation to obtain an extract with ≥95% steviol glycosides, of which ≥50% is rebaudioside A, as described in the flow chart in Figure 3. All raw materials and processing aids Shandong uses to manufacture the raw material steviol glycosides extract are food grade.

2. Prostevia Enzyme Modified Steviol Glycosides

Shandong uses the purified stevia extract product, maltodextrin, and cyclomaltodextrin glucanotransferase enzyme to manufacture high purity enzyme modified steviol glycosides. The starting materials are mixed in a reactor and heated to 76±1°C for 24 hours. The mixed starting material is deactivated at 100°C for 30 minutes and the resulting glucosylated steviol glycosides and unglycosylated steviol glycosides are obtained using sterile filtration. The resulting material is spray dried to obtain the Prostevia preparation, which is a mixture of glucosylated steviol glycosides and unmodified steviol glycosides, with ≤5% unreacted maltodextrin.

The enzyme used to glucosylate the purified stevia extract is Toruzyme 3.0L, which is a CGTase enzyme produced by a genetically modified strain of *Bacillus licheniformis*.[3] Supporting documentation for the raw materials and processing aids are provided in Appendix 1. The manufacturing process for Prostevia is summarized in the flow charts provided in Figure 4.

Shandong's high purity glucosylated steviol glycosides preparations are prepared in accordance with CGMP.

[3] Toruzyme 3.0L, manufactured by Novozymes, is a cyclomaltodextrin glucanotransferase produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. It is a food grade product and complies with JECFA and FCC recommended specifications for food grade enzymes, and is GRAS as defined in 21 CFR 170.30(a).

Figure 3. Flow Chart of Manufacturing Process for Shandong's Steviol Glycosides Raw Material (≥95% Steviol Glycosides)

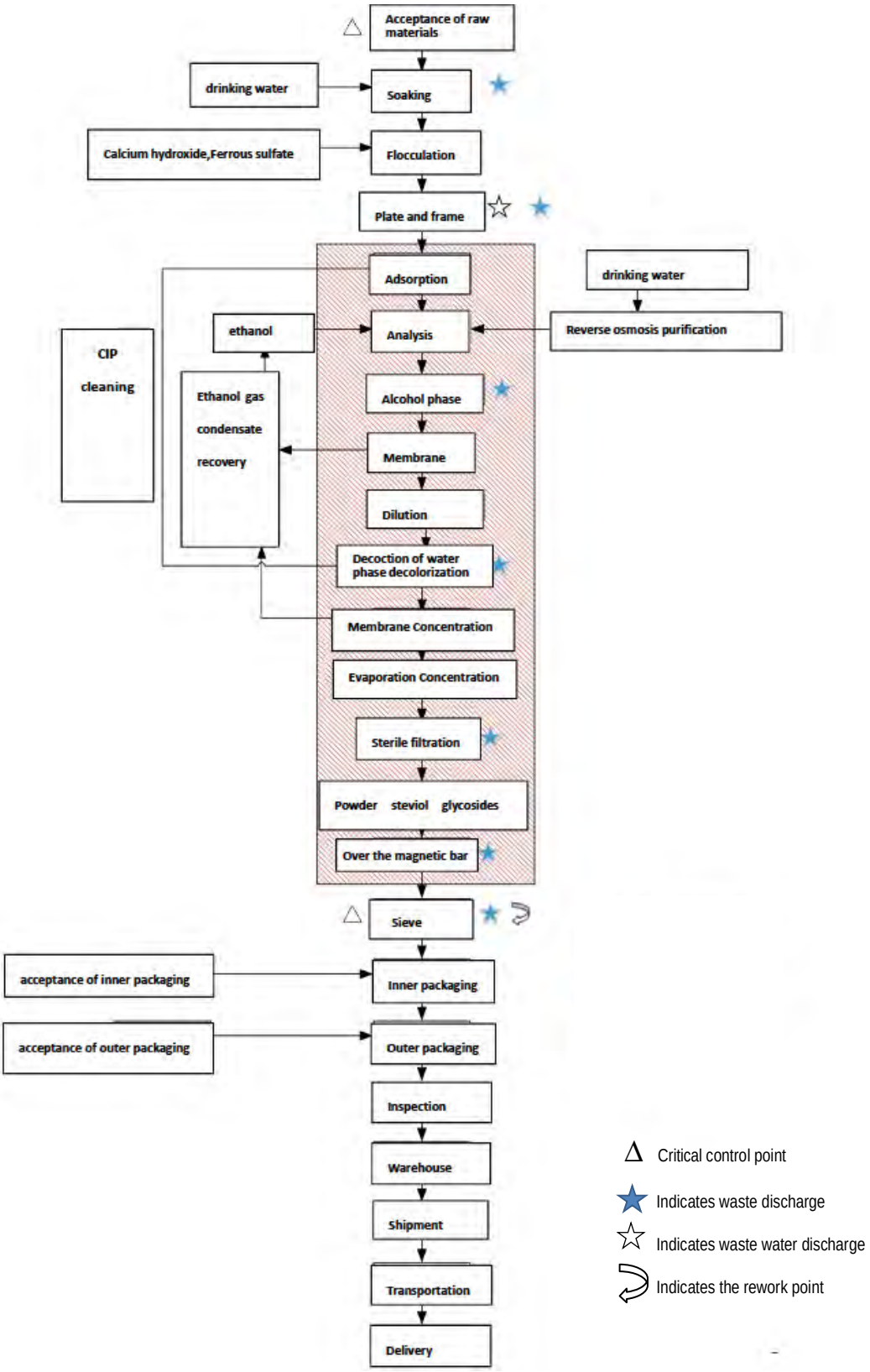
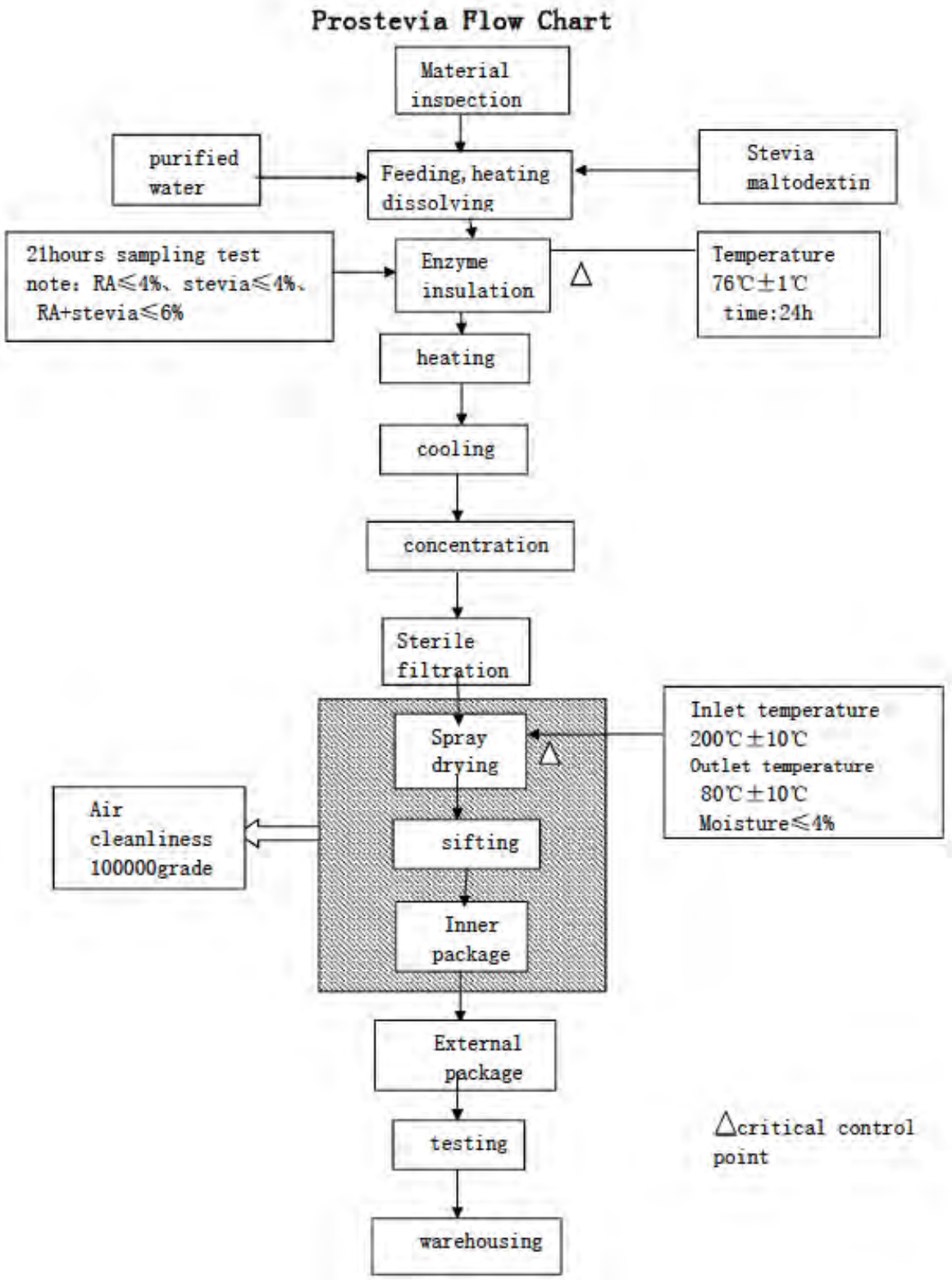


Figure 4. Flow Chart of Manufacturing Process for Shandong's Prostevia High Purity Glucosylated Steviol Glycosides Preparations



RA – rebaudioside A

C. Product Specifications

1. JECFA Specifications for Steviol Glycosides

The compositions of extracts of *Stevia rebaudiana* Bertoni depend upon the compositions of the harvested leaves, which are, in turn, influenced by soil, climate, and the manufacturing process itself (FAO, 2007b).

In the most recent JECFA monograph, published in 2017 (FAO, 2017), steviol glycosides specifications were modified to include a minimum requirement of not less than 95% total steviol glycosides, on a dry basis, "determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni."

JECFA's 2017 monograph describes steviol glycosides as white-to-yellow powders that are odorless or have a slight characteristic odor and exhibit a sweetness that is 200 - 300 times greater than that of sucrose. The ingredient must consist of a minimum of 95% total steviol glycosides, as defined above. The steviol glycosides are freely soluble in a 50:50 mixture of ethanol and water, and the 1 in 100 solutions exhibit pH values between 4.5 and 7.0. The product should not have more than 1% ash, with no more than a 6% loss on drying at 105 °C after 2 hours. Any residual methanol levels should not exceed 200 mg per kg, and ethanol residues should not exceed 5,000 mg per kg. Arsenic and lead levels should not exceed 1 mg per kg. Microbiological criteria have also been established, with specifications of no more than 1,000 colony forming units (cfu) per g total plate count, not more than 200 cfu per g yeasts and molds, and *E. coli* and *Salmonella* negative in 1 g and 25 g, respectively.

Shandong has adopted specifications for our purified steviol glycosides extract starting material, which are compared with the current JECFA specifications in Table 2. The typical glycosides content of production batches is provided in Table 3.

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Table 2. Specifications for Steviol Glycosides Starting Material

Physical and Chemical Parameters	JECFA[a] Specifications Steviol Glycosides	Shandong's Specifications for Steviol Glycosides Starting Material
Appearance Form	Powder	Powder
Appearance Color	White to light yellow	White
Solubility	Freely soluble in 50:50 water: ethanol	Conform
Assay	Not less than 95% total steviol glycosides[b]	≥ 95% (on dry basis)
Residual Ethanol	NMT 5,000 mg/kg	≤5,000 ppm
Residual Methanol	NMT 200 mg/kg	≤200 ppm
Loss on Drying	NMT 6.0%	≤4.00%
pH, 1% Solution	4.5 - 7.0	4.5-7.0
Total Ash	NMT 1%	≤0.1%
Arsenic	NMT 1 mg/kg	≤0.1 ppm
Lead	NMT 1 mg/kg	≤0.1 ppm
Total Plate Count	NMT 1,000 cfu/g	≤1,000 cfu/g
Yeast & Mold	NMT 200 cfu/g	Negative
Salmonella	Negative in 25 g	Negative
Escherichia coli	Negative in 1 g	Negative

cfu – colony forming units; g – gram; kg – kilogram; mg – milligram; NMT – not more than; ppm – parts per million

[a] Prepared at 84th JECFA (2017)

[b] Total steviol glycosides as the sum of all compounds containing a steviol backbone conjugated to any number, combination, or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose xylose, galactose, arabinose, and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni

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Table 3. Typical Levels of Steviol Glycosides in Unmodified Stevia Extract and Prostevia High Purity Glucosylated Steviol Glycosides Preparations

Component	Unmodified Stevia Extract (%)	Prostevia High Purity Glucosylated Steviol Glycosides (%)
Rubusoside	0.85	--
Steviolbioside	0.00	--
Dulcoside A	0.97	--
Rebaudioside B	0.00	--
Stevioside	29.95	1.75
Rebaudioside C	8.45	--
Rebaudioside F	1.85	--
Rebaudioside A	52.35	3.39
Rebaudioside D	0.72	--
Monoglucosyl stevioside m/z 966	--	11.21
Monoglucosyl rebaudioside A m/z 1128	--	13.25
Diglucosyl stevioside m/z 1128	--	4.28
Diglucosyl rebaudioside A m/z 1290	--	11.33
Triglucosyl stevioside m/z 1290	--	9.82
Triglucosyl rebaudioside A m/z 1452	--	8.75
Tetraglucosyl stevioside m/z 1452	--	6.61
Tetraglucosyl rebaudioside A m/z 1614	--	5.32
Pentaglucosyl stevioside m/z 1614	--	1.66
Pentaglucosyl rebaudioside A m/z 1776	--	4.37
Hexaglucosyl stevioside m/z 1776	--	0.55
Hexaglucosyl rebaudioside A m/z 1938	--	1.89
Heptaglucosyl rebaudioside A m/z 2100	--	0.96
Octaglucosyl rebaudioside A m/z 2262	--	0.63
Nonaglucosyl rebaudioside A m/z 2424	--	0.37
Unidentified glucosylated steviol glycosides m/z > 2424	--	7.44

2. Specifications for Shandong's Prostevia High Purity Glucosylated Steviol Glycosides Preparations and Supporting Methods

Shandong has adopted product specifications for our Prostevia high purity glucosylated steviol glycosides preparation based upon current JECFA recommendations, while also complying with relevant Food Chemicals Codex (FCC) specifications for steviol glycosides as a consumable human food substance. The compositions of five non-consecutive lots of Shandong's Prostevia (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) preparation are compared with the JECFA and FCC specifications in Table 4.

Details of the analytical methodology employed to characterize and quantitate the steviol glycosides are provided in Appendix 2. The certificates of analysis and representative chromatograms for five representative lots of material are presented in Appendix 3 and Appendix 4. A test report for the analysis of pesticides residues in Prostevia is provided in Appendix 5. The collection of these reports demonstrates that Shandong's high purity enzyme modified steviol glycosides products are well-characterized and meet the established purity criteria.

D. Physical or Technical Effect

Shandong conducted sweetness equivalence evaluations for Prostevia high purity glucosylated steviol glycosides preparations. A taste panel determined that Prostevia is 200 times sweeter than sucrose (Appendix 6).

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Table 4. Specifications for Shandong's Prostevia Enzyme Modified Stevia Preparation

Physical & Chemical Parameters	JECFA[A] Specifications for Steviol Glycosides	FCC[B] Specifications for Steviol Glycosides	Shandong's Minimum Specifications for Prostevia Enzyme Modified Stevia	Results for Prostevia Enzyme Modified Stevia Preparations				
				Batch 20200503	Batch 20200510	Batch 20200517	Batch 20200522	Batch 20200528
Appearance Form	Powder	Powder, flakes, or granules	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder
Appearance Color	White to light yellow	White to light yellow	White	White	White	White	White	White
Solubility	Freely soluble in water: ethanol (50:50)	Freely soluble in water: ethanol (50:50)	Freely soluble in water and ethanol (1:1)	Conform	Conform	Conform	Conform	Conform
Purity (HPLC Area) ≥ 95% Total Steviol Glycosides ≥ 80% Glucosylated Steviol Glycosides ≤ 15% Unreacted Steviol Glycosides ≤ 5% Maltodextrin	≥ 95% Steviol Glycosides	≥ 95% Steviol Glycosides		95.18% 89.33% 5.85% 4.82%	95.13% 88.64% 6.49% 4.87%	95.16% 89.26% 5.90% 4.84%	95.10% 89.24% 5.86% 4.90%	95.15% 89.22% 5.93% 4.85%
Residual Ethanol	NMT 5,000 mg/kg	NMT 0.50%	NS[c]	NA	NA	NA	NA	NA
Residual Methanol	NMT 200 mg/kg	NMT 0.020%	NS[c]	NA	NA	NA	NA	NA
Loss on Drying	NMT 6.0%	NMT 6.0%	≤ 6%	3.12%	3.24%	3.34%	3.33%	3.41%
pH, 1% Solution	4.5 - 7.0	4.5 - 7.0	4.5-7.0	5.0	5.0	5.0	5.0	5.0
Total Ash	NMT 1%	NMT 1%	≤ 1%[d]	0.10%	0.09%	0.10%	0.10%	0.10%
Arsenic	NMT 1 mg/kg	NMT 1 mg/kg	≤ 0.1 ppm	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Lead	NMT 1 mg/kg	NMT 1 mg/kg	≤ 0.1 ppm	0.040 ppm	0.040 ppm	0.040 ppm	0.040 ppm	0.040 ppm
Cadmium	NS	NS	≤ 0.1 ppm	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Mercury	NS	NS	≤ 0.1 ppm	0.030 ppm	0.030 ppm	0.030 ppm	0.030 ppm	0.030 ppm
Total Plate Count (cfu/g)	NMT 1,000	NS	≤ 1,000	<1 ,000	< 1,000	< 1,000	< 1,000	< 1,000
Yeast & Mold (cfu/g)	NMT 200	NS	Negative	Negative	Negative	Negative	Negative	Negative
E. coli	Negative in 1 g	NS	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella spp.	Negative in 25 g	NS	NS	NA	NA	NA	NA	NA
Coliform (cfu/g)	NS	NS	Negative	Negative	Negative	Negative	Negative	Negative

cfu – colony forming units; g – gram; HPLC – high-performance liquid chromatography; kg – kilogram; mg – milligram; NA – not applicable; NMT – not more than; NS – not specified; ppm – parts per million

[a] Prepared at 84th JECFA (2017)

[b] Steviol Glycosides monograph. Food Chemicals Codex (12th Ed.) (FCC, 2020)

[c] No solvents are used to prepare Shandong's Prostevia Enzyme Modified Stevia preparation; therefore, no residual solvent specifications have been established.

[d] Reported as burned residue

E. Stability

1. Stability Data on Steviol Glycosides

The stabilities of steviol glycosides and enzyme modified steviol glycosides have previously been reviewed in a number of GRAS Notifications, including GRN 337 (NOW Foods, 2010), GRN 667 (Blue California, 2016), and GRN 715 (Blue California, 2017).

Stevioside has been reported to be stable over the pH range 3-9 and can be heated at 100ºC for 1 hour without decomposing, but, at pH values greater than 9, it rapidly decomposes (Kinghorn, 2002). A series of stability studies in food applications was conducted on stevioside by Kroyer (2010). Solid stevioside was reported to be stable at up to 120ºC for 1 hour. In aqueous solution, stevioside was reported to be stable at pH levels ranging from 2 to 10 for 2 hours at 60ºC. No degradation was observed after 4 months at room temperature for 1 g per L solutions of stevioside in acetic acid (pH 3.1), citric acid (pH 2.6), and tartaric acid (pH 2.6). A 30% loss of stevioside was observed in a 1 g per L solution of phosphoric acid (pH 1.6) stored under the same conditions. In addition, degradation was observed in 10 g per L solutions of stevioside in acetic acid (pH 2.6, 2% loss), citric acid (pH 2.1, 22% loss), tartaric acid (pH 2.1, 33% loss), and phosphoric acid (pH 1.6, 75% loss) after 4 months.

Kroyer (2010) reported no significant changes in the concentrations of B-vitamins incubated with stevioside in aqueous solution at 80ºC for 4 hours. A decrease in the degradation rate of vitamin C was observed after 4 hours under the same conditions, indicating that stevioside provides a protective effect. No stability effects or interactions were observed between mixtures of stevioside and saccharin, cyclamate, aspartame, acesulfame, and neohesperidin stored at 80ºC for 4 hours or room temperature for 4 months. Furthermore, no stability effects or interactions were observed between stevioside and caffeine in coffee and tea beverages at 80ºC for 4 hours. These results indicate that stevioside is stable under the intended conditions of use.

Buniowska et al. (2020) studied the stability of steviol glycosides (from an aqueous *Stevia rebaudiana* Bertoni leaf extract) in a fruit juice beverage after thermal treatment ranging from 60.0 to 99.0ºC. The authors reported that decreases in rebaudioside A, rebaudioside C, and rebaudioside F concentrations were observed after thermal processing at all temperatures, independent of initial concentration. However, for stevioside, both temperature and concentration affected the concentration after thermal processing, where stevioside was stable in solution at temperatures up to 80ºC.

In a shelf stability study conducted by Salar et al. (2020), a stevia sweetener of unknown composition that was purchased from Agriestevia S.L (Molina de Segura, Murcia, Spain) was used to sweeten a fruit juice prepared with maqui powder and lemon and other citrus juices at a concentration of 4 mg per 100 mL. The samples were pasteurized at 85ºC for 15 seconds, after which aliquots were drawn and used in studies that investigated the effects of light and

temperature on the stabilities of vitamin C and phenolic compounds. The stabilities of vitamin C and phenolic compounds in the beverages prepared with sucrose and stevia were similar. Storage temperature had more of an effect on the analyzed bioactive compounds than light exposure, which was not deemed to be a "critical factor." Compared with the sucrose-containing juice samples, there was a greater reduction in total flavonones content under light exposure at room temperature and a slightly higher loss of vitamin C during the first month was observed in juice containing the stevia sweetener. The authors noted that "stevia could be considered as an alternative sweetener by the industry," even with these observations.

Previously submitted GRAS Notices GRN 252 (Merisant, 2008), GRN 253 (Cargill, 2008), and GRN 304 (Sunwin/WILD, 2010) reported data indicating that rebaudioside A is stable under the intended conditions of use.

Furthermore, in the 64 GRAS Notices that have been submitted to FDA and have received "no questions" letters to date, the presented stability data have supported the position that steviol glycosides are stable and well-suited for the intended uses in foods (FDA, 2020).

2. Stability Data for Shandong's Prostevia High Purity Glucosylated Steviol Glycosides Preparation

Shandong is conducting ongoing long-term stability studies on three lots of our Prostevia high purity glucosylated steviol glycosides preparation. Samples were stored at 25°C ± 2°C at a relative humidity of 60% ± 5%. A summary of the stability results is provided in Table 5.

The stability data for steviol glycosides in the scientific literature, the JECFA report, and the extensive stability testing for rebaudioside A as presented by Merisant, Cargill, and Sunwin & WILD Flavors, along with Shandong's stability testing results, support the position that Shandong's Prostevia preparation is well-suited for the intended food uses.

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Table 5. Shandong's Prostevia Stability Data

BATCH NO: 20150523						
Duration	Appearance	Total Steviol Glycosides (%)	Glucosylated Steviol Glycosides (%)	Loss on Drying (%)	Total Plate Count (cfu/g)	Yeast & Mold
t=0	White Powder	95.21	83.22	4.66	< 1,000	Negative
6 months	White Powder	95.22	83.19	4.65	< 1,000	Negative
12 months	White Powder	95.18	83.20	4.62	< 1,000	Negative
18 months	White Powder	95.20	83.17	4.65	< 1,000	Negative
24 months	White Powder	95.18	83.15	4.68	< 1,000	Negative
BATCH NO: 20150627						
Duration	Appearance	Total Steviol Glycosides (%)	Glucosylated Steviol Glycosides (%)	Loss on Drying (%)	Total Plate Count (cfu/g)	Yeast & Mold
t=0	White Powder	95.49	82.55	4.55	< 1,000	Negative
6 months	White Powder	95.46	82.55	4.56	< 1,000	Negative
12 months	White Powder	95.48	82.53	4.58	< 1,000	Negative
18 months	White Powder	95.46	82.54	4.56	< 1,000	Negative
24 months	White Powder	95.47	82.51	4.58	< 1,000	Negative
BATCH NO: 20150623						
Duration	Appearance	Total Steviol Glycosides (%)	Glucosylated Steviol Glycosides (%)	Loss on Drying (%)	Total Plate Count (cfu/g)	Yeast & Mold
t=0	White Powder	97.22	95.12	4.61	< 1,000	Negative
6 months	White Powder	97.20	95.11	4.65	< 1,000	Negative
12 months	White Powder	97.20	95.14	4.63	< 1,000	Negative
18 months	White Powder	97.18	95.12	4.66	< 1,000	Negative
24 months	White Powder	97.17	95.11	4.66	< 1,000	Negative

cfu – colony forming unit; g – gram

F. Calculation of Steviol Equivalents of Prostevia Glucosylated Steviol Glycosides

For comparative purposes, the content of steviol glycosides is often expressed as steviol or steviol equivalents. Each component steviol glycoside has a steviol equivalence factor that is calculated based upon the ratio of the molecular weights (MW) of steviol and a particular steviol glycoside, as shown in Table 6.

Table 6. Steviol Equivalency Factors for Various Steviol Glycosides

Component Steviol Glycoside	Molecular Weight	Steviol Equivalency Factor[A]
Rubusoside	643	0.495
Steviolbioside	643	0.495
Dulcoside A	789	0.403
Rebaudioside B	805	0.395
Stevioside	805	0.395
Rebaudioside C	951	0.334
Rebaudioside F	937	0.339
Rebaudioside A	967	0.329
Rebaudioside D	1129	0.282

[a] Calculated by dividing the molecular weight of steviol (MW=318) by the molecular weight of each glycoside.

Using these steviol equivalency factors, along with the percent composition of the stevia extract starting material, it is possible to determine the steviol equivalency of the raw material steviol glycosides extract, as presented in Table 7.

Table 7. Steviol Equivalency of Steviol Glycosides Extract Raw Material

Component Steviol Glycoside	Typical Composition[A] (%)	Steviol Equivalents[B] (%)
Rubusoside	0.85	0.42
Steviolbioside	0.00	0.00
Dulcoside A	0.97	0.39
Rebaudioside B	0.00	0.00
Stevioside	29.95	11.83
Rebaudioside C	8.45	2.82
Rebaudioside F	1.85	0.627
Rebaudioside A	52.35	17.22
Rebaudioside D	0.72	0.20
Total Steviol Equivalence	--	33.51

[a] Based on the typical levels of steviol glycosides in the raw material steviol glycosides extract, as reported in Table 3.
[b] Calculated by multiplying the % of the steviol glycoside by the steviol equivalency factor.

The stevia extract starting material is enzymatically glycosylated as described in Part 2.B, in a process in which a glucosyltransferase enzyme adds glucose moieties, obtained from a maltodextrin source, to the steviol glycosides present in the raw material. It is reasonable to assume that all steviol glycosides and glucosylated steviol glycosides will maintain the same level of steviol equivalence described above since no other reactions are known to occur from the

known chemistry of the enzyme. Therefore, the steviol equivalency of the Prostevia preparation is expected to be no greater than 33.51 g steviol per 100 g Prostevia.

PART 3. DIETARY EXPOSURE

The subject Prostevia preparation is intended to be used as a table top sweetener and general purpose non-nutritive sweetener in various foods other than infant formulas and meat and poultry, as defined in 21 CFR 170.3(o)(19).[4] The intended use levels will vary by actual food category, but the actual levels are self-limiting due to organoleptic factors and consumer taste considerations. However, the amounts of Prostevia to be added to foods will not exceed the amounts reasonably required to accomplish the intended technical effect in foods as required by FDA regulation.[5]

A. Estimate of Dietary Exposure to the Substance

Many scholarly estimates of potential dietary intake replacement of sweeteners, including steviol glycosides have been published (FSANZ, 2008; WHO, 2003; Renwick, 2008) or submitted to FDA (Merisant, 2008). These are summarized in Appendix 7. In GRAS Notice 301, a simplified estimate was proposed to, and accepted by, FDA based on the estimates of exposure in "sucrose equivalents" (Renwick, 2008) and the sweetness intensity of any particular sweetener (BioVittoria, 2009). As summarized in GRN 301, the intake of a sweetener that is 100 times as sweet as sucrose when used as a total sugar replacer for a 90th percentile would be a maximum of 9.9 mg per kg body weight (bw) per day for any population subgroup.

The sweetness intensity of Prostevia is approximately 200 times that of sucrose. A weighted sum estimate was used to determine the steviol equivalency factor for Prostevia, which was determined to be 33.51 g steviol per 100 g Prostevia (as described in Part 2.F).

The highest 90th percentile consumption by any population subgroup of Prostevia would be approximately 4.95 mg per kg steviol glycosides bw per day. Based on a weighted sum estimate for steviol equivalents provided in Table 7, consumption would be less than 1.66 mg per kg bw per day on a steviol equivalents basis for any population group for Shandong's Prostevia preparation described herein. These calculations are summarized in Table 8.

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[4] Non-nutritive sweeteners: Substances having less than 2 percent of the caloric value of sucrose per equivalent unit of sweetening capacity.
[5] See 21 CFR 182.1(b)(1).

Table 8. Daily Intake of Sweeteners (in Sucrose Equivalents) and Estimated Daily Intakes of Prostevia

Population Group	Intakes of Sweeteners (mg sucrose/kg bw/day)[A]		Calculated Intake of Prostevia (mg/kg bw/day)[B]		Calculated Intake of Prostevia as Steviol Equivalents (mg/kg bw/day)	
	Low	High	Low	High	Low	High
Healthy Population	255	675	1.28	3.38	0.43	1.13
Diabetic Adults	280	897	1.40	4.49	0.47	1.50
Healthy Children	425	990	2.13	4.95	0.71	1.66
Diabetic Children	672	908	3.36	4.54	1.13	1.52

bw – body weight; kg – kilogram; mg – milligram

[a] From Renwick (2008)

[b] Calculated by dividing the sucrose intake by the minimum average relative sweetness value of 200 for Prostevia.

The values in Table 8 are based on the assumption that Shandong's Prostevia preparation constitutes the entire sweetener market, which makes these estimates extremely conservative since the likelihood of that occurrence is minimal. For the general healthy adult population, the estimated maximum intake of purified steviol glycosides is 3.38 mg per kg bw per day (1.13 mg per kg steviol equivalents) for Prostevia. For healthy children, the estimated maximal intake is 4.95 mg per kg bw per day (1.66 mg per kg as steviol equivalents) for Prostevia. In all population groups, the estimated daily intake of purified steviol glycosides, expressed as steviol equivalents, is below the JECFA-established acceptable daily intake (ADI) of 4.0 mg per kg bw per day steviol equivalents.

B. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed In or On Food

This section is not applicable to Shandong's Prostevia products, which would be chemically stable under conditions of use.

C. Dietary Exposure to Contaminants or Byproducts

While a recent publication by Kumari et al. (2016) investigated the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Antioxidant Capacity (TAC) of *S. rebaudiana* leaf --- and the observed activity has been attributed to naturally-occurring phytochemicals such as phenolics, flavonoids, and pigments in the plant --- the study has minimal relevance with regard to the safety considerations of highly purified stevia extract, of which ≥ 95% consists of the most familiar steviol glycosides and their glucosylated steviol glycosides. These phytochemical contaminants, if

present, are in low amounts and were likely similarly present in purified test materials that were used in the toxicology studies summarized in Appendix 8.

Furthermore, no concerns regarding dietary exposure to contaminants or byproducts have been raised by expert regulatory bodies, including the World Health Organization/Joint FAO/WHO Expert Committee on Food Additives (WHO/JECFA), European Food Safety Authority (EFSA), Food Standards Australia New Zealand (FSANZ), and FDA, since JECFA's first steviol glycosides review was performed in 2000 (WHO, 2000).

PART 4. SELF-LIMITING LEVELS OF USE

It has been well-documented in the published literature that the use of steviol glycosides is self-limiting due to organoleptic factors and consumer taste considerations (Kochikyan et al., 2006; Carakostas et al., 2008; Brandle et al., 1998; Prakash et al., 2008; Gupta et al., 2016; Gerwig et al., 2016). These organoleptic factors include bitterness and astringency, as well as a lingering metallic aftertaste (Gerwig et al., 2016).

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

A. Other Information on Dietary Exposure

1. History of Traditional Medicinal and Human Food Use

Stevia has been used as a traditional medicine and sweetener by native Guarani tribes for centuries (Esen, 2016; Gerwig et al., 2016; Brusick, 2008; Brandle et al., 1998). Hawke (2003) reported that stevia is commonly used as a treatment for type 2 diabetes in South America**.** However, therapeutic doses of 1 gram per person per day or more were reported to be necessary to achieve the desired effects (Gregersen et al., 2004).

For about 30 years, consumers in Japan and Brazil, where stevia has long been approved as a food additive, have been using stevia extracts as non-caloric sweeteners (Raintree, 2012). It was previously reported that 40% of the artificial sweetener market in Japan had been stevia based and that stevia is commonly used in processed foods in Japan (Lester, 1999). Use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and use as a natural health product is permitted in Canada. It has wide use in China and Japan in food and in dietary supplements. In 2005, it was estimated that sales of stevia in the US reached $45 million (Newsday, 2006).

NewHope360 reported that the global market for stevia in 2014 was $347 million and is expected to increase to $565.2 million by 2020. In addition, consumption was expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).

Most recently, Nutritional Outlook reported that Mintel data indicated a 48% increase in stevia-containing products over the last five years (Decker and Prince, 2018).

B. Summary of Regulatory History of Enzyme Modified Steviol Glycosides

Stevia-derived sweeteners are permitted as food additives in South America and in several countries in Asia, including China, Japan, and Korea. In recent years, these sweeteners have received food usage approvals in Mexico, Australia, New Zealand, Switzerland, France, Peru, Uruguay, Colombia, Senegal, Russia, Malaysia, Turkey, Taiwan, Thailand, Israel, Canada, and Hong Kong (EFSA, 2010; Watson, 2010; Health Canada, 2012). In the United States, steviol glycosides have been used as a dietary supplement since 1995 (Geuns, 2003).

A brief overview of the most recent regulatory activity regarding steviol glycosides is presented below in Part 5.B Sections 1-5; a more detailed historical overview is provided in Appendix 9.

1. U.S. Regulatory History

Based on available information from FDA's GRAS Notice Inventory website (FDA, 2020) as of June 23, 2020, FDA has issued 64 "no questions" letters on GRAS notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme modification.

In addition, the Flavor and Extract Manufacturers Association (FEMA) includes 20 steviol glycosides preparations, as detailed in Appendix 9, six of which are for enzymatically modified stevia extracts, on their GRAS lists.

2. Canadian Regulatory History

On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012). In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (Health Canada, 2014). On January 15, 2016, Health Canada approved the use of rebaudioside M as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (Health Canada, 2016).

Health Canada's Food Directorate updated its List of Permitted Sweeteners to allow for the use of steviol glycosides as a sweetener in "unstandardized snack bars," including granola bars, cereal bars, fiber bars, and protein isolate-based bars (Health Canada, 2017b). Health Canada (2017a) also modified the List of Permitted Sweeteners to include "all the steviol glycosides in the *Stevia rebaudiana* Bertoni plant (stevia plant)."

In April 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides from *Stevia rebaudiana* Bertoni in canned fruit products (Health Canada, 2019c). In May 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides derived from *Saccharomyces cerevisiae* strains CD15380 and CD15407 at the same maximum levels of use as steviol glycosides derived from *Stevia rebaudiana* Bertoni (Health Canada, 2019b). Most recently, on June 27, 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides from various sources in "standardized flavoured milks" (Health Canada, 2019a).

3. European Regulatory History

An amendment to the European Union (EU) food additives regulation 231/2012, which became active on November 3, 2016, removed the previous requirement for stevia blends to contain at least 75% Reb A or stevioside. In addition, the updated regulation ---(EU) 2016/1814---now permits the following steviol glycosides in stevia blends: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside, and dulcoside (Searby, 2016).

In 2017, JECFA updated the steviol glycosides specifications to include a minimum requirement of not less than 95% total steviol glycosides, on a dry basis, "determined as the sum of all compounds containing a steviol backbone conjugated to any number, combination or orientation of saccharides (glucose, rhamnose, fructose, deoxyglucose, xylose, galactose, arabinose and xylose) occurring in the leaves of *Stevia rebaudiana* Bertoni." Microbiological criteria were also established, with specifications of no more than 1,000 cfu per g total plate count, not more than 200 CFU per g yeasts and molds, and *E. coli* and *Salmonella* negative in 1 g and 25 g, respectively (FAO, 2017).

The European Food Safety Authority (EFSA) Panel of Food Additives and Nutrient Sources reviewed an application for glucosylated steviol glycoside preparations for use as a new food additive. The Panel concluded that the data supplied by the applicant were "insufficient to assess the safety" of the glucosylated steviol glycosides preparation. It should be noted that no safety concerns were raised in a more recent review by the EFSA Panel where their decision was based on the "limited" data provided in the dossier submitted by the applicant (EFSA, 2018).

On September 24, 2019, the EFSA Panel on Food Additives and Flavourings concluded that there is no safety concern for Rebaudioside M produced via enzymatic bioconversion and recommended that the European Commission consider establishing specifications for the preparation (EFSA, 2019).

On March 24, 2020, EFSA published a scientific opinion in response to a proposed amendment of the specifications for steviol glycosides, stating that all steviol glycosides share the same metabolic fate, and therefore the safety of 60 steviol glycosides identified in the leaves of *Stevia rebaudiana* Bertoni can be based on "read-across" from previously evaluated toxicological data. EFSA maintained that the ADI of 4 mg per kg bw applies to all 60 steviol glycosides. The EFSA Panel

noted that the inclusion of more steviol glycosides, “whilst maintaining the assay value of not less than 95%, would allow less pure preparations” onto the market. The Panel stated that they “cannot conclude on the safety of the proposed amendment to the specifications of steviol glycosides (E 960) as [a] food additive if the purity assay value of not less than 95% for the total content of steviol glycosides is maintained.” Furthermore, the Panel noted that it is possible to manufacture steviol glycosides with a purity higher than 95% total steviol glycosides (EFSA, 2020).

4. Asian Regulatory History

No regulatory updates have been identified in recent years. The Asian regulatory history for steviol glycosides through 2014 is presented in Appendix 9.

5. Other Regulatory History

FSANZ called for submissions on permitting all minor steviol glycosides extracted from stevia leaf to be included in the definition of steviol glycosides in the Food Standards Code, noting that “[no] evidence was found to suggest that the proposed changes pose any public health and safety concerns.” The submission period ended on December 19, 2016 (FSANZ, 2016b). Subsequently, on February 8, 2017, FSANZ approved a draft variation of the definition of steviol glycosides to include all steviol glycosides present in the *Stevia rebaudiana* leaf (FSANZ, 2017).

FSANZ called for comments on the production of Reb M using enzymes derived from genetically modified yeast. The comment period closed on August 31, 2018 (FSANZ, 2018b). Subsequently, on October 31, 2018, FSANZ approved a draft variation to include a reference to the production method (FSANZ, 2018a).

On May 14, 2020, FSANZ published an approval report for a draft variation to amend the specification for steviol glycosides from *Stevia rebaudiana* Bertoni in section S3—35 of the Australia New Zealand Food Standards Code to include rebaudioside E produced by enzymatic conversion from stevia leaf extract. The approved draft variation allows for the use of high purity rebaudioside E (≥85% rebaudioside E; ≥95% total steviol glycosides) within the existing permissions and limits for steviol glycosides (FSANZ, 2020a). Subsequently, on July 28, 2020, Amendment No. 193 was published to include rebaudioside E produced by enzymatic conversion from stevia leaf extract (FSANZ, 2020b).

PART 6. NARRATIVE

The biological, toxicological, and clinical effects of stevia and steviol glycosides have been extensively reviewed (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002). Additionally---and as noted earlier--- national and international regulatory agencies have thoroughly reviewed the safety of stevia and its glycosides. Most notably, over the years, JECFA has evaluated purified steviol glycosides multiple times (WHO, 2000; WHO, 2006; WHO, 2007; WHO, 2008), and their findings have been summarized in Part 5.B.3. FSANZ (2008) also evaluated steviol glycosides for use in

food. The JECFA reviews, as well as the other reviews completed before 2008, primarily focused on mixtures of steviol glycosides. These studies are summarized in Appendix 10.

Since the JECFA evaluation (WHO, 2008), FDA has received and not objected to over fifty-five GRAS notifications for steviol glycosides or enzyme modified steviol glycosides, many of which were discussed by Perrier et al. (2018). In each case, FDA has agreed with the conclusions that steviol glycosides are GRAS based largely on the 0-4 mg per kg bw per day ADI on a steviol equivalence basis that was established by JECFA. A publication by Roberts et al. (2016) indicates that the ADI could be higher, as discussed further in Appendix 7. Among the GRAS notifications submitted to FDA, several assessed purified preparations of rebaudioside A, and they were supported by additional toxicology and clinical studies that are summarized in Appendix 8.

Because of their sweetness characteristics, steviol glycosides have viable uses as a non-nutritive sweetener in foods.[6] Periodic reviews by JECFA over the years indicate the progression of knowledge on the toxicology of steviol glycosides. Several early safety-related studies on these compounds were performed on crude extracts of stevia. These studies also included multiple investigations with *in vivo* and *in vitro* models, which explored the biological activity of stevia extracts at high doses or high concentrations. These early investigations raised several concerns, including impairment of fertility, renal effects, interference with glucose metabolism, and inhibition of mitochondrial enzymes. In recent years, as more and more studies were performed on purified glycosides, the toxicology profile of steviol glycosides eventually proved to be rather unremarkable. A number of subchronic, chronic, and reproductive studies have been conducted in laboratory animals. These studies were well designed with appropriate dosing regimens and adequate numbers of animals to maximize the probability of detection of important effects. Notably, the initially reported concerns related to the effects of stevia leaves or crude extracts on fertility were refuted by the well-designed reproductive studies with purified steviol glycosides. All other concerns failed to manifest themselves at the doses employed in the long-term rat studies.

As discussed in Appendix 10 and elsewhere, at its 51st meeting, JECFA determined that there were adequate chronic studies in rats, particularly the study by Toyoda et al. (1997), to establish a temporary ADI of 0 - 2 mg per kg bw per day with an adequate margin of safety (Toyoda et al., 1997). The Committee also critically reviewed the lack of carcinogenic response in well-conducted studies. These studies validated the Committee's conclusion that the *in vitro* mutagenic activity of steviol did not present a risk of carcinogenic effects *in vivo* and, therefore, all common steviol glycosides that likely share the same basic metabolic and excretory pathway and that use high purity preparations of various steviol glycosides, are safe as sugar substitutes. Subsequently, the

[6] It has also been reported that steviol glycosides may have pharmacological properties, which can be used to treat certain disease conditions such as hypertension and type 2 diabetes. Chatsudthipong and Muanprasat (2009), as well as others, have published reviews where they note that such therapeutic applications have not been firmly established as being due to steviol glycosides. The reviewers point out that the effects occur at higher doses than would be used for sweetening purposes. Furthermore, many effects noted in older studies may have been due to impurities in preparations that do not meet the contemporary purity specifications established by JECFA for use as a sweetener. If oral doses of steviol glycosides impart pharmacological effects, such effects would undoubtedly occur due to actions of the principal metabolite, steviol, but the pharmacological effects of steviol have not been comprehensively investigated.

additional clinical data reviewed by JECFA allowed the Committee to establish a permanent ADI of 0 - 4 mg per kg bw per day (based on steviol equivalents).

More recently, JECFA published a safety evaluation of a number of food additives, including steviol glycosides (WHO, 2017). The JECFA Committee reviewed information supporting the safety of a *Yarrowia lipolytica* fermentation-produced rebaudioside A, which included a 90-day rat toxicity study and two *in vitro* genotoxicity studies, as well as *in vitro* colonic microflorae hydrolysis studies in several steviol glycosides, toxicokinetic studies of stevioside in humans and rats, and literature published since the 69th meeting.

The Committee noted that the most recent short-term toxicity studies were consistent with those reviewed at or prior to the 69th meeting, and that the new toxicokinetic study in humans did not have a large enough subject pool to provide reliable toxicokinetic estimates to derive an update ADI for steviol glycosides. The Committee confirmed the current ADI of 0 - 4 mg per kg bw steviol. In addition, the Committee prepared new “tentative” specifications for steviol glycosides, which were expanded to include “any mixture of steviol glycosides compounds derived from *S. rebaudiana* Bertoni” while retaining the requirement that the total percentage of steviol glycosides is ≥ 95% (WHO, 2017).

Shandong critically reviewed the JECFA assessments and agrees with the calculation of the ADI for steviol glycosides.

Several published and unpublished studies (summarized in Appendix 8) on purified preparations of rebaudioside A showed an absence of toxicological effects in rats (Curry and Roberts, 2008; Nikiforov and Eapen, 2008) and dogs (Eapen, 2008) in subchronic studies, and an absence of reproductive (Curry et al., 2008; Sloter, 2008a) and developmental effects (Sloter, 2008b) in rats. Most notably, pharmacokinetic studies in rats (Roberts and Renwick, 2008) and humans (Wheeler et al., 2008) on purified rebaudioside A follow the same pathway of being degraded to steviol by intestinal bacteria with subsequent rapid glucosylation and elimination in urine and feces.

Most recently, Purkayastha and Kwok (2020) investigated the metabolic fate of steviol glycosides in fecal homogenates collected from adults and children. Steviol glycosides obtained from stevia leaf extract (composed of more than 20 steviol glycosides, with Reb D and Reb M as the principal components), bioconversion reaction product (composed of Reb D and Reb M), minor steviol glycosides extracted from a stevia leaf extract (composed of Reb AM, Reb W2, Reb U2, Reb V, Reb N, and Reb O), enzyme modified steviol glycosides, and rebaudioside A standard were used as test samples for *in vitro* incubation in pooled human fecal homogenate samples obtained from adult and pediatric donors. Purkayastha and Kwok reported that all steviol glycosides preparations tested “shared qualitatively similar *in vitro* metabolic fates.” In addition, the authors concluded that “safety data for individual steviol glycosides can be used to support safety of all steviol glycosides produced by extraction and enzymatic conversion of stevia leaf extract.”

Shandong concludes that these studies on rebaudioside A and other enzyme modified steviol glycosides preparations strengthen the argument that all steviol glycosides that follow the same metabolic pathway are safe at the JECFA established ADI.

Shandong has also reviewed the findings from human clinical studies. Shandong noted that the clinical effects reported in humans occurred in patients with either elevated blood glucose or blood pressure (or both). JECFA called for studies in individuals that are neither hypertensive nor diabetic (WHO, 2006). The supplemental data presented to JECFA and also published by Barriocanal et al. (2008) demonstrate the lack of pharmacological effects of steviol glycosides at 11 mg per kg bw per day in normal individuals, or approximately slightly more than 4 mg per kg bw on the basis of steviol equivalents (Barriocanal et al., 2008). Clinical studies on purified rebaudioside A showed an absence of effects on blood pressure (Maki et al., 2008a) and blood glucose levels (Maki et al., 2008b) at doses slightly higher than the exposures expected in food. Shandong concludes that there will be no effects on blood pressure and glucose metabolism in humans at the doses of steviol glycosides expected from its use in food as a non-nutritive sweetener.

Two previously published studies summarized in Appendix 8 raised a potential concern regarding the toxicological effects of steviol glycosides. In one study, DNA damage was seen in a variety of organs as assessed by Comet assay in rats given drinking water containing 4 mg per mL steviol glycosides for up to 45 days (Nunes et al., 2007a). Several experts in the field have since questioned the methodology used in this study (Geuns, 2007; Williams, 2007; Brusick, 2008). Shandong has reviewed the cited publications, along with the responses made by the authors (Nunes et al., 2007b; Nunes et al., 2007c), and concurs with the challenges to the methodology utilized by Nunes et al. (2007a), thereby discounting the validity and relevance of this study.

In another study with stevioside in rats, tartrate-resistant alkaline phosphatase (TRAP) levels were measured and found to be significantly decreased at doses as low as 15 mg per kg bw (Awney et al., 2011). TRAP is an enzyme that is expressed by bone-resorbing osteoclasts, inflammatory macrophages, and dendritic cells. This enzyme was not measured in any previous toxicology studies on steviol glycosides, nor has it been adequately vetted for application in toxicological studies. Critical reviews of this study by Carakostas (2012) and Waddell (2011) revealed a poor study design that included: insufficient numbers of animals; group-housing with the potential for stress-related changes; unreliable access to steviol *via* drinking water resulting in suspect dosing calculations in group-housed cages; no indication of fasting prior to blood collection (which affects many chemistry and hematological values); no urine collection; and no histopathological evaluations for confirmation of findings beyond the controls. Additionally, the report did not adequately describe mean or individual organ weight data, and it lacked comparison of study findings against laboratory historical control data. Shandong concurs with Carakostas (2012) and Waddell (2011) evaluations of the Awney et al. (2011) study and concludes that it does not impact the safety discussion of Shandong's Prostevia high purity glucosylated steviol glycosides.

Urban et al. (2013) examined the extensive genotoxicity database on steviol glycosides because some concern has been expressed in two publications (Brahmachari et al., 2011; Tandel, 2011) in which the authors concluded that additional testing is necessary to adequately address the genotoxicity profile (Urban et al., 2013). The review aimed to address this matter by evaluating the specific genotoxicity studies of concern, while evaluating the adequacy of the database that includes more recent genotoxicity data not noted in these publications. The results of this literature review showed that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside A is genotoxic. This finding, combined with a paucity of evidence for neoplasm development in rat bioassays, establishes the safety of all steviol glycosides with respect to their genotoxic/carcinogenic potential. Shandong agrees with the Urban et al. (2013) conclusions.

In addition, a paper by Shannon et al. (2016) raises a possible concern of endocrine disruption by steviol. Shandong reviewed the publication and noted that the effects on progesterone production and on the action of progesterone (both antagonistic and agonistic) were observed *in vitro* in sperm cells. Shandong concludes that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at receptors and that no adverse effects were observed in well-conducted reproductive toxicology studies. Therefore, this study does not alter Shandong's opinion that steviol glycosides preparations are generally recognized as safe. A summary of this study is provided in Appendix 8.

A commercially available steviol glycoside extract (>99%, composition and brand unknown) was used to investigate genotoxicity in human peripheral blood lymphocytes. Uçar et al. (2017) observed no significant differences in chromosomal aberration induction or micronuclei between the control and treatment groups at 24 and 48 h. These data support previous findings that steviol glycosides are not genotoxic.

Thøgersen et al. (2018) investigated the effect of rebaudioside A, stevioside, and steviol on porcine cytochrome p450 (CYP) expression and activity to assess their potential food-drug interactions in the IPEC-J2 cell line, which is a non-transformed cell line derived from intestinal porcine epithelial cells and in primary hepatocytes. The authors reported that there were no changes in CYP messenger ribonucleic acid (mRNA) expression following treatment of IPEC-J2 cells with rebaudioside A, stevioside, and steviol compared with control. Treatment of primary hepatocytes resulted in increases in CYP329 mRNA at low concentrations of rebaudioside A and steviol, and at all concentrations of stevioside. The authors reported that while treatment with the steviol glycosides tested over 24 hours resulted in minor increases in CYP3A29 mRNA expression (< 2.0 fold), "no direct effect on CYP activity" was observed. The authors concluded that rebaudioside A, stevioside, and steviol are unlikely to cause a food-drug interaction but noted that the study could not predict long term effects and effects *in vivo*.

A recently published study addressed the genotoxic activity of stevia (Svetia™, purity not reported[7]). Human lymphocytes were treated with 5% and 0.5% Svetia™ for 2 hours. No statistically significant difference in genetic damage was observed in the 0.5% treatment concentration compared with the negative control, while the 5% treatment concentration resulted in a statistically significant difference ($P<0.0001$) compared with the control, with a decrease in migration average. The authors described the effect as being beneficial. Human lymphocytes treated with 10% Svetia™ demonstrated significant ($P<0.0001$) genotoxic activity compared to the control; however, at treatment concentrations of 0.05%, 0.5%, and 5% Svetia™, a significant ($P<0.0001$) decrease in average migration of DNA was observed compared with the control. The authors conclude that these results demonstrate the absence of genotoxicity at concentrations up to 5% Svetia™ (Silva et al., 2018). It should be noted that these observations are inconsistent with data reported by Nunes et al. (2007a); however, as discussed above, the validity and importance of the Nunes et al. study has been discounted given the questions surrounding the methodology.

In a seven-week study, 13 subjects (ages 18 to 30 years) without a history of hypertension or hyperglycemia were supplemented with four commercial 1 g packets per day of steviol glycosides (equivalent to 0.1 g steviol glycosides per day, brand and composition not reported) for six weeks. Decreases in triglyceride, cholesterol, and serum TNF-α concentrations were observed. No adverse effects were reported (Sánchez-Delgado et al., 2019).

Wang and Wu (2019) investigated the angiotensin-converting enzyme (ACE) inhibiting activity of a 95% pure steviol glycosides extract (composition not reported) obtained from an ethanol extract of stevia leaves. Steviol glycosides were reported to have double the ACE inhibitory activity of an ethanolic extract of steviol leaves, were well-accepted in a sensory test in decaffeinated coffee, decaffeinated tea, and peanut protein beverages, and had a significant antihypertensive effect in spontaneously hypertensive rats. No adverse events in humans or rats were reported.

The interaction between select prescription drugs and steviol acyl glucuronide, the major metabolite of rebaudioside A, was investigated by Zhou et al. (2019). Organic anion transporter 3 (OAT3) – mediated update of steviol acyl glucuronide was examined *in vitro* using human embryonic kidney 293 (HEK293) cells. HEK293 cells were transfected with human organic anion transporter 3 (hOAT3) and rat organic anion transporter 3 (rOAT3). Both probenecid and glimepiride were potent inhibitors of hOAT3 and rOAT3 with no apparent species differences observed. Pharmacokinetic studies in male Sprague Dawley rats revealed that both probenecid and glimepiride significantly elevated plasma steviol acyl glucuronide concentrations, particularly between 6 and 8 hours after oral administration of rebaudioside A. The inhibition of OAT3 is a potential mechanism for the interaction between steviol acyl glucuronide and probenecid and glimepiride, which could be clinically relevant. The authors concluded that “care should be given to populations with concomitant use of stevia leaf extracts and probenecid or glimepiride.”

[7] While the purity of the material used for the study was not reported by Silva et al. (2018), a search of the manufacturer’s website (www.svetia.us) indicates that the trademarked material is a blend of cane sugar and 97% pure Reb A.

Shandong has reviewed the Zhou et al. (2019) publication in detail and notes that the pharmacokinetic oral dose used in the study was 15 mg per kg rebaudioside A. Plasma concentrations of steviol acyl glucuronide were observed to be dose-dependent after oral administration of rebaudioside A, with an average concentration maximum (C_{max}) of approximately 39 ng per mL at 5 mg per kg rebaudioside A and 170 ng per mL at 15 mg per kg rebaudioside A, respectively, observed at 6 hours post-dosing. Given the observed dose-dependency, it is possible that a reduced interaction would be observed between steviol acyl glucuronide and probenecid and glimiperide at lower doses of rebaudioside A. Based on a steviol equivalence factor of 0.329 for rebaudioside A as listed in Table 6, the 15 mg per kw dose corresponds to 4.9 mg per kg steviol equivalents, which is higher than the JECFA-established ADI of 4.0 mg per kg bw per day. Given that the investigational dose is higher than the accepted ADI for steviol glycosides and the history of safe use of steviol glycosides, including the paucity of reported case studies regarding the concomitant ingestion of steviol glycosides and probenecid and glimiperide, Shandong concludes that the use of steviol glycosides as proposed herein remains safe for the general population and agrees with Zhou et al. (2019) that care should be exercised in the small subset of the population for which probenecid and glimiperide are prescribed.

Halasa et al. (2020) published a case study vignette on the investigation of the presence of steviol glycosides metabolites in plasma, cerebrospinal fluid, amniotic fluid, and cord blood in samples collected as early as 2004. The end date was not provided. Steviol glucuronide, the primary steviol metabolite, was detected in all types of samples, but was observed primarily in the plasma. Of the samples, seven of the 38 adults (18%) had detectable steviol glucuronide concentrations, while two of 13 (15%) amniotic fluid samples and one of 15 (7%) cord blood samples contained steviol glucuronide. The authors noted that steviol glucuronide was detected only in samples obtained in and after 2008, which corresponds to the dates of the first GRAS Notices submitted to FDA for steviol glycosides.

It should be noted that Halasa et al. (2020) did not discuss their findings in relation to the time of consumption and intake levels of steviol glycosides. As steviol glucuronide is a known metabolite of steviol glycosides and is expected to be observed in plasma following steviol glycosides ingestion, this study serves to support previous published findings.

Recent studies in rats have been identified in the published literature. Assi et al. (2020) investigated the use of an ethanolic extract of dried *S. rebaudiana* leaves (chemical composition not reported) to treat diabetic rats. No adverse effects or unplanned animal deaths were reported.

Cho et al. (2018) investigated the impact of stevia and obesity on fertility and reproductive outcomes in Sprague Dawley rats. Rats were administered 2-3 mg per kg bw per day rebaudioside A in drinking water starting two weeks prior to mating and throughout lactation. The authors reported that obese rats supplemented with rebaudioside A displayed a lower fertility index than untreated obese rats (53.3% vs. 85.7%, respectively); however, the rate of successful pregnancies

was higher in obese rats supplemented with rebaudioside A than untreated obese rats (100% vs. 60.7%). No animal deaths were reported.

In a follow up study, the impact of maternal low-dose rebaudioside A consumption on adiposity, glucose tolerance, gut microbiota, and the mesolimbic pathway in obese dams and their offspring was investigated (Nettleton et al., 2020). Pregnant obese rats and their offspring were fed high fat/sucrose diet plus 3 mg per kg bw per day rebaudioside A (Sigma-Aldrich) through 18 weeks postpartum. The authors noted that rebaudioside A consumption reduced the fertility of dams, as previously reported (Cho et al., 2018). The study supports findings that low-calorie sweeteners may not be metabolically inert. No animal deaths were reported.

Shandong notes that the effect of steviol glycosides on fertility and reproductive outcomes has been the subject of a number of investigations as discussed further in Appendix 9, and that these recent publications by Cho et al. (2018) and Nettleton et al. (2020) corroborate previous findings by Planas and Kuć (1968), where 5% crude stevia leaf extract was observed to reduce fertility to 21% in female rats.

The effects of non-nutritive low-calorie sweeteners on gut microbiota were reviewed by Plaza-Diaz et al. (2020). It was noted that there have been no reports of negative interactions between steviol glycosides and colonic microbiota; however, it is possible that steviol glycosides modify the gut microbiota. The authors note further studies are necessary to “clarify its specific effects.”

A recent review by Ray et al. (2020) focused on the effects of *Stevia rebaudiana* on glucose homeostasis, blood pressure, and inflammation. The authors reported that no hypersensitivities or allergies were reported since 2008, and that the few prior reports were for “improperly filtered stevia extracts.” Furthermore, Ray et al. noted that additional randomized controlled trials are needed to confirm the beneficial effects of stevia. No significant adverse effects were noted from any study included in the review.

Zhao et al. (2020) reported that stevioside improved hyperglycemia-induced cardiac dysfunction in male C57BL/6 mice. Stevioside supplementation reduced the expression levels of cardiac fibrosis producing lysyl oxidase family and weakened the collagen cross-linking lysyl oxidase-like 2 caused by hyperglycemia, as well as promoted the elimination of existing fibrosis via the regulation of matrix metalloproteinase and tissue inhibitors of metalloproteinase. No adverse events or unplanned deaths were reported.

The effect of steviol on cytotoxicity, adipogenesis, ROS concentration, and gene expression were studied in the murine 3T3-L1 cell line. Kurek et al. (2020) reported that there was no observed effect on the proliferation of cells, lipid accumulation, or intracellular ROS generation at steviol concentrations up to 100 μM. Furthermore, it was reported that steviol reduced the expression of genes regulating the adipogenesis and lipogenesis process. Results of this study further supports the safety of steviol—and by extension—steviol glycosides.

Abolhasani et al. (2020) evaluated the *in vitro* cytotoxicity of stevioside on cancerous liver (HepG2), colon (HT29), and breast (MCF7) cells, as well as normal kidney cells (Hek293), compared to cisplatin. Stevioside was reported to display higher cell growth inhibition on the HepG2 cell line and was not observed to have high toxicity on the Hek293 normal cell line. The authors concluded that stevioside "showed less cytotoxic effects compared to cisplatin" (abstract only).

Shandong agrees with the safety conclusions of the 64 GRAS Expert Panels in the notices for steviol glycosides previously submitted to FDA that resulted in "no questions" responses from FDA, JECFA (WHO, 2006; WHO, 2008), and Renwick (2008) that a sufficient number of good quality health and safety studies exist to support the determination that purified preparations of steviol glycosides, when added to food at levels up to full replacement of sucrose on a sweetness equivalency basis, meet FDA's definition of safe.

Shandong concludes that it is reasonable to apply the JECFA ADI of 4 mg per kg bw per day for steviol glycosides (expressed on a steviol basis) to Prostevia high purity glucosylated steviol glycosides. Therefore, with the steviol equivalence values shown in Table 8, Shandong concludes that, for the general population, the estimated maximum daily intake of the Prostevia preparation described herein is 4.95 mg per kg bw or 1.66 mg per kg expressed as steviol equivalents. Based upon these calculations, the intake of Shandong's Prostevia high purity glucosylated steviol glycosides preparation described herein safely aligns with the 4 mg per kg bw per day ADI expressed as steviol equivalents as determined by JECFA.

The raw material steviol glycosides extract used to manufacture Shandong's Prostevia high purity glucosylated steviol glycosides preparation contain not less than 95% total steviol glycosides. The finished high purity glucosylated steviol glycosides preparations are a mixture of glucosylated steviol glycosides, unreacted steviol glycosides, and unreacted maltodextrin, where Prostevia is ≥95% total glucosylated steviol glycosides and unreacted steviol glycosides. Given the structural similarities with rebaudioside A, stevioside, and other steviol glycosides, and considering analogous metabolic pathways for all these substances, the safety data on stevia and its other components have a direct bearing on the present safety assessment for Prostevia. This is further supported by over a decade and a half of scientific studies on the safety of these substances, along with the fact that the major regulatory bodies view the results of toxicology studies on either stevioside or rebaudioside A as applicable to the safety assessment of all known steviol glycosides, since all are metabolized and excreted by similar pathways, with steviol being the common metabolite for each. The foundational safety of Reb A, other steviol glycosides and steviol has been summarized, with key studies summarized in Appendix 8.

Furthermore, Shandong has reviewed this safety information and have concluded that Prostevia high purity glucosylated steviol glycosides is generally recognized as safe for the proposed uses.

A. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "…reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use."[8]

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "…General recognition of safety requires common knowledge, throughout the expert scientific community knowledgeable about the safety of substances directly or indirectly added to food, that there is reasonable certainty that the substance is not harmful under the conditions of its intended use."
>
> "'Common knowledge' can be based on either "scientific procedures" or on experience based on common use of a substance in food prior to January 1, 1958." [9]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[10]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally

[8] See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 6/22/20).
[9] See 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 9/8/18).
[10] See Footnote 1.

available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

The apparent imprecision of the terms “appreciable,” “at the time,” and “reasonable certainty” demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

As noted below, this safety assessment to ascertain GRAS status for high purity steviol glycosides for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Expert Panel Findings on Safety of Prostevia High Purity Glucosylated Steviol Glycosides

An evaluation of the safety and GRAS status of the intended use of Shandong’s Prostevia high purity glucosylated steviol glycosides (≥95% total unreacted steviol glycosides and glucosylated steviol glycosides) preparation has been conducted by an Expert Panel convened by GRAS Associates; the Panel consisted of Robert Kapp, Ph.D., Fellow Academy of Toxicological Sciences (ATS), Fellow Royal Society of Biology (FRSB) and European Registered Toxicologist (ERT, UK); Kara Lewis, Ph.D.; and Katrina Emmel, Ph.D., as Panel Chair. The Expert Panel reviewed Shandong’s dossier as well as other publicly available information available to them. The individuals who served as Expert Panelists are qualified to evaluate the safety of foods and food ingredients by merit of scientific training and experience.

The GRAS Expert Panel report is provided in Appendix 11.

C. Common Knowledge Elements for GRAS Conclusions

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS conclusion requires that consensus exists within the broader scientific community.

1. Public Availability of Scientific Information

The majority of the studies reviewed on steviol glycosides and steviol have been published in the scientific literature as summarized in Appendix 8. Most of the literature relied upon by JECFA has also been published---most importantly the chronic rat studies on steviol glycosides. JECFA did make limited use of unpublished studies, and they were summarized in the two JECFA monographs. Moreover, JECFA publicly releases the results of their safety reviews, and their meeting summaries and monographs are readily available on their website.

With regard to the safety documentation, the key pharmacokinetic data establish that steviol glycosides are not absorbed through the gastrointestinal (GI) tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but rapidly metabolized and excreted (Gardana et al., 2003; Koyama et al., 2003b). The action of bacteria in the large intestine is directly supported by the published study that showed that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003b; Renwick and Tarka, 2008).

The ADI for steviol glycosides has been set largely based on a published chronic study in rats (Toyoda et al., 1997) and several published clinical studies that report no pharmacological effects in humans at doses several fold higher than the ADI (Barriocanal et al., 2006; Barriocanal et al., 2008; Wheeler et al., 2008). As mentioned above, Roberts et al. (2016) noted that the ADI could be higher using a chemical-specific adjustment factor (CSAF), as defined by the WHO in 2005, determined by comparative studies in rats and humans, which they conclude can justify an ADI value of 6-16 mg per kg bw per day for steviol glycosides.

The toxicity of the metabolite, steviol, has been well reviewed in the published literature (Geuns, 2003; WHO, 2006; Urban et al., 2013).

In addition, there is a large, publicly available, collection of GRNs regarding steviol glycosides on FDA's website.

2. Scientific Consensus

The second common knowledge element for a GRAS conclusion requires that there be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use.

A number of well-respected regulatory agencies, including JECFA, EFSA, FSANZ, the Switzerland Office of Public Health, and Health Canada, as well as numerous well-respected individual scientists, have indicated that steviol glycosides are safe for human consumption at doses in the range of the JECFA ADI (FAO, 2010; EFSA, 2010; FSANZ, 2008; Switzerland Federal Office of Public Health, 2008; Health Canada, 2012; Xili et al., 1992; Toyoda et al., 1997; Geuns, 2003; Williams, 2007). Since December 2008, over fifty-five GRAS notifications have been submitted to FDA for highly purified stevia-derived sweetener products, and FDA detailed reviews have consistently yielded "no questions" letters.

In summary, a compelling case can be made that scientific consensus exists regarding the safety of steviol glycosides when of sufficiently high purity. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which the various steviol glycosides molecules are metabolized and eliminated from the body. While the scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, Shandong believes that a wide consensus does exist in the scientific community to

support a GRAS conclusion as evidenced by several publications (Carakostas, 2012; Geuns, 2007; Urban et al., 2013; Waddell, 2011; Williams, 2007; Brusick, 2008) that refute safety concerns expressed by a minority of scientists. Roberts et al. (2016) suggests that the ADI could be higher than has been previously accepted by the scientific community.

D. Conclusion

In consideration of the aggregate safety information available on naturally occurring steviol glycosides, Shandong concludes that Prostevia high purity glucosylated steviol glycosides (≥95% total unreacted steviol glycosides and glucosylated steviol glycosides) defined in the subject notification are safe for use as a general purpose non-nutritive sweetener in foods other than infant formulas and meat and poultry products. The JECFA ADI for steviol glycosides of 4 mg per kg bw per day (as steviol equivalents) can be applied to Shandong's Prostevia preparation. Based on published dietary exposure data for other approved sweeteners and adjusting for relative sweetness intensity, intake was estimated for healthy non-diabetic children and adults, and diabetic children and adults with the following findings.

The worst-case estimated intakes of Shandong's Prostevia preparation for several population groups summarized in Part 3.A. are no greater than 1.66 mg per kg steviol equivalents per bw per day, which is well below the ADI of 4 mg per kg bw expressed as steviol equivalents as established by JECFA. The dietary levels from anticipated food consumption are not likely to exceed the ADI when high purity glucosylated steviol glycosides composed of at least 95% total unreacted steviol glycosides and glucosylated steviol glycosides mixed with unreacted maltodextrin are used as a general non-nutritive sweetener.

Accordingly, Prostevia high purity glucosylated steviol glycosides (≥95% total unreacted steviol glycosides and glucosylated steviol glycosides) as produced by Shandong and declared within the subject notification meet FDA's definition of safety in that there is "reasonable certainty of no harm under the intended conditions of use" as described herein and, therefore, are generally recognized as safe (GRAS).

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PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE.

A. References

1. List of Acronyms

ACE	Angiotensin-converting enzyme
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism and Excretion
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATS	Academy of Toxicological Sciences
AUC	Area under the plasma-concentration time curve
AVA	Agri-food and Veterinary Authority of Singapore
BP	Blood pressure
bw	Body Weight
CFR	Code of Federal Regulations
cfu	Colony Forming Unit
CGMP	Current Good Manufacturing Practice
CGTase	Cyclomaltodextrin glucanotransferase
C_{max}	Maximum (peak) serum concentration of substance is observed
C_{max}	Maximum serum concentration
CSAF	Chemical-Specific Adjustment Factor
CYP	Cytochrome P450
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic Acid
EDI	Estimated daily intake
EFSA	European Food Safety Authority
ERT	European Registered Toxicologist
EU	European Union
FAO/WHO	Food and Agriculture Organization of the United Nations/World Health Organization
FCC	Food Chemicals Codex
FD&C Act	Federal Food Drug and Cosmetics Act
FDA	Food and Drug Administration
FEMA	Flavor Extract Manufacturers Association
FOIA	Freedom of Information Act
FRSB	Fellow Royal Society of Biology
FSANZ	Food Standards Australia New Zealand
FSSAI	Food Safety and Standards Authority of India
g	Gram
GA	GRAS Associates
GEMS	Global Environment Monitoring System
GGT	Gamma-glutamyltransferase
GI	Gastrointestinal
GRAS	Generally Recognized as Safe
GRNs	GRAS Notices
h or hr	Hour
HbA1c	Glycated hemoglobin
HEK293	Human embryonic kidney 293

hOAT3	Human organic anion transporter 3
HPLC	High-Performance Liquid Chromatography
IADSA	International Alliance of Dietary/Food Supplement Associations
INS	International Numbering System
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Killogram
kg	Kilogram
LD_{50}	Median (50%) lethal dose
mg	Milligram
mL	Milliliter
MPL	Maximum permitted level
mpn	Most probable number
mRNA	Messenger ribonucleic acid
MW	Molecular Weight
NA	Not applicable
ng	Nanogram
NHANES	National Health and Nutrition Examination Surveys
NHPs	Natural Health Products
NMT	Not more than
NOAEL	No observed adverse effect level
NS	Not specified
OAT3	Organic anion transporter 3
OECD	Organisation for Economic Co-operation and Development
ppm	Parts per million
Reb A	Rebaudioside A
Reb M	Rebaudioside M
rOAT3	Rat organic anion transporter 3
SBP	Systolic blood pressure
TAC	Total antioxidant capacity
TFC	Total flavonoid content
T_{max}	Time at which maximum (peak) plasma concentration (C_{max}) of substance is observed
TPC	Total phenolic content
TRAP	Tartrate-resistant alkaline phosphatase
UDS	Unscheduled DNA synthesis
ug	Microgram
WHO	World Health Organization
WHO/JECFA	World Health Organizaiton/Joint FAO/WHO Expert Committee on Food Additives

2. References

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B. Appendices

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Appendix 1 Specifications and Certificates of Analyses for Raw Materials and Production Processing Aids

Appendix 1.1 Steviol Glycosides Extract

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Rebaudioside A 50%/stevia 95% Manufacture Date: 2020.01.04
Latin Name: Stevia Rebaudiana Bertoni Batch No: 20200102
Plant part used: Stevia leaves Expire Date: 2022.01.03

ITEM	SPECIFICATION	TEST RESULTS	Standards
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic	Visual Gustation
CHEMICAL TESTS			
Steviol glycosides (% dry basis)	≥95	95.14	HPLC
Rebaudioside D %		0.72	HPLC
Rebaudioside A %	≥50	52.35	HPLC
Stevioside %		29.95	HPLC
Rebaudioside F %		1.85	HPLC
Rebaudioside C %		8.45	HPLC
DulcosideA %		0.97	HPLC
Rubusodide %		0.85	HPLC
Rebaudioside B%		0.00	HPLC
Steviobioside %		0.00	HPLC
Loss on Drying (%)	≤4.00	3.12	CP/USP
Ash (%)	≤0.1	0.08	GB(1g/580C/2hrs)
PH (1% solution)	4.5-7.0	5.0	
Specific Optical Rotation	-30°~-38°	-34.5°	GB
Specific Absorbance	≤0.05	0.020	GB
Lead (ppm)	≤0.1	0.04	CP
Arsenic(ppm)	≤0.1	Negative	CP
Cadmium (ppm)	≤0.1	Negative	CP
Mercury (ppm)	≤0.1	0.03	CP
Microbiological Data			
Total Plate Count(cfu/g)	≤1000	<1000	CP/USP
Coliform(cfu/g)	Negative	Negative	CP/USP
Yeast&Mold(cfu/g)	Negative	Negative	CP/USP
Salmonella(cfu/g)	Negative	Negative	CP/USP
Staphylococcus(cfu/g)	Negative	Negative	CP/USP

Solvents

Methanol (ppm)	≤200	62	CP/USP
Ethanol (ppm)	≤5000	275	CP/USP

Country of Original: China

Analyst: Nie Junming Checker: Guo Changmin Auditor: Peng Baojuan

Appendix 1.2 Maltodextrin

Analysis Sheet

Serial No: REG-ZL-00303-00

Product Name		Maltodextrin	Testing No:	FMJ20-05-001
From		Shandong Xiwang Sugar co.,ltd	Test category	Raw material
Batch No		202005011	Batch Quantity	1500kg
Identifier No		GF001	Test quantity	500g
Spec		25kg/bag	package	-------
Test date		2020.05.16	Report Date	2020.05.17
Standard		《Enterprise internal control quality standard 》		
Test Items		Standard	Test Result	Conclusion
Sensory index	Appearance Colour	white or light yellow amorphous powder without visible impurities	White amorphous powder without visible impurities	Conform
	Odor	Special smell of maltodextrin, no peculiar smell	Special smell of maltodextrin, no peculiar smell	Conform
	Taste	Not sweet or slightly sweet, no peculiar smell	slightly sweet, no peculiar smell	Conform
Physical and chemical indicators	DE（%）	16≤DE＜20	17.6	Conform
	Moisture（%）≤	6.0	5.70	Conform
	Solubility（%）≥	98.0	99.0	Conform
	PH	4.5～6.5	5.30	Conform
	Sulfated ash（%）≤	0.5	0.10	Conform
	Sulfur dioxide（%）≤	0.0025	0.0009	Conform
	Total Solids（%）≥	90	93.2	Conform
	Protein（%）≤	0.5	0.003	Conform
	Lead (mg/kg)≤	0.5	0.001	Conform
	Iodine test	No blue reaction	No blue reaction	Conform
Conclusion: the product was tested according to the enterprise internal control quality standard, and the results were in conformity with the regulations.				

Analyst: Xiaoli Chen Checker: Mengmeng Guo Auditor: Li Li

Appendix 1.3 Toruzyme 3.0 L Cyclodextrin Glucosyltransferase Enzyme

Certificate of Analysis
分析证书

novozymes
Rethink Tomorrow

Date 证书打印日期
Mar 10, 2020
Purchase order item 客户采购定单号

Delivery 送货号
7937354
Sales order 销售定单号
5765493

Sold-to 客户名及地址

济南海润浩达生物科技有限公司
济南
济南市历下区解放路122号

Ship-to 收货单位
曲阜市创业大道12号
曲阜
曲阜市创业大道12号

Material: **Toruzyme 3.0 L**
产品名: 诺维信环糊精葡萄糖苷转移酶3.0 L

Batch: **ACN00257** **Quantity:** **250 KG**
批号 数量
Production Date: **Aug 27, 2019** **Best before:** **Aug 26, 2021**
生产日期: 保质期:

Characteristic 分析项目	Unit 单位	Value 结果
环麦芽糊精葡萄糖苷基转移酶单位 KNU-CP Cyclod.Glycosyl Transf. KNU-CP	/g	3.38
pH值为25° C pH at 25° C		6.60
密度 Density	g/ml	1.033
菌落总数 Total viable count	/g	< 100
大肠菌群 Coliform bacteria	/g	< 4
大肠杆菌 E.coli		未检出/25g Not Detected, 25 g
沙门氏菌 Salmonella		未检出/25g Not Detected, 25 g

本产品同时符合食品化学品法典（FCC）和联合国粮农组织(FAO)/世界卫生组织(WHO)食品添加剂联合专家委员会（JECFA）推荐的食品级酶制剂纯度标准。

产品符合《GB1886.174-2016 食品安全国家标准 食品添加剂 食品工业用酶制剂》关于砷、铅的指标要求。
砷：≤3mg/kg；铅：≤5mg/kg。
产品抗菌活性未检出。
产品合格。

Novozymes (China)
Biotechnology Co. Ltd.
诺维信(中国)
生物技术有限公司

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1 / 2

Certificate of Analysis
分析证书

Delivery
7937354

novozymes
Rethink Tomorrow

Ship-to 收货单位
曲阜市创业大道12号
曲阜
曲阜市创业大道12号

The product complies with current FAO/WHO JECFA and FCC recommended purity specifications for food grade enzymes.

诺维信(中国)生物技术有限公司 质量保证部

Quality Assurance
质量保证部

Novozymes (China)
Biotechnology Co., Ltd.
诺维信(中国)生物技术有限公司

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葡萄糖基甜菊糖苷生产

Enzyme Information

1、Product characteristics

Toruzyme®3.0 L、 NOVOZYMES	
Nominal Enzyme	Cyclomaltodextrin, glucosyltransferase
Nominal Activity	3 KNU-CP/g
Colour	Brown
Physical shape	Liquid
Approximate density（g/ml）	1.04
Viscosity （cPs)	1-25

2、Specification

	Lower limit	Upper limit	Unit
Alpha-amylase unit KNU-CP	3		/g
25C hours pH	6	7	
Bacterial total account	-		/g
Coliform	-		/g
Escherichia coli	Negative		/25g
Salmonella	Negative		/25g
Heavy metal		Max30	mg/kg
Pb		Max5	mg/kg
Arsenic		Max 3	mg/kg
Cadmium		Max0.5	mg/kg
Mercury		Max0.5	mg/kg

3、Ingredients

Ingredients	Approximate %（weight /weight）
water, CAS no. 7732-18-5	73
Propylene glycol, CAS no. 57-55-6	23
Cyclomaltodextrin glucan transferase, CAS no. 9030-09-5*	4
*Defined as enzyme concentration (dry matter basis)	

4、Production strain

Name of production strain	Bacillus licheniformis
It is produced by deep fermentation of genetically modified microorganisms. The enzyme protein, as far as it is concerned, is not genetically modified, but isolated and purified from these microorganisms.	

5、Storage condition

The packaging must be kept in good condition, stored in a dry environment, and avoid direct sunlight. 0 – 10 ° C / 32 – 50 ° f this product has the best effect before the best use period. If stored at a maximum storage temperature of 25 ° C / 77 ° F, the product shall be used for the best effect within 3 months from the date of delivery.

6、Safety and use precautions

An enzyme is a protein. Inhalation of the powder or suspension of the enzyme may induce allergies and may lead to allergic reactions in sensitive individuals. Prolonged exposure to certain enzymes may cause irritation to the skin, eyes and mucous membranes.

7、Compliance

The product meets the purity standard of food grade enzyme preparation recommended by the world food and Agriculture Organization (FAO) / World Health Organization (who), the Joint Expert Committee on food additives (JECFA) and the food chemical code (FCC), as well as the relevant requirements of China's food safety and food grade enzyme preparation product standards. The customer center or sales representative can provide kosher and Halal certificates.

Appendix 2 Analytical Method

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Inspection method

(1) Identification experiment

White or light yellow powder, soluble in water, slightly soluble in ethanol.

(2) Determination of glucosyl stevioside, stevioside and maltodextrin

A. 1 Principle

The total content of stevioside (TSG), residual maltodextrin (RD), unreacted stevioside and the ratio of glucosyl stevioside can be determined by adsorption chromatography and high performance liquid chromatography.

A. . 2 Scope

The final product containing a mixture of α - 1,4-glucosyl stevioside (GSG) is suitable for solid samples with total stevioside content in the range of 60-102% on a dry basis.

A. 3 equipment and reagents

A. 3.1 high performance liquid chromatography (HPLC); the equipment shall be equipped with binary pump, automatic sampler, column temperature box and dad detector, interface and data acquisition software;

A. 3.2 HPLC amino column, 4.6m m x 250mm, 5 μ m particles;

A. 3.3 analytical balance with an accuracy of 0.0001g;

A. 3.4 Karl Fischer coulometric titrator;

A. 3.5 vacuum rotary evaporator for laboratory;

A. 3.6 vacuum oven;

A. 3.7 moisture meter;

A. 3.8 vacuum solvent filtration system, all glass material;

A. 3.9 vacuum system filter: polypropylene material, 0.2 μ m, 47mm;

A. 3.10 class a volumetric flask and pipette;

A. 3.11 glass column filled with 200ml macroporous adsorption resin (inner diameter: 25mm);

A. 3.12 acetonitrile, HPLC grade;

A. 3.13 water, HPLC grade;

A. 3.14 ethanol, reagent grade, system equipment, or other equivalent;

A. 3.15 ribavirin a standard;

A. 3.16 stevioside standard;

A. 3.17 Rebaudioside C standard;

A. 3.18 Rebaudioside F standard;

A. 3.19. Dulcoside A standard;

A. 3.20 standard product of stevioside;

A. 3.21 ammonium acetate, reagent grade;

A. 3.22 glacial acetic acid, reagent grade.

A. Safety precautions

A. 6.1 when handling materials and cleaning up spilled liquid and waste, safety measures and emergency disposal principles for hazardous chemicals shall be followed at all times.

A. 6.1 for the chemicals used in the above steps, all precautions and hazard precautions listed in the material safety data sheet shall be followed.

A. 4.3 stevioL glycosides is usually in powder form. In the process of shaking, feeding and mixing, it is easy to generate air dust, which may cause discomfort when inhaled into people's mouth and nose. Therefore, it is necessary to operate carefully to avoid dust generation.

A. 5 steps

A.5.1 TSG

Test solution - accurately weigh about 5g of GSG and pour it into 250ml of water for dissolution. At a rate of less than 15 ml / min, add the solution to the glass column containing 200 ml of macroporous resin (inner diameter: 25 mm), and then wash the resin with 1000 ml of water. The stevioside adsorbed was eluted with 1000 ml 50% (volume) ethanol at a rate of 15 ml / min or less. Evaporate the collected ethanol eluate and water wash solution to dryness, then place them in a vacuum oven and dry for two hours at 105 ℃. Weigh and record the dry weight of each component. The content (%) of TSG and RD was calculated by formula.

The mass fraction W1 of TSG is calculated according to formula (a.1), and the mass fraction W2 of RD content is calculated according to formula (A.2):

Calculation of mass fraction W1 of TSG

Where:

M1 -- total amount of ethanol components after drying, unit: G;

M2 - wet weight of the original sample, unit: G;

Wh - water content (%);
Calculate the mass fraction W2 of RD content:

=

Where:

M3 - total amount of water components after drying, unit: G;

M2 - wet weight of the original sample, unit: G;

Wh water content (%);

Sample recovery calculation:/

Where:

W1 - mass fraction (%) of total TSG content;

W2 - mass fraction of RD content (%);

A. 5.2 unreacted stevioside content

Weigh about 3G of GSG, and pour it into buffer solution (a.3.6.1.2) to dissolve it, so as to prepare 100ml of solution as test solution. The content of unresponsive stevioside (SG) was determined by HPLC according to the HPLC determination step (a.6.1). The

chromatogram of the sample conforms to the example chromatogram. Calculate the content of α - glucosyl stevioside from the total content of stevioside (a.5.1) as follows. The mass fraction w α of α - glucosyl stevioside content is as follows

(a.4) calculation:

Where:

wα =w1 - w4 ..... （A.4）

W1 -- TSG mass fraction (%);

W4 - mass fraction (%) of unreactive stevioside content;

A. 5.3 ratio of α - glucosyl stevioside

Weigh about 5g of GSG and dissolve it in water to prepare 100ml of solution as test solution.

HPLC analysis the area ratio (%) of α - glucostevioside was determined according to the HPLC determination step of glucostevioside (a.3.6.2).

The ratio of α - glucostevioside was calculated from the content of α - glucostevioside (a.3.5.2).
Calculate the mass fraction W2 of RD content:

=

Where:

M3 - total amount of water components after drying, unit: G;

M2 - wet weight of the original sample, unit: G;

Wh water content (%);

Sample recovery calculation:/

Where:

W1 - mass fraction (%) of total TSG content;

W2 - mass fraction of RD content (%);

A. 5.2 unreacted stevioside content

Weigh about 3G of GSG, and pour it into buffer solution (a.3.6.1.2) to dissolve it, so as to prepare 100ml of solution as test solution. The content of unresponsive stevioside (SG) was determined by HPLC according to the HPLC determination step (a.6.1). The chromatogram of the sample conforms to the example chromatogram. Calculate the content of α - glucosyl stevioside from the total content of stevioside (a.5.1) as follows. The mass fraction w α of α - glucosyl stevioside content is as follows

(a.4) calculation:

Where:

wα =w1 - w4 ..... （A.4）

W1 -- TSG mass fraction (%);

W4 - mass fraction (%) of unreactive stevioside content;

A. 5.3 ratio of α - glucosyl stevioside

Weigh about 5g of GSG and dissolve it in water to prepare 100ml of solution as test solution.

HPLC analysis the area ratio (%) of α - glucostevioside was determined according to the HPLC determination step of glucostevioside (a.3.6.2).

The ratio of α - glucostevioside was calculated from the content of α - glucostevioside (a.3.5.2).
A. 6.1.5 analysis steps

A. 6.1.5.1 system startup / applicability

Detector sensitivity test: inject 2.5mg/l stevioside standard solution, and confirm that the signal-to-noise ratio between stevioside peak value and noise is ≥ 3;

If not, check the instrument to ensure that the signal-to-noise ratio reaches ≥ 3 before the next operation.

Tailing factor: inject the standard solution of reb-a2000mg / L, and calculate the tailing factor-t with this peak. Tailing factor: 0.8 ≤ t ≤ 2.

Signal to noise ratio: calculate the signal to noise ratio of stevioside standard solution injection. The detection limit (LOD) is 5 mg / L stevioside standard solution: the signal-to-noise ratio of the standard solution must be ≥ 10. The detection limit (LOD) is 2.5mg/l stevioside standard solution: the signal-to-noise ratio must be ≥ 3.

Separation of stevioside: inject M6 standard solution, stevioside and rebaudioside C peaks should be clearly separated. The retention time of each stevioside was recorded (a.8.1).

A. 6.1.5.2 analysis sequence

After the system suitability check, according to the principle of low to high concentration, inject all the remaining standard solutions in turn, followed by sample injection; after up to 12 sample injections and after the end of sample analysis sequence, inject 2000 mg / L stevioside and REB a standard solution respectively for backup calibration.

A. 6.1.5.3 integral parameters

Use the software tool of the liquid chromatography analyzer to complete the integration.
A. 6.1.6 calculation

A. . 6.1.6.1 relative standard deviation of peak area relative standard deviation of peak area R1 is calculated according to formula (a.6):

r1 =s1/x*100% ..... （A.6）

Where:

S1 -- standard deviation value = (∑ (x-x) 2) / (n-1)) 1 / 2;

X -- average value = (x1 + x2 + X3 + xn) / N;

Xn -- peak area;

N - total number of samples.

A. 6.1.6.2 a tailing factor (T)

The tailing factor t is calculated according to formula (a.7):

T =W 0.05/2 f ..... （A.7）

Where:

W0.05 - peak width at 5% height;

F -- the distance from the maximum peak to the value on the x-axis at the peak front, and 5% above the peak baseline

Line measurement.

A. . 6.1.6.3 Standard Recovery P is calculated according to formula (A.8):

p = c1/ c2×100% ..... （A.8）

Where: C1 -- concentration calculation value in the curve;

C2 -- theoretical concentration.

A. 6.1.6.4 analysis and calculation

The target analyte was determined by matching the retention time of M6 standard solution. Determine the peak response area of the target analyte in the standard solution and sample.

Determine the system drift of REB a standard. Determine the response area of REB a at 2000 mg / L, and calculate the relative standard deviation, which is required to be ≤ 2.0%.

Using REB a or stevioside concentration (in mg / L) as ordinate and its corresponding response area as abscissa to draw fully

The fitted standard curve of linear regression. Alternatively, data acquisition software can be used to plot calibration curves. From the linear regression equation of the standard curve, calculate the concentration of the analyte in the sample (in mg / L) (REB a

REB a curve was used and stevioside curve was used for all other analytes. Or use the data acquisition software to calculate the analyte concentration (using the calibration curve drawn by the software). The concentration y of analyte is calculated according to formula (A.9):

$Y = AX + B$ ..... (A.9)

Where: X -- peak response area;

A -- slope;

B -- Y-axis intercept.

Correct the concentration of each analyte in the sample as follows: multiply the concentration of each glycoside (stevioside, Duke glycoside a, rebaudioside C, rebaudioside f) by the calibration factor of the glycoside

To correct the molecular weight difference between it and stevioside (see table A.2).
Table A.2 R1 and R2 groups, molecular formula and corresponding molecular weight of stevioside

Name	Abbreviation	R1	R2	Mol weight (g/mol)	Correction factor
Dulcoside A	Dul A	β glc-	α rha- β glc-	788.88	0.98
Rebaudioside A	Reb A	β glc-	(β glc) 2- β glc-	967.03	-
Rebaudioside C	Reb C	β glc-	(β glc, α rha) - β glc-	951.02	1.18
Rebaudioside F	Reb F	β glc-	(β glc, β xyl) - β glc-	936.99	1.16
Rubusoside	Rub	β glc- β glc-	β glc- β glc-	642.73	0.80
Stevioside	Stev	β glc-	β glc- β glc-	804.88	-

The weight percentage w of REB A and other glycosides in the sample is calculated according to formula (a.10):

$w = c3 / c4 \times 100$ ..... (A.10)

Where: C3 -- analyte concentration, mg / L;

C4 -- sample concentration, mg / L.

The weight percentage of Reba and all other glycosides can be corrected by multiplying the following factor (f) by W

(excluding moisture), the correction factor F is calculated according to formula (a.11):

$F = 100 / (100 - M)$ ..... (A.11)

Where:

M -- sample moisture,%.

The weight percentage of stevioside (SG) in the sample WSG is calculated according to formula (a.12):

wSG = wRub + wDulA + wRebC + wRebF + wStev + wRebA ..... （A.12）

Where: wdula - weight percentage of Dula in the sample, (%);

Wreb C -- weight percentage of REB C in the sample, (%);

Wreb F -- weight percentage of REB f in the sample, (%);

Wstev -- weight percentage of Stev in the sample, (%);

Wreb a - weight percentage of REB a in the sample, (%).

A. 6.1.7 acceptance criteria

A. 6.1.7.1 standard curve acceptance standard

Standard curve of Reba - for different concentration levels of Reba used in all calibration curves, the recovery of the standard must be 100 ± 3%, and the acceptable standard of the correlation coefficient of the standard curve is ≥ 0.9900.

Stevioside standard curve - for different stevioside concentration levels used in all calibration curves, the standard recovery must be within 100.0 ± 10%, except for the minimum concentration level (2.5mg / L) when the standard recovery must be within 100.0 ± 20%. The acceptable standard of correlation coefficient of standard curve is ≥ 0.9900.

A. 6.1.7.2 sequence standard (standard inspection) - the recovery rate of stevioside and REB a sequence standard (see a.. 6.1.6.3) must be within 100.0 ± 2%.

A. 6.1.7.3 samples the% RSD of SG and reb-a test results of parallel samples shall not exceed 2.0%. The% relative standard deviation of other glycosides shall not exceed 50% when the content is lower than 5mg / L (0.1% in the sample) and 20% when the content is higher than 5mg / L. When the% RSD of the sample does not fall within the above range, the fresh sample shall be prepared again until the new sample passes the quality control inspection.

A. 6.2 determination of glucostevioside by gradient HPLC

A. 6.2.1 the mobile phase (a-acetonitrile, B-Water) filters and degasses acetonitrile and water.

A. 6.2.2 filter 1000ml of water with diluent (100% water) and use it immediately.

A. 6.2.3 preparation of standard (M6) weigh each standard of rubusoside, ducoside a, stevioside, rebaudioside C, rebaudioside F and rebaudioside a

About 100mg / L of seed is prepared into mixed standard solution with diluent.

A. 6.2.4 sample preparation prepare sample solution (about 5%) according to the method described in a.5.3.

A. 6.2.5 see table A.3 for the service conditions of the instrument.

Table A.3 service conditions of instrument

chromatographic column	Amino column，250 x 4.6 mm，5μm
temperature	30° C
Gradient mobile phase	A-acetonitrile，B-water 0 min A：B-80：20 0～2 min A：B-80：20 2～70 min A：B-50：50

Current speed	1.0 mL/min
Ingection volume	10 μL
Detection wavelength	UV210 nm（4 nm bw），reference：260 nm（100 nm bw）
Running time	70 min
Autosampler temperature	Room temperature

A. 6.2.6 analysis steps

TSG separation: inject M6 solution. There should be clear separation between stevioside and rebaudioside C. The retention time of each stevioside was recorded (a.8.2).

A. 6.2.7 analyze the advanced sample samples of the sequence, and then inject the standard samples for quantitative detection after the injection of up to 12 samples and the end of the sample sequence test.

A. 6.2.8 the integral parameters shall be completed with the software tool provided by the liquid chromatography analyzer.

A. 6.2.9 calculation

Each α - glucosylstevioside is identified by comparing the elution profile with the example chromatogram.

All peaks were integrated except unreacted glycosides. The ratio of α - glucosyl stevioside (in% area) was determined by using the data acquisition software of chromatograph.

The ratio of each α - glucosyl stevioside was recorded.

A. 7 Results Report

Concentrations of unresponsive stevioside and TSG should be reported as% by weight of the dry basis. The proportion of α - glucostevioside was reported on the basis of area%. The average value of the repeated test results of two samples is used as the reported value.

(3) Measurement of optical rotation

A. Instrument used: polarimeter with accuracy of at least 0.5

B. Measurement method:

Accurately weigh 1g of the dried sample according to the regulations, add water to dissolve, and fix the volume to 100ml. Adjust the sample and polarizing tube to 20 ℃± 1 ℃, measure with polarimeter at the wavelength of 589.3nm ± 0.3nm, and measure the deflection angle degree at least three times, and the difference between the three measurements is not more than 0.08 °

And calculate as follows:

Specific rotation (α) = α 20d / C = 100A / LC

Where: (α) 20d ------ optical rotation of sample solution at 20 ℃

α - specific rotation of sample solution at 20 ℃.

A -------- degree of deflection angle

L -------- length of polarizing tube mm.

C ------- concentration of perfume solution (g / ml)
The allowable deviation of the parallel experiment results is 0.2 °. The specific rotation of the sample is the rotation of 1G / 100ml concentration.

(4) Determination of relative density

A. Instrument: analytical balance, low temperature constant temperature water bath, density bottle

B. Operation method:

Place the density bottle processed according to the standard in the balance room. When the temperature reaches the balance, weigh the mass of the density bottle including the cork to M0, accurate to 0.0002g. Fill the density bottle with distilled water and immerse it in the water bath. If necessary, adjust the water level to the scale after 30min. Close the cork and dry the outer wall with a dry cloth or filter paper. Put it in the balance chamber, and when the temperature reaches equilibrium, weigh the mass M1 of the density bottle including the cork. Empty the density bottle, replace the distilled water with the sample, repeat the above operation, and weigh the mass m2 of the density bottle including the cork. The allowable difference of two results of parallel determination is 0.0004.

Standard: 0.2 - 0.6

（5） Ph

A. Instrument and equipment: acidity meter of analytical balance

B. Detection method

Accurately weigh 5g of the test sample, dissolve it with water without carbon dioxide, fix the volume to 100ml, and divide it into two equal parts for standby. Calibrate the acidimeter with phthalate buffer solution and phosphate standard buffer solution, wash the electrode with water, wash the electrode with test sample solution, insert the electrode into the test sample solution for determination, and record the value after the reading is stable for more than 1min. The allowable error of two determinations shall not be greater than ± 0.02. Take the average value as the final result.

Standard specification: pH: 4.5-7.0

Appendix 3 Certificates of Analysis for Multiple Batches of Prostevia

Appendix 3.1 Certificate of Analysis for Prostevia Batch 20200503

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Prostevia 95% Expire Date: 2022.05.02

Manufacture Date: 2020.05.03 Batch Quantity: 2000kgs

Batch No: 20200503

ITEM	SPECIFICATION	TEST RESULTS
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic
Total steviol glycosides %	≥95	95.18
Glucosylated Steviol Glycosides %	≥80	89.33
Unreacted steviol Glycosides %	≤15	5.85
Maltodextrin	≤5	4.82
Burned residue %	≤1	0.10
Solubilty	Freely soluble in water and ethanol 1:1	Conform
Loss on Drying %	≤6	3.12
PH	4.6-7.0	5.0
Lead (ppm)	≤0.1	0.040
Arsenic(ppm)	≤0.1	Negative
Cadmium (ppm)	≤0.1	Negative
Mercury (ppm)	≤0.1	0.030
Microbiological Data		
Total Plate Count(cfu/g)	≤1000	<1000
E.Coli(cfu/g)	Negative	Negative
Coliform (cfu/g)	Negative	Negative
Yeast&Mold(cfu/g)	Negative	Negative

Package:25kg drum or carton (two food grade bags inside)

Country of Original : China

Note:NON-GMO NON-ALLERGEN

Analyst: Nie Junming Checker: Guo Changmin Auditor: Peng Baojuan

Appendix 3.2 Certificate of Analysis for Prostevia Batch 20200510

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Prostevia 95% Expire Date: 2022.05.09

Manufacture Date: 2020.05.10 Batch Quantity: 2000kgs

Batch No: 20200510

ITEM	SPECIFICATION	TEST RESULTS
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic
Total steviol glycosides %	≥95	95.13
Glucosylated Steviol Glycosides %	≥85	88.64
Unreacted steviol Glycosides %	≤10	6.49
Maltodextrin	≤5	4.87
Burned residue %	≤1	0.09
Solubilty	Freely soluble in water and ethanol 1:1	Conform
Loss on Drying %	≤6	3.24
PH	4.5-7.0	5.0
Lead (ppm)	≤0.1	0.040
Arsenic(ppm)	≤0.1	Negative
Cadmium（ppm）	≤0.1	Negative
Mercury（ppm）	≤0.1	0.030
Microbiological Data		
Total Plate Count(cfu/g)	≤1000	<1000
E.Coli(cfu/g)	Negative	Negative
Coliform (cfu/g)	Negative	Negative
Yeast&Mold(cfu/g)	Negative	Negative

Package:25kg drum or carton (two food grade bags inside)

Country of Original: China

Note:NON-GMO NON-ALLERGEN

Analyst: Nie Junming Checker: Guo Changmin Auditor: Peng Baojuan

Appendix 3.3 Certificate of Analysis for Prostevia Batch 20200517

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Prostevia 95% Expire Date: 2022.05.16

Manufacture Date: 2020.05.17 Batch Quantity: 2000kgs

Batch No: 20200517

ITEM	SPECIFICATION	TEST RESULTS
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic
Total steviol glycosides %	≥95	95.16
Glucosylated Steviol Glycosides %	≥85	89.26
Unreacted steviol Glycosides %	≤10	5.90
Maltodextrin	≤5	4.84
Burned residue %	≤1	0.10
Solubilty	Freely soluble in water and ethanol 1:1	Conform
Loss on Drying %	≤6	3.34
PH	4.5-7.0	5.0
Lead (ppm)	≤0.1	0.040
Arsenic(ppm)	≤0.1	Negative
Cadmium (ppm)	≤0.1	Negative
Mercury (ppm)	≤0.1	0.030
Microbiological Data		
Total Plate Count(cfu/g)	≤1000	<1000
E.Coli(cfu/g)	Negative	Negative
Coliform (cfu/g)	Negative	Negative
Yeast&Mold(cfu/g)	Negative	Negative

Package:25kg drum or carton (two food grade bags inside)

Country of Original: China

Note:NON-GMO NON-ALLERGEN

Analyst: Nie Junming Checker: Guo Changmin Auditor: Peng Baojuan

Appendix 3.4 Certificate of Analysis for Prostevia Batch 20200522

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Prostevia 95% Expire Date: 2022.05.21

Manufacture Date: 2020.05.22 Batch Quantity: 2000kgs

Batch No: 20200522

ITEM	SPECIFICATION	TEST RESULTS
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic
Total steviol glycosides %	≥95	95.10
Glucosylated Steviol Glycosides %	≥85	89.24
Unreacted steviol Glycosides %	≤10	5.86
Maltodextrin	≤5	4.90
Burned residue %	≤1	0.10
Solubilty	Freely soluble in water and ethanol 1:1	Conform
Loss on Drying %	≤6	3.33
PH	4.5-7.0	5.0
Lead (ppm)	≤0.1	0.040
Arsenic(ppm)	≤0.1	Negative
Cadmium（ppm）	≤0.1	Negative
Mercury（ppm）	≤0.1	0.030
Microbiological Data		
Total Plate Count(cfu/g)	≤1000	<1000
E.Coli(cfu/g)	Negative	Negative
Coliform (cfu/g)	Negative	Negative
Yeast&Mold(cfu/g)	Negative	Negative

Package:25kg drum or carton (two food grade bags inside)

Country of Original: China

Note:NON-GMO NON-ALLERGEN

Analyst：Nie Junming Checker：Guo Changmin Auditor：Peng Baojuan

Appendix 3.5 Certificate of Analysis for Prostevia Batch 20200528

Shandong Shengxiangyuan Biotechnology Co.,Ltd
山东圣香远生物科技有限公司
Address: No.58,East haiguan Rd,qufu,jining,shandong province,china
Tel:0086-537-4487369 Fax:0086-537-4400999

Certificate Of Analysis

Product Name: Prostevia 95% Expire Date: 2022.05.27

Manufacture Date: 2020.05.28 Batch Quantity: 2000kgs

Batch No: 20200528

ITEM	SPECIFICATION	TEST RESULTS
Appearance Odor	White fine powder Characteristic	White fine powder Characteristic
Total steviol glycosides %	≥95	95.15
Glucosylated Steviol Glycosides %	≥85	89.22
Unreacted steviol Glycosides %	≤10	5.93
Maltodextrin	≤5	4.85
Burned residue %	≤1	0.10
Solubilty	Freely soluble in water and ethanol 1:1	Conform
Loss on Drying %	≤6	3.41
PH	4.5-7.0	5.0
Lead (ppm)	≤0.1	0.040
Arsenic(ppm)	≤0.1	Negative
Cadmium (ppm)	≤0.1	Negative
Mercury (ppm)	≤0.1	0.030
Microbiological Data		
Total Plate Count(cfu/g)	≤1000	<1000
E.Coli(cfu/g)	Negative	Negative
Coliform (cfu/g)	Negative	Negative
Yeast&Mold(cfu/g)	Negative	Negative

Package:25kg drum or carton (two food grade bags inside)

Country of Original : China

Note:NON-GMO NON-ALLERGEN

Analyst: Nie Junming Checker: Guo Changmin Auditor: Peng Baojuan

Appendix 4 Representative Chromatograms for Multiple Batches of Prostevia

Appendix 4.1 Representative Chromatogram for Prostevia Batch 20200503

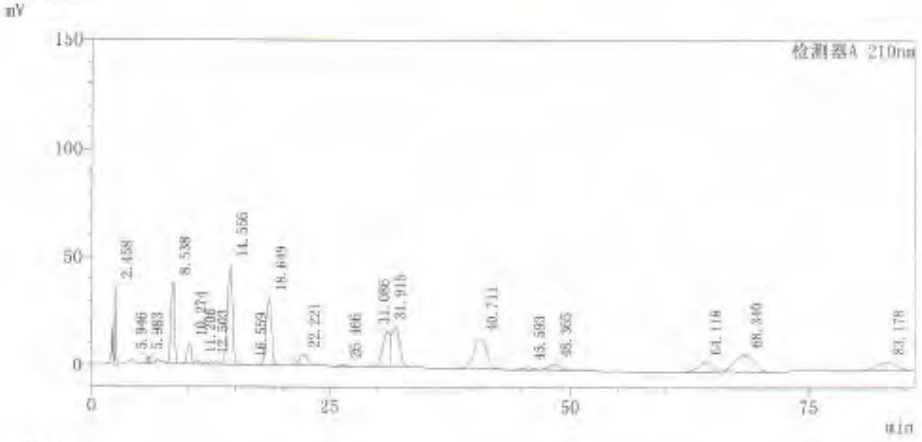
2020/6/5 16:11:06 1 / 1

SHIMADZU LabSolutions 分析报告

〈样品信息〉

分析者	: System Administrator
样品名称	:
样品ID	: 批号 20200503
样品架#	: 1
样品瓶号	: 3
数据文件名	: 0530-3.lcd
方法文件名	: 葡萄糖基甜菊糖苷.lcm
批处理文件名	: 1.lcb
报告文件名	: 无标题.lsr
分析日期	: 2020/5/30 20:33:11

〈色谱图〉



〈峰表〉

检测器A 210nm

峰号	保留时间	面积	高度	浓度	浓度单位	标记	化合物名
1	2.458	227470	36496	2.114			
2	5.946	46268	3063	0.430		M	
3	5.983	28253	3137	0.263		V M	
4	8.538	1010347	37873	9.390			
5	10.274	304924	9636	2.834			
6	11.206	59834	1748	0.556		V	
7	12.503	40054	1224	0.372		V	
8	14.556	1721116	45983	15.995			
9	16.559	7967	262	0.074			
10	18.649	1316550	31013	12.235			
11	22.221	292273	4589	2.716			
12	26.466	34898	577	0.324			
13	31.086	923714	16333	8.584			
14	31.915	1048392	18422	9.743		V	
15	40.711	1334973	14326	12.406			
16	45.593	71317	979	0.663			
17	48.365	184832	2286	1.718			
18	64.118	443679	4569	4.123			
19	68.340	1127455	7810	10.478			
20	83.178	536056	3797	4.982			
总计		10760370	244124				

葡萄糖基甜菊糖苷 - 1-28/1-73 - 0530-3.lcd

Appendix 4.2 Representative Chromatogram for Prostevia Batch 20200510

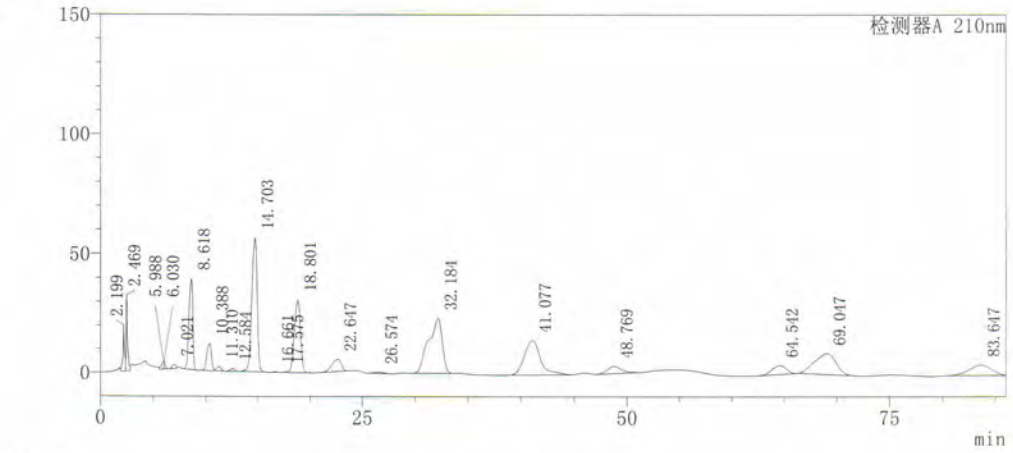
2020/6/5 16:12:05 1 / 1

SHIMADZU LabSolutions 分析报告

〈样品信息〉

分析者	: System Administrator
样品名称	:
样品ID	: 批号: 20200510
样品架#	: 1
样品瓶号	: 5
数据文件名	: 0530-5.lcd
方法文件名	: 葡萄糖基甜菊糖苷.lcm
批处理文件名	: 1.lcb
报告文件名	: DEFAULT.lsr
分析日期	: 2020/5/30 23:26:02

〈色谱图〉



〈峰表〉

检测器A 210nm

峰号	保留时间	面积	高度	浓度	浓度单位	标记	化合物名
1	2.199	196032	19687	1.639			
2	2.469	263936	32277	2.207		V	
3	5.988	47312	2989	0.396		M	
4	6.030	30264	3053	0.253		V M	
5	7.021	40565	1647	0.339			
6	8.618	1001555	38509	8.374			
7	10.388	358846	11545	3.000			
8	11.310	57394	1883	0.480		V	
9	12.584	35631	1181	0.298			
10	14.703	2052376	56331	17.161			
11	16.661	9085	305	0.076			
12	17.575	5760	228	0.048			
13	18.801	1275863	30804	10.668		V	
14	22.647	319224	4999	2.669			
15	26.574	32264	527	0.270			
16	32.184	2113130	23355	17.669			
17	41.077	1550027	14536	12.961			
18	48.769	276657	2994	2.313			
19	64.542	359948	3761	3.010			
20	69.047	1292545	8953	10.808			
21	83.647	641200	4343	5.361			
总计		11959615	263907				

葡萄糖基甜菊糖苷 - 1-28/1-75 - 0530-5.lcd

Appendix 4.3 Representative Chromatogram for Prostevia Batch 20200517

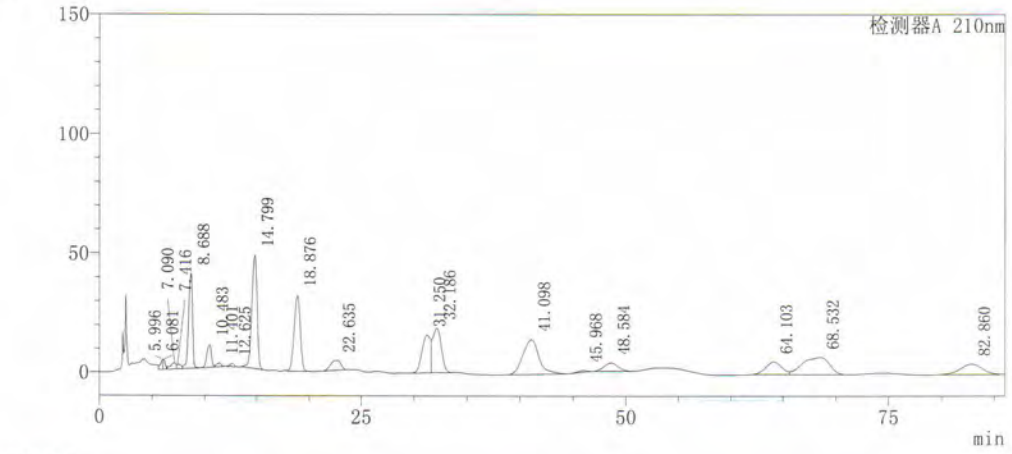
2020/6/5 16:16:46 1 / 1

SHIMADZU LabSolutions 分析报告

〈样品信息〉

分析者	: System Administrator
样品名称	:
样品ID	: 批号 20200517
样品架#	: 1
样品瓶号	: 8
数据文件名	: 0530-8.lcd
方法文件名	: 葡萄糖基甜菊糖苷.lcm
批处理文件名	: 1.lcb
报告文件名	: DEFAULT.lsr
分析日期	: 2020/5/31 3:45:19

〈色谱图〉



〈峰表〉

检测器A 210nm

峰号	保留时间	面积	高度	浓度	浓度单位	标记	化合物名
1	5.996	67135	4113	0.585		M	
2	6.081	59357	4293	0.517		V M	
3	7.090	90723	2620	0.790			
4	7.416	49513	1840	0.431		V	
5	8.688	1054082	39950	9.181		V	
6	10.483	293702	9697	2.558			
7	11.401	49224	1629	0.429		V	
8	12.625	37496	1274	0.327			
9	14.799	1719504	47742	14.977			
10	18.876	1322988	32161	11.524			
11	22.635	282405	4080	2.460			
12	31.250	887015	15994	7.726			
13	32.186	1102305	18934	9.601		V	
14	41.098	1570923	14696	13.683			
15	45.968	42676	670	0.372			
16	48.584	318501	3492	2.774			
17	64.103	591566	5464	5.153			
18	68.532	1314154	7368	11.447		V	
19	82.860	627428	4346	5.465			
总计		11480698	220364				

葡萄糖基甜菊糖苷 - 1-28/1-78 - 0530-8.lcd

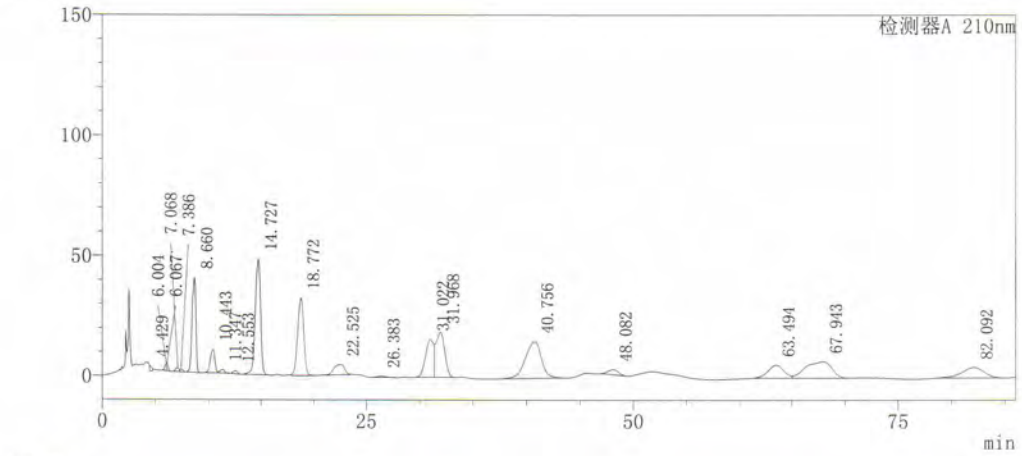
Appendix 4.4 Representative Chromatogram for Prostevia Batch 20200522

SHIMADZU LabSolutions 分析报告

〈样品信息〉

分析者	: System Administrator
样品名称	:
样品ID	: 批号 20200522
样品架#	: 1
样品瓶号	: 9
数据文件名	: 0530-9.lcd
方法文件名	: 葡萄糖基甜菊糖苷.lcm
批处理文件名	: 1.lcb
报告文件名	: DEFAULT.lsr
分析日期	: 2020/5/31 5:11:45

〈色谱图〉



〈峰表〉

检测器A 210nm

峰号	保留时间	面积	高度	浓度	浓度单位	标记	化合物名
1	4.429	29196	2349	0.263		M	
2	6.004	34543	2970	0.312		M	
3	6.067	34076	3160	0.307		V M	
4	7.068	39495	1628	0.356			
5	7.386	21050	1009	0.190		V	
6	8.660	1003750	39667	9.052			
7	10.443	306900	9917	2.768			
8	11.347	48402	1590	0.437		V	
9	12.553	36844	1271	0.332			
10	14.727	1726079	48210	15.567			
11	18.772	1318148	32348	11.888			
12	22.525	297985	4186	2.687			
13	26.383	37210	560	0.336			
14	31.022	873520	15934	7.878			
15	31.968	1085365	18931	9.788		V	
16	40.756	1614942	15303	14.564			
17	48.082	157057	2146	1.416			
18	63.494	584457	5479	5.271			
19	67.943	1183518	6914	10.673		V	
20	82.092	655853	4380	5.915			
总计		11088389	217953				

葡萄糖基甜菊糖苷 - 1-28/1-79 - 0530-9.lcd

Appendix 4.5 Representative Chromatogram for Prostevia Batch 20200528

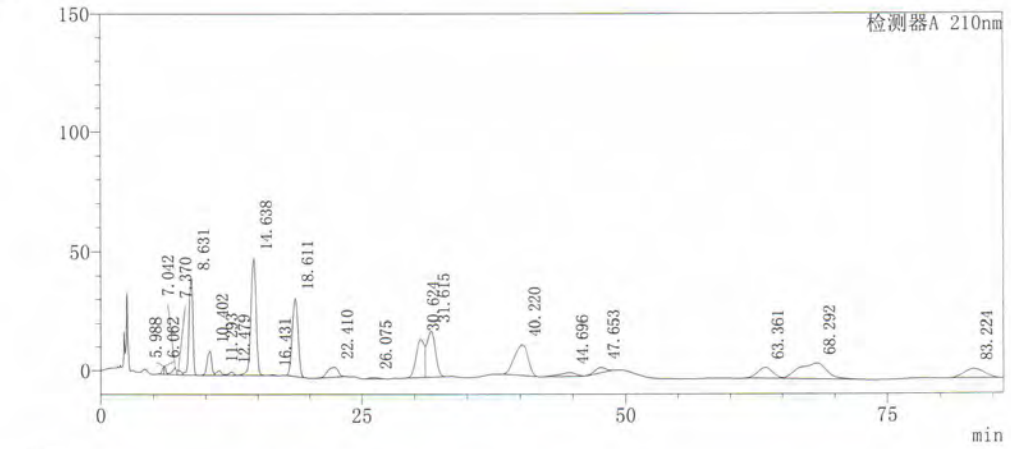
2020/6/5 16:17:35 1 / 1

SHIMADZU LabSolutions 分析报告

〈样品信息〉

分析者	: System Administrator
样品名称	:
样品ID	: 批号: 20200528
样品架#	: 1
样品瓶号	: 11
数据文件名	: 0530-11.lcd
方法文件名	: 葡萄糖基甜菊糖苷.lcm
批处理文件名	: 1.lcb
报告文件名	: DEFAULT.lsr
分析日期	: 2020/5/31 8:04:38

〈色谱图〉



〈峰表〉

检测器A 210nm

峰号	保留时间	面积	高度	浓度	浓度单位	标记	化合物名
1	5.988	42918	3292	0.396		M	
2	6.062	42051	3525	0.388		V M	
3	7.042	80778	2581	0.746			
4	7.370	47237	1817	0.436		V	
5	8.631	1045276	40749	9.655		V	
6	10.402	314085	10218	2.901			
7	11.293	70372	1987	0.650		V	
8	12.479	44477	1399	0.411		V	
9	14.638	1737534	49120	16.049			
10	16.431	10368	336	0.096			
11	18.611	1309456	33074	12.095			
12	22.410	289550	4053	2.675			
13	26.075	35209	554	0.325			
14	30.624	895487	16236	8.271			
15	31.615	1107519	19492	10.230		V	
16	40.220	1186902	13075	10.963			
17	44.696	165959	1589	1.533			
18	47.653	150473	2107	1.390			
19	63.361	471513	4612	4.355			
20	68.292	1256138	6676	11.603		V	
21	83.224	523015	3859	4.831			
总计		10826318	220350				

葡萄糖基甜菊糖苷 - 1-28/1-81 - 0530-11.lcd

Appendix 5 Prostevia Pesticide Testing Report

eurofins

Analytical Report

Sample Code	128-2019-00005295	Report date	24-Mar-2019
Certificate No.	AR-19-VV-005426-01		

SHANDONG SHENGXIANGYUAN
BIOTECHNOLOGY CO.,LTD
BaojuanPeng
No.58 East Haiguan Rd.,Qufu

Our reference:	128-2019-00005295/ AR-19-VV-005426-01
Client Sample Code:	20190201
Sample described as:	STEVIA
Sample Packaging:	Sealed plastic bag
Sample reception date:	18-Mar-2019
Analysis starting date:	18-Mar-2019
Analysis ending date:	24-Mar-2019
Arrival Temperature (°C)	18.1
Sample Weight	210g
Sample Type	Powder

		Results	Unit	LOQ	LOD
☆SUS17	Pesticide Screening(LC) Method: BS EN 15662:2018				
Screened pesticides		Not Detected	mg/kg		
☆SUS36	Pesticide Screening(GC) Method: BS EN 12393:2013				
Screened pesticides		Not Detected	mg/kg		

List of screened molecules (* = limit of quantification)

SUS17 Pesticide Screening(LC) (LOQ* mg/kg)

2,6-Dimorpholino (Aldoxycarb Metabolite) (0.01)	3,4,5-Trimethacarb (0.01)	3-Hydroxycarbofuran (0.01)	4-CPA (0.01)	Abamectin (sum) ()	Acephate (0.05)
Acetamiprid (0.01)	Acibenzolar-s-methyl (0.01)	Aclfluorfen (0.01)	Acrinathrin (0.01)	Alachlor (0.05)	Aldicarb (0.05)
Aldicarb (sum) ()	Aldicarb-sulfone (0.01)	Aldicarb-sulfoxide (0.05)	Ametoctradin (0.01)	Amitraz (0.01)	Amitraz (sum) ()
Amitrol (sum) ()	Anilofos (0.05)	Avermectin B1a (0.01)	Avermectin B1b (0.01)	Azaconazole (0.01)	Azamethiphos (0.01)
Azinphos-ethyl (0.01)	Azinphos-methyl (0.05)	Azoxystrobin (0.01)	Barban (0.05)	Benalaxyl (0.01)	Benalaxyl-M (0.01)
Bendiocarb (0.01)	Benfuracarb (0.01)	Benoxacor (0.01)	Bensulfuron-methyl (0.01)	Bentazone (0.01)	Bifenazate (0.01)
Bioresmethrin (0.01)	Bitertanol (0.01)	Boscalid (0.01)	Bromacil (0.01)	Bromoxynil (0.01)	Bromuconazole (Sum) ()
Bromuconazole, cis- (0.01)	Bromuconazole, trans- (0.01)	Bupirimate (0.01)	Buprofezin (0.01)	Butocarboxim (0.05)	Butocarboxim-sulfoxide (0.01)
Butoxycarboxim (0.01)	Butylate (0.05)	Carbaryl (0.01)	Carbendazim/Benomyl (sum) (0.01)	Carbetamide (0.01)	Carbofuran (0.01)
Carbofuran (Sum) ()	Carbosulfan (0.01)	Carfentrazone-ethyl (0.01)	Chlorantraniliprole (0.01)	Chlorfluazuron (0.01)	Chlorbufam (0.01)
Chlorbenzuron (0.01)	Chlorotoluron (0.01)	Chlorpropham (0.01)	Chlorpyrifos (-ethyl) (0.01)	Chlorthiophos (0.01)	Cinerin-ethyl (0.01)
Cinidon-ethyl (0.01)	Clethodim (0.01)	Clomazone (0.01)	Clothianidin (0.01)	Cloxacillin (0.01)	Cyanophos (0.01)
Cyclohexidi (0.01)	Cycloheximid (0.01)	Cycloxydim (0.01)	Cymoxanil (0.02)	Cyproconazole (0.01)	Cyprodinil (0.01)
Cyromazine (0.05)	Daimuron (0.01)	Dazomet (0.01)	Deltamethrin (0.01)	Demeton-S-methyl-sulfone (0.01)	Desmedipham (0.01)
Diafenthiuron (0.05)	Diazinon (0.01)	Dichlobenil (0.01)	Dichlofluanid (0.01)	Dichlorophen (0.01)	Diclocymet (0.01)
Diethofencarb (0.01)	Difenoconazole (0.01)	Diflubenzuron (0.01)	Dimefuron (0.01)	Dimepiperate (0.02)	Dimethachlor (0.01)
Dimethenamid (0.01)	Dimethoate (0.01)	Dimethomorph (0.01)	Dimethylvinphos (0.01)	Dinocap (0.01)	Dinocap (0.01)
Dinotefuran (0.05)	Diovacarb (0.01)	Diphenamid (0.01)	Disulfoton (0.05)	Disulfoton sulfone (0.01)	Disulfoton-PS-sulfone (0.01)
Ditalimfos (0.01)	Diuron (0.01)	Dodine (0.01)	Emamectin B1a (0.01)	Emamectin B1b (0.02)	Epoxiconazole (0.01)
EPTC (0.01)	Etaconazole (0.05)	Ethiofencarb (0.01)	Ethiofencarb (Sum) ()	Ethiofencarb-sulfone (0.01)	Ethiofencarb-sulfoxide (0.01)
Ethiprole (0.01)	Ethirimol (0.01)	Ethofumesate (0.01)	Ethoprophos (0.01)	Ethoxyquin (0.02)	Ethoxysulfuron (0.01)
Etofenprox (0.01)	Etoxazole (0.05)	Fenamidone (0.01)	Fenamiphos (0.01)	Fenazaquin (0.01)	Fenbuconazole (0.01)
Fenbuconazol (0.01)	Fenobucarb (0.01)	Fenoxycarb (0.01)	Fenpyroximate (0.01)	Fenpyrrolimin (0.01)	Fensulfothion (0.01)
Fensulfothion-PS-sulfone (0.01)	Fensulfothion-PS-sulfoxide (0.01)	Fenthion (0.01)	Fenthion (sum) ()	Fenthion-oxon (0.01)	Fenthion-oxon-sulfone (0.01)
Fenthion-oxon-sulfoxide (0.01)	Fenthion-PS-sulfoxide (0.01)	Fenthion-sulfone (0.01)	Fipronil (0.01)	Fipronil (sum) ()	Fipronil-sulfone (0.01)
Fipronil-sulfone (0.01)	Flamprop-methyl (0.01)	Flazasulfuron (0.01)	Fluazifop-P-butyl (0.01)	Fluazinam (0.01)	Fludioxonil (0.01)
Flufenacet (0.01)	Flufenoxuron (0.01)	Fluometuron (0.05)	Fluopicolide (0.01)	Fluridone (0.01)	Flusilazole (0.01)
Flutriafol-methyl (0.01)	Fluvalinate (0.01)	Fluxofenim (0.05)	FM-6-1 (0.01)	Forchlorfenuron (0.01)	Forchlorfenuron (0.01)
Formetanate (0.05)	Fosthiazate (0.01)	Furathiocarb (0.01)	Halosulfuron-methyl (0.01)	Hexaconazole (0.01)	Hexaflumuron (0.01)
Hexythiazox (0.01)	Hexythiazox (0.01)	Imazalil (0.01)	Imazaquin (0.01)	Imibenconazole (0.01)	Imidacloprid (0.01)
Indoxacarb (0.02)	Iodosulfuron-methyl (0.01)	Ioxynil (0.01)	Ipconazole (0.01)	Iprovalicarb (0.01)	Isoprocarb (0.01)
Isoprothiolane (0.01)	Isoxaflutole (0.01)	Isoxathion (0.01)	Linuron (0.01)	Lufenuron (0.01)	Lufenuron (0.01)
Malathion (0.01)	Mefenacet (0.01)	Mepanipyrim (0.01)	Mephosfolan (0.01)	Mesosulfuron (0.01)	Metalaxyl (0.01)
Metconazole (0.01)	Methabenzthiazuron (0.01)	Methamidophos (0.02)	Methiocarb (0.01)	Methiocarb (Sum) ()	Methiocarb-sulfoxide (0.01)
Methiocarb-sulfone (0.01)	Methomyl (0.01)	Methoxyfenozide (0.01)	Metolachlor (0.01)	Metolcarb (0.01)	Metosulam (0.05)
Molinate (0.01)	Monocrotophos (0.01)	Monolinuron (0.01)	Myclobutanil (0.01)	Naled (0.05)	Napropamide (0.01)
Neburon (0.01)	Nicosulfuron (0.01)	Nitenpyram (0.05)	Norflurazon (0.01)	Novaluron (0.01)	Nuarimol (0.01)
Omethoate (0.01)	Oxadixyl (0.01)	Oxamyl (0.01)	Oxamyl-oxime (0.02)	Oxycarboxin (0.01)	Oxydemeton-methyl (0.02)
Oxydemeton-methyl (sum) ()	Paraoxon (0.01)	Paraoxon-methyl (0.01)	Penconazole (0.01)	Pencycuron (0.01)	Pendimethalin (0.01)
Phenmedipham (0.05)	Phorate (Sum) ()	Phorate-sulfone (0.01)	Phorate-sulfoxide (0.01)	Phosalone (0.01)	Phosmet (0.01)

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Procetn (0.01)	Prothioconazole (0.01)	Piperonyl butoxide (0.01)	Primicarb (0.01)	Prothioconazole-desmethyl (0.01)	Pirimicarb-Desmethylformamido (0.01)
Pirimiphos-methyl (0.01)	Primisulfuron-Methyl (0.01)	Prochloraz (0.01)	Promecarb (0.01)	Propachlor (0.01)	Propamocarb (0.01)
Propaphos (0.01)	Propargite (0.01)	Propetamphos (0.01)	Propham (0.01)	Propiconazole (0.01)	Propoxur (0.01)
Propoxycarbazone (0.05)	Propyzamide (0.01)	Prosulfocarb (0.01)	Prosulfuron (0.01)	Prothioate (0.01)	Pyrimethanil (0.05)
Pyraclofos (0.01)	Pyraclostrobin (0.01)	Pyrethrins (0.01)	Pyridaben (0.01)	Pyridate (0.01)	Pyrimethanil (0.01)
Pyrimidifen (0.01)	Pyriproxyfen (0.01)	Quinoxyfen (0.01)	Resmethrin (0.05)	Rimsulfuron (0.01)	Rotenone (0.01)
Sebuthylazine (0.01)	Sethoxydim (0.01)	Simazine (0.01)	Simeconazole (0.01)	Spinosad ()	Spinosyn A (0.01)
Spinosyn D (0.01)	Spirodiclofen (0.01)	Spiromesifen (0.01)	Spirotetramine (0.01)	Sulfentrazone (0.02)	Sulfotep (0.01)
Sulprofos (0.01)	TCMTB (0.01)	Tebuconazole (0.01)	Tebufenozide (0.01)	Tebufenpyrad (0.01)	Tebufenzuron (0.01)
TEPP (0.01)	Tepraloxydim (0.01)	Terbacil (0.01)	Terbufos (0.01)	Terbuthylazine (0.01)	Terbuthyn (0.01)
Tetraconazole (0.01)	Thiabendazole (0.01)	Thiacloprid (0.05)	Thiamethoxam (0.02)	Thifensulfuron methyl (0.01)	Thiophanate-methyl (0.01)
Thiodicarb (0.01)	Thiofanox sulfone (0.01)	Thiofanox sulfoxide (0.05)	Thionazin (0.01)	Thiophanate-methyl (0.01)	Tolclofos-methyl (0.01)
Tolfenpyrad (0.01)	Tolylfluanid (0.01)	Triadimefon (0.1)	Triadimenol (0.01)	Tralkoxydim (0.01)	Triasulfuron (0.01)
Triasulfuron methyl (0.01)	Tribenuron-methyl (0.01)	Triclopyr (0.01)	Tricyclazole (0.01)	Tridemorph (0.01)	Trifloxystrobin (0.01)
Trifloxysulfuron (0.01)	Triflumizol-FM-6-1 (Sum) ()	Triflumizole (0.01)	Triflumuron (0.01)	Trifolsulfuron-methyl (0.01)	Trimesapac-ethyl (0.05)
Vamidothion (0.01)	Vamidothion-sulfone (0.01)	Vamidothion-sulfoxide (0.01)	XMC (0.05)	Zoxamide (0.01)	

SUS36 **Pesticide Screening(GC) (LOQ* mg/kg)**

2-Phenylphenol (0.01)	Acetochlor (0.01)	Aclonifen (0.05)	Aldrin (0.01)	Ametryne (0.01)	Anthraquinone (0.01)
Atrazine (0.02)	Atrazine (0.01)	Benfluralin (0.01)	Bifenox (0.02)	Bitertanol (0.01)	Bupirimate (0.01)
Bromfenvinfos (0.01)	Bromophos (0.01)	Bromophos-ethyl (0.01)	Bromopropylate (0.01)	Butachlor (0.01)	Butylamacil (0.01)
Cadusafos (0.02)	Captafol (0.05)	Captan (0.25)	Captan/THPI (Sum: calculated as Captan) ()	Carbophenothion (0.01)	Carbophenothion-methyl (0.01)
Carbofuran (0.02)	Chlomenside (0.01)	Chlordane (Sum) ()	Chlordane, alpha (0.01)	Chlordane, gamma (0.01)	Chlorfenapyr (0.03)
Chlorfenson (0.01)	Chlorfenvinphos (0.01)	Chlormephos (0.05)	Chloroneb/date (0.01)	Chloroneb (0.02)	Chloropropylate (0.01)
Chlorothalonil (0.01)	Chlorpyrifos-methyl (0.01)	Chlorthal-dimethyl (0.01)	Chlorthiron (0.05)	Chlozolinate (0.01)	Clofibrate (0.01)
Cyanazine (0.02)	Cyanofenphos (0.01)	Cyanophos (0.02)	Cyfluthrin (0.05)	Cyhalothrin, lambda-(incl. Cyhalothrin, gamma-) (0.02)	Cypermethrin (0.05)
Cyphenothrin (0.02)	DDD, o,p'- (0.01)	DDD, p,p'- (0.01)	DDE, o,p'- (0.01)	DDE, p,p'- (0.01)	DDT (Sum) ()
DDT, o,p'- (0.01)	DDT, p,p'- (0.01)	Deltamethrin (0.02)	Dichlofenthion (0.01)	Dichlofenthion (0.01)	Dichlorfluanid (0.01)
Dichlorobenzophenone o,p' (0.01)	Dichlorobenzophenone p,p' (0.01)	Dichlorvos (0.05)	Dicloran (0.02)	Dicofol (Sum) ()	Dicofol, o,p'- (0.02)
Dicofol, p,p'- (0.02)	Dieldrin (0.02)	Dienochlor (Sum) ()	Dienochlor (0.05)	Diflubenzuron (0.05)	Dimoxystrobin (0.01)
Dioxathion (0.02)	Diphenylamine (0.01)	Edifenphos (0.01)	Endosulfan (Sum) ()	Endosulfan, alpha- (0.02)	Endosulfan, beta- (0.01)
Endosulfan, sulfat- (0.01)	Endrin (0.01)	EPN (0.01)	Ethalfluralin (0.01)	Ethion (0.01)	Ethiozazole (0.01)
Etrimfos (0.01)	Famoxadone (0.02)	Fenarimol (0.05)	Fenchlorphos (0.01)	Fenchlorphos (sum) ()	Fenchlorphos oxon (0.01)
Fenfluthrin (0.02)	Fenitrothion (0.01)	Fenpropathrin (0.01)	Fenson (0.01)	Fenvalerate & Esfenvalerate (Sum of RR,SS,RS,SR isomers) (0.01)	Fenvalerate & Esfenvalerate (sum of RR,SS,RS,SR) ()
Fenvalerate & Esfenvalerate (sum of RR,SS isomers) (0.01)	Fluchloralin (0.01)	Flucythrinate (0.02)	Flumetralin (0.02)	Flumioxazin (0.02)	Fluotrimazole (0.01)
Fluquinconazole (0.01)	Fluvalinate-tau (0.02)	Folpet (0.25)	FolpetPh (Sum: calculated as Folpet) ()	Forclofa (0.01)	Formothion (0.01)
Halfenprox (0.01)	HCB (0.01)	HCH gamma(Lindan) (0.01)	HCH, alpha- (0.01)	HCH, beta- (0.01)	HCH, delta- (0.01)
HCH, epsilon- (0.01)	Heptachlor (0.01)	Heptachlor (Sum) ()	Heptachlor epoxide cis (0.01)	Heptachlor epoxide trans (0.01)	Heptenophos (0.01)
Iprobenfos (0.01)	Isazofos (0.01)	Isocarbophos (0.02)	Isodrin (0.01)	Isofenphos (0.01)	Isofenphos-methyl (0.01)
Isoprothiolane (0.01)	Jodfenphos (0.01)	Kresoxim-methyl (0.01)	Lindrin (0.02)	Malaoxon (0.05)	Malathion (Sum) ()
Mecarbam (0.02)	Mepronil (0.01)	Methacrifos (0.01)	Methidathion (0.02)	Methoxychlor (0.02)	Methyl-Pentachlorophenylsulfide (0.01)
Metribuzin (0.02)	Mevinphos (0.02)	Mirex (0.01)	N-Desethyl-pirimiphos-methyl (0.01)	Nitrapyrin (0.01)	Nitrofen (0.01)
Nitrothal-isopropyl (0.01)	Octachlorodipropyl ether (S-421) (0.02)	Ofurace (0.02)	Oxadixyl (0.01)	Oxychlordane (0.01)	Oxyfluorfen (0.01)
Paclobutrazol (0.01)	Parathion (0.01)	Parathion-methyl (0.01)	Parathion-methyl (Sum) ()	PCB 101 (0.01)	PCB 118 (0.01)
PCB 138 (0.01)	PCB 153 (0.01)	PCB 180 (0.01)	PCB 28 (0.01)	PCB 52 (0.01)	Pencycuron-urea (0.01)
Pentachloroaniline (0.01)	Pentachlorobenzene (0.01)	Permethrin (0.02)	Phenkaptor (0.05)	Phenothrin (0.02)	Phenthoate (0.02)
Phorate (0.02)	Phosphamidon (0.02)	Phthalimide (PI) (0.05)	Picoxystrobin (0.01)	Piperophos (0.01)	Pirimiphos-ethyl (0.01)
Procymidone (0.01)	Profenofos (0.01)	Profluralin (0.01)	Prometryn (0.01)	Propanil (0.02)	Propazine (0.01)
Prothiofos (0.01)	Pyrazophos (0.01)	Pyridaphenthion (0.01)	Pyrimiphos-methyl (0.01)	Pyrifenox (0.01)	Pyrimethanil (0.01)
Quinalphos (0.01)	Quintozene (0.01)	Quintozene (Sum) ()	Quizalofop-P-ethyl (0.01)	Silafluofen (0.01)	Vinclozolin (0.01)
Tebufenpyrad (0.01)	Tecnazene (0.01)	Teflubenzuron (0.01)	Terbufos (0.01)	Tetrachlorvinphos (0.01)	Tetradifon (0.01)
Tetrahydrophthalimide (THPI) (0.05)	Tetramethrin (0.01)	Tetrasul (0.01)	Tolyfluanid (0.01)	Triadimefon (0.01)	Triazamate (0.01)
Trichlorfon (0.01)	Trifloxystrobin (0.01)	Triflusulfuron (0.01)	Triforine (0.02)	Triticonazole (0.01)	Vinclozolin (0.01)

SIGNATURE

Kevin Fu
Authorized Signatory

EXPLANATORY NOTE

LOQ: Limit of Quantification
< LOQ: Below Limit of Quantification
N/A means Not applicable
☆ means the test is subcontracted within Eurofins group
* means the test is subcontracted outside Eurofins group
Sum compounds results are calculated from the results of each quantified compound as set by regulation

For and on behalf of Eurofins Technology Service (Qingdao) Co., Ltd.

END OF REPORT

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Appendix 6 Sweetness Intensity Report

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Sweetness equivalency of Glucosylated steviol glycosides

INTRODCUTION:

Sucrose,more commonly known as table sugar,is the standard by which sugar substitutes are compared to in terms of taste,texture,and caloric values, Glucosylated steviol glycosides is made from stevia and detrin ,in order to create a non-caloric sweetener than can be used in similar applications to sucrose.

PURPOSE:

To determine the sweetness equivalence of glucosylated steviol glycosides in comparison to sucrose.

TEST SAMPLES:

Sample of purified steviol glycoside and sucrose were prepared in water at room temperature respectively for comparison.

EQUIPMENT AND MATRIALS:

glucosylated steviol glycosides Sucrose

Purified water

Analytical scales

100ml beakers

Glass stirrers

Plastic cups

PROCEDURE

1.40 participants were pre-screened for taste acuiy prior to completing the taste panel

2.Serial dilutions of Glucosylated steviol glycosides in water were prepared by mixing increasing amounts of Glucosylated steviol glycosides in equal amounts of water.

3.Serial dilutions of sucrose in water were prepared by mixing increasing amounts sucrose in water,making sure that all sugar granules were completely dissolved.

4.Participants were given samples ,starting with plain water,followed by the series of serial dilutions from lowest to highest concentration .The sample in which they first noticed a change was selected by each participants for both sets of solutions.

5.The concentration at which at least 50% of the participants first noted detection of a change in the samples was used to determine the threshold value for each sweetener.

RESULTS

The threshold values found were 0.0017 solution for glucosylated steviol glycosides and 0.5% solution for sucrose,The sweetness equivalence of glucosylated steviol glycosides solution compared to the sucrose solution was determined to be 200 times sweeter than sucrose.

The following obsevations were made on the taste portion of the test:

Taste:High sweetness with Little after taste

Sweetness onset: Aquick offset of sweetness was noted

Sweetness level:Sweetness was comparatively high against sucrose solution

Sweet Linger: long linger

NAME OF SUPPLIER: Shandong Shengxiangyuan Biotechnology Co.,ltd

NAME OF PRODUCT:Glucosylated steviol glycosides (prostevia)

April 30, 2020

Appendix 7 Estimated Daily Intake Levels of Steviol Glycosides Preparations

Food Uses as Addressed by JECFA, EFSA, FSANZ & Others

JECFA reviewed various estimates of possible daily intake of steviol glycosides (WHO, 2006). Merisant (2008) also listed intended use levels of rebaudioside A for various food applications in their GRAS Notice. Cargill (2008) estimated the possible daily intake of rebaudioside A assuming the use levels would be comparable to aspartame and (Renwick, 2008). BioVittoria (2009) used an exposure estimate of "sucrose equivalents" and the sweetness intensity of Luo Han Guo fruit extract.

A. Estimated Daily Intake

Using different approaches, JECFA (WHO, 2006), Merisant (2008), and Cargill (2008) estimated daily intakes (EDIs) ranging from 1.3 – 4.7 mg per kg bw per day.

1. JECFA

- JECFA (WHO, 2006) evaluated information on exposure to steviol glycosides as submitted by Japan, China, and the European Commission by the Scientific Committee on Food. They used the Global Environment Monitoring System (GEMS)/Food consumption database to prepare international estimates of exposure to steviol glycosides (as steviol). JECFA assumed that steviol glycosides would replace all dietary sugars at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, which is 200:1.
- The intakes ranged from 1.3 mg per kg bw per day with the African diet to 3.5 mg per kg bw per day with the European diet. Exposures to steviol glycosides assumed full replacement of all dietary sugars in the diets for Japan and the US.
- JECFA concluded that the replacement estimates were highly conservative. Furthermore, the calculated dietary exposures were overestimates and would probably be 20 – 30% of these values, or 1.0 - 1.5 mg per kg bw per day on a steviol basis or 3.0 – 4.5 mg per kg bw per day for rebaudioside A, based on the molecular weight adjustment.

2. EFSA

- On January 13, 2011, EFSA revised its dietary exposure assessment of steviol glycosides. For high consumers, revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent). For European children aged 1-14 years, revised intake estimates ranged from 1.7 to 16.3 mg per kg bw per day, and for adults, the range was reported to be from 5.6 to 6.8 mg per kg bw per day (EFSA, 2011b).

3. FSANZ

- FSANZ (2008) estimated the steviol glycoside dietary intake for adult consumers in New Zealand, assuming a full sugar replacement scenario. The estimated exposure to rebaudioside A ranged from 0.3 – 1.0 mg per kg bw per day for a consumer at the mean and 0.5 – 1.5 mg per kg bw per day for a consumer in the 90th percentile. FSANZ concluded that there were no safety concerns for either adults or children.
- In 2009, Cargill applied to FSANZ to increase the maximum usage levels of steviol glycosides in the high-volume food categories with increased usage levels by presenting market share analyses that overestimate actual intake while remaining well below the generally accepted ADI.
- FSANZ (2010) accepted the increased usage levels as requested from Cargill since no public health and safety issues were identified.

4. MERISANT

- Merisant (2008) utilized food consumption survey data from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) to determine the estimated daily intake from the proposed uses of rebaudioside A.
- On a per user basis, the mean and 90th precentile daily consumption levels of rebaudioside A were estimated as 2.0 and 4.7 mg per kg bw per day, respectively.
- On a steviol equivalent basis, the Merisant estimates were calculated to be 0.7 and 1.6 mg per kg bw per day, respectively.
- On December 17, 2008, Merisant (2008) received a “no questions” letter from FDA for the use of rebaudioside A using NHANES food consumption data.

5. CARGILL

- Cargill (2008) estimated dietary intake figures for rebaudioside A by assuming that use levels of rebaudioside A would be comparable to those of aspartame in the US via post-market surveillance consumption data and published data for consumption of aspartame and other high intensity sweeteners (Renwick, 2008).
- On December 17, 2008, Cargill (2008) received a “no questions” letter from FDA for the use of rebaudioside A using comparative aspartame data.
- On May 13, 2011, FSANZ approved a Cargill application to increase the allowed maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages up to 200 mg per kg and in plain soy beverages up to 100 mg per kg (FSANZ, 2011).

6. BIOVITTORIA

- BioVittoria Ltd (2009) used an exposure estimate of “sucrose equivalents” and the sweetness intensity of any particular sweetener based upon data published by Renwick (2008).
- These data resulted in a maximum of 9.9 mg per kg bw per day for any population.
- BioVittoria (2010) received a “no questions” letter from FDA for the use of Luo Han Guo fruit extract using Renwick’s “sucrose equivalents.”

7. Other Publications

- Roberts et al. (2016) suggested that a higher ADI is justified based on metabolic factors to reduce the 100X safety factor. A chemical-specific adjustment factor (CSAF), as defined by the WHO in 2005, is determined by comparative studies in rats and humans.
    - A CSAF that is less than the standard 100X safety factor will result in an increase in the ADI, independent of the no observed adverse effect level (NOAEL).
    - The authors determined that using a CSAF can justify an ADI value of 6-16 mg per kg bw per day for steviol glycosides, depending on whether area under the plasma-concentration time curve (AUC) or C_{max} data are used when considering the 1,000 mg per kg bw per day NOAEL (which is equivalent to 400 mg per kg bw per day of steviol) for stevioside reported by Toyoda et al. (1997).

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Appendix 8 Studies on Steviol Glycosides Preparations

Part 1. Preparations that are Primarily Mixtures of Stevioside & Rebaudioside A

A. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

1. *In vivo* and *In vitro* Studies

- Studies investigating the hydrolysis of steviol glycosides by intestinal microflora have demonstrated that both stevioside and rebaudioside A are hydrolyzed to steviol following *in vitro* incubation with various cecal microflora (Wingard Jr. et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Geuns et al., 2003a).
- Various animal studies that show stevioside is not readily absorbed from the GI tract:
    - Rats - Wingard Jr. et al. (1980); Nakayama et al. (1986); Koyama et al. (2003b);
    - Hamsters - Hutapea et al. (1999);
    - Pigs - Geuns et al. (2003a); and
    - Chickens - Geuns et al. (2003b).
- *In vitro* metabolism studies show that steviol glycosides are transformed to steviol which is better absorbed in rats and humans (Geuns, 2003; Koyama et al., 2003b; Gardana et al., 2003; Wang et al., 2004).
- *In vitro* hydrolysis of rebaudioside A to steviol was found to be slower than that of stevioside (Koyama et al., 2003a).
    - The major pathway for rebaudioside A is conversion to stevioside with a minor pathway of conversion to rebaudioside B prior to being ultimately converted to steviol. Stevioside is further converted to steviolbioside, steviolmonosides, and finally steviol, with glucose being released with each subsequent hydrolysis.
- Koyama et al. (2003b) showed steviol can be converted to various glucuronides.
- Roberts and Renwick (2008) identified free steviol (82 to 86%), steviol, glucuronide (10 to 12%), and two unidentified metabolites (5-6%) in rat plasma following treatment with either stevioside or rebaudioside A eight hours post oral administration. Steviol T_{max} for plasma was noted within 30 minutes of oral administration as opposed to rebaudioside A, which has a T_{max} of 2 to 8 hours.
    - Following rebaudioside A treatment, significant amounts of unchanged rebaudioside A (29% in males and 19% in females) and stevioside (3% in males and 4% in females) were excreted in the feces.
    - Urinary excretion accounted for less than 2% of the administered dose.
    - Steviol was the predominant component found in plasma samples after oral administration of rebaudioside A, stevioside, and steviol in rats. The majority of all samples were found to be excreted rapidly---primarily in the feces---within 48 hours.
    - The predominant compound detected in the bile was steviol glucuronide, while the prominent material in the intestine was steviol.

  - The authors concluded that the overall data on toxicokinetics and metabolism indicate that rebaudioside A and stevioside are handled in an almost identical manner in the rat after oral dosing.
- Wheeler et al. (2008) assessed the comparative pharmacokinetics of steviol and steviol glucuronide following single oral doses of rebaudioside A and stevioside.
  - Following administration of rebaudioside A or stevioside, steviol glucuronide appeared in the plasma of all subjects, with median T_{max} values of 12.0 and 8.00 hours post-dose, respectively.
  - Administration of rebaudioside A resulted in a significantly (~22%) lower steviol glucuronide geometric mean C_{max} value (1,472 ng per mL) than administration of stevioside (1,886 ng per mL). The geometric mean AUC_{0-t} value for steviol glucuronide after administration of rebaudioside A (30,788 ng*h per mL) was approximately 10% lower than after administration of stevioside (34,090 ng*h per mL).
  - The authors concluded that rebaudioside A and stevioside underwent similar metabolic and elimination pathways in humans, with steviol glucuronide excreted primarily in the urine and steviol in the feces.
  - No safety concerns were noted as determined by reporting of adverse events, laboratory assessments of safety, or vital signs.
- Excretion of metabolites of stevioside after oral doses has been shown in urine and feces in rats (Sung, 2002) and hamsters (Hutapea et al., 1999).
- Oral doses in pigs led to the detection of metabolites in feces but not in urine (Geuns et al., 2003a).
- Since the individual steviol glycosides show similar pharmacokinetics in the rat and humans, the results of toxicology studies on individual steviol glycosides are applicable to the safety of steviol glycosides in general.

2. Human Studies

- Geuns et al. (2006) measured blood, urine, and fecal metabolites in 10 healthy subjects who received 3 doses of 250 mg of purified stevioside (>97%) three times per day for 3 days:
  - Free steviol was detected in feces but not in blood or urine. Steviol glucuronide was detected in blood, urine, and feces. Approximately 76% of the total steviol equivalents dosed were recovered in urine and feces.
  - The authors concluded that there was complete conversion of stevioside in the colon to steviol, which was absorbed and rapidly converted to the glucuronide.
- Renwick and Tarka (2008) reviewed studies on microbial hydrolysis of steviol glycosides and concluded that stevioside and rebaudioside A are not absorbed directly but are converted to steviol by gut microbiota in rats and in humans. This hydrolysis occurs more slowly for rebaudioside A than for stevioside.

B. Acute Toxicity Studies

A summary of the studies that investigated the acute toxicity of stevioside (96% pure) is presented in Table 8.1.

Table 8.1. Acute Toxicity of Stevioside (Purity 96%) Given Orally to Rodents

SPECIES	SEX	LD_{50} (G/KG BW)	REFERENCE
Mouse	Male and Female	>15	Toskulkac et al. (1997)
Mouse	Male	> 2	Medon et al. (1982)
Rat	Male and Female	>15	Toskulkac et al. (1997)
Hamster	Male and Female	>15	Toskulkac et al. (1997)

bw – body weight; g – gram; kg – kilogram

No lethality was noted within 14 days after administration, and no clinical signs of toxicity, or morphological or histopathological changes were found, indicating that stevioside is essentially nontoxic in acute oral exposures.

C. Subchronic Toxicity Studies

- Akashi and Yokoyama (1975) dosed rats with up to 2,500 mg per kg bw per day of stevioside for 3 months and reported no adverse effects.
- Mitsuhashi (1976) added up to 7% stevioside to the diets of F344 rats for 3 months and report no adverse effects.
- Aze et al. (1990) added stevioside at 0, 0.31, 0.62, 1.25, 2.5, and 5% to the diets of F344 rats for 13 weeks and reported no adverse effects. The apparent NOAEL was >5% dietary stevioside.
- The Awney et al. (2011) study revealed toxicity in rats dosed at 15 and 1,500 mg per kg stevioside, which resulted in a NOAEL of 15 mg per kg per day. This study is considered to be an outlier in critical reviews by Carakostas (2012) and Waddell (2011) for the following reasons:
    - Insufficient number of animals;
    - Animals were group housed leaving unreliable drinking water quantification;
    - No evidence of fasting before blood collection;
    - No urinalyses;
    - No histopathological confirmation of effects;
    - No organ weight data;
    - No laboratory historical control comparisons; and
    - Use of tartrate-resistant alkaline phosphatase (TRAP) enzyme, which has not been properly vetted for application on toxicological studies.

In summary, the data presented by Awney et al. (2011) are probably not representative of changes due to the subchronic dietary administration of steviol glycosides because of overall inadequate study design and reliance on the findings of the untested enzyme TRAP.

D. Chronic Toxicity Studies

- Yamada et al. (1985) added stevioside to the diets of F344 rats at 0.1, 0.3, and 1.0% with 95.2% steviol (75% stevioside/16% rebaudioside A) for 22 months in males and 24 months in females. Differences were noted in some parameters; however, the authors concluded that after 2 years of exposure, there were no significant changes that could be attributed to the administration of stevioside and reported no adverse effects. The calculated NOAEL was 550 mg per kg bw per day.
- Xili et al. (1992) added stevioside (86%) to the diets of F344 rats at 0, 0.2, 0.6, and 1.2% for 3 months and report no adverse effects. The calculated NOAEL was 794 mg per kg bw per day (high dose – 1.2%).
- Toyoda et al. (1997) added stevioside (96.5%) to the diets of F344 rats at 0, 2.5, and 5% for 104 weeks. The authors reported dose-dependent body weight gain decreases in both sexes. Kidney weights were significantly lower in 5% stevioside males; ovary, kidney and brain weights were significantly increased in 5% stevioside females; and there were decreased survival rates in males receiving 5% stevioside. However, stevioside was not carcinogenic at any level. The apparent NOAEL was the dietary level of 2.5%.
- No treatment-related increase in tumor incidence was seen in any of these studies.

E. Reproductive & Developmental Toxicity Studies

- No effects were observed in rats at doses of 96% stevioside dosed at 0, 0.15, 0.75, or 3% (equivalent to 2,000 mg per kg bw per day). The NOAEL was determined to be 2,000 mg per kg bw per day (Mori et al., 1981).
- No effect on fertility or reproductive parameters was seen in a three-generation study in hamsters at doses of 90% stevioside at 0, 500, 1,000, and 2,500 mg per kg bw per day (Yodyingyuad and Bunyawong, 1991). The NOAEL was determined to be 2,500 mg per kg bw per day.
- No teratogenic effects were observed in an additional rat study that was reviewed by Geuns (2003) in which pregnant female Wistar rats were administered stevioside (95.6%) at 0, 250, 500 or 1,000 mg per kg bw per day for 10 days (Usami et al., 1994). The NOAEL was determined to be 1,000 mg per kg bw per day.
- No effects on pregnancy or developmental parameters were observed in Swiss albino mice administered stevioside or aqueous stevia extract at doses of 500 and 800 mg per kg bw per day for 15 days to female mice (Kumar and Oommen, 2008).

F. Genotoxicity Studies

The following key genotoxicity studies have been conducted on stevia extracts and stevioside and showed negative responses:

- Bacterial mutagenicity studies negative for mutagenic response:
  - Medon et al. (1982).
  - Pezzuto et al. (1985);
  - Suttajit et al. (1993);
  - Matsui et al. (1996); and
  - Klongpanichpak et al. (1997);
- Mouse lymphoma (L5178Y/TK+/) study negative for mutagenic response:
  - Oh et al. (1999)
- Chromosome aberration studies negative for mutagenic response:
  - Human lymphocytes – Suttajit et al. (1993)
  - Chinese hamster lung fibroblasts – Nakajima (2000a); Ishidate et al. (1984)
- DNA damage (Comet assay) negative for mutagenic response:
  - Sekihashi et al. (2002); and
  - Sasaki et al. (2002)
- Mouse bone marrow/liver micronucleus studies negative for mutagenic response:
  - Oh et al. (1999)
- In two separate reviews by Carakostas et al. (2008) and Brusick (2008), research on rebaudioside A was summarized and combined with the body of knowledge on stevioside. These authors noted the following:
  - Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro.*
  - Steviol glycosides, rebaudioside A, and stevioside have not been shown to be genotoxic *in vivo* in well-conducted assays.
  - The Nunes et al. (2007a) study was improperly interpreted as positive.
  - Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
- Urban et al. (2013) examined the genotoxicity database on steviol glycosides concluding that the current database of *in vitro* and *in vivo* studies for steviol glycosides is robust and does not indicate that either stevioside or rebaudioside A is genotoxic.

G. Cytotoxicity

Abolhasani et al. (2020) evaluated the *in vitro* cytotoxicity of stevioside on cancerous liver (HepG2), colon (HT29), and breast (MCF7) cells, as well as normal kidney cells (Hek293), compared to cisplatin. Stevioside was reported to display higher cell growth inhibition on the HepG2 cell line and was not observed to have high toxicity on the Hek293 normal cell line. The authors concluded that stevioside “showed less cytotoxic effects compared to cisplatin.”

H. Clinical Studies & Other Reports in Humans

In South America, stevioside is used as a treatment for type 2 diabetes. These effects were key concerns for JECFA. In 2006, JECFA summarized the available clinical studies on stevioside and further studies were recommended (WHO, 2006). Subsequently, several additional studies were conducted and, in 2009, JECFA again reviewed these new studies (WHO, 2009). JECFA's summaries of the key studies are included in Table 8.2.

Table 8.2: Human Studies with Stevioside Preparations

AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY DESIGN AND DURATION	NOTED EFFECTS SAFETY PARAMETER RESULTS
Curi et al. (1986)	Aqueous extracts *S. rebaudiana* leaves	5 g at 6 h intervals for 3 days = 20 g/day	16 healthy patients – extract/ 6 healthy patients – arabinose	3-day glucose tolerance in healthy adults	The extract of *Stevia rebaudiana* increased glucose tolerance. The extract decreased plasma glucose levels during the test and after overnight fasting in all volunteers. No adverse effects were reported.
Chan et al. (2000)	Stevioside (purity not stated)	750 mg (11 mg per kg bw/day)	60 hypertensive Chinese men and woman (aged 28-75 years) + 46 patients were given placebo.	Multicenter randomized, double-blind, placebo-controlled for 12 months	3 months: mean systolic and diastolic BP decreased and continued through the 12 months. Minor side effects occurred with 2 test group and 1 placebo group patient withdrawing. Other side effects were minor and resolved.
Hsieh et al. (2003)	Stevioside (purity not stated)	1,500 mg (21 mg/kg bw/day)	85 hypertensive Chinese men and woman (aged 20-75 years) + 89 patients were given placebo.	Multicenter randomized, double-blind, placebo-controlled for 24 months	Mean systolic and diastolic blood pressures were decreased commencing from about 1 week after the start of treatment. At 2 years test group patients had a ↓ in incidence of left ventricular hypertrophy. 3 patients withdrew. Other side effects were minor and resolved.
Anonymous (2004a)	Steviol extract: (~73% stevioside ~24% Reb A)	100 mg (3.3 mg/kg bw/day)	48 hyperlipidemic volunteers (24/24)	Randomized, double-blind, placebo-controlled for 3 months	Analyses of serum concentrations of triglycerides, liver-derived enzymes, and glucose indicated no adverse effects. 3 patients withdrew. No adverse side effects were reported.
Anonymous (2004b)	Steviol extract: (~73% stevioside ~24% Reb A)	3.25, 7.5, or 5 mg/kg bw/day	12 patients per test group	Randomized, double-blind, placebo-controlled for 30 days	No adverse responses in blood and urine biochemical parameters.

AUTHOR/ YEAR	SUBSTANCE TESTED	TOTAL DAILY DOSE	POPULATION CHARACTERISTICS	STUDY DESIGN AND DURATION	NOTED EFFECTS SAFETY PARAMETER RESULTS
Gregersen et al. (2004)	Stevioside - 91% + 9% other stevia glycosides	1 g stevioside or 1 g starch	12 patients with type 2 diabetes total	Acute paired cross-over study, single dose study	18% ↓ in glucose concentrations: Systolic and diastolic blood pressure were unchanged. No adverse effects
Temme et al. (2004)	Stevioside 97%	750 mg/kg bw/day (288 mg/kg bw steviol)	4 male and 5 female healthy patients	Short term study – 3 days	Blood chemistry, blood pressure and urinalyses were unremarkable.
Barriocanal et al. (2006)	Stevioside – 64.5% + 18.9% Reb A	750 mg/kg bw/day	Type 1 (n=8) + Type 2 (n=15) diabetics + non-diabetics (n=15) + matching controls - placebo	Double-blind, placebo-controlled trial study for 3 months	Blood chemistry, glycated hemoglobin (HbA1c), blood pressure and urinalyses were unremarkable. No adverse effects
Barriocanal et al. (2008)	Stevioside - >92%	250 mg/kg bw/day	Type 1 and Type 2 diabetics, placebo controls	Randomized, double-blind, placebo-controlled for 3 months	No changes in systolic BP, diastolic BP, glucose, or glycated hemoglobin from baseline. No adverse effects
Ferri et al. (2006)	Stevioside (purity not stated)	3.75, (7 weeks), 7.5 (11 weeks), 15 (6 weeks) + placebo (24 weeks) mg/kg bw/day	Patients with mild hypertension	Randomized 24 week study	No changes in systolic BP, diastolic BP. No adverse effects.
Silva et al. (2006)	Stevioside: 70%	Equivalent to 1.04 mg steviol/kg bw/day + placebo	49 Mild hyperlipidemic patients: Stevioside group (n=24) placebo controls (n=25) Age: 20-70 years	Placebo-controlled double-blind trial for 90 days	No effects of treatment on ALT, AST, or GGT were found. No relevant adverse effects were noted.
Jeppesen et al. (2006)	Stevioside (purity not stated)	1,500 mg/kg bw/day or maize starch placebo	55 patients with Type 2 diabetes:	Randomized, double blinded, placebo-controlled study	No effects on the HbA1c fasting blood glucose levels, lipids, or blood pressure

ALT – alanine aminotransferase; AST – aspartate aminotransferase; BP – blood pressure; bw – body weight; g – gram; GGT – gamma-glutamyltransferase; h – hour; HbA1c – glycated hemoglobin; kg – kilogram; mg – milligram

I. Other Studies

- Thøgersen et al. (2018) investigated the effect of rebaudioside A, stevioside, and steviol on porcine cytochrome p450 (CYP) expression and activity to assess their potential food-drug interactions in the IPEC-J2 cell line.
    - There were no changes in CYP messenger ribonucleic acid (mRNA) expression following treatment of IPEC-J2 cells with rebaudioside A, stevioside, and steviol compared with control.
    - Treatment of primary hepatocytes resulted in increases in CYP329 mRNA at low concentrations of rebaudioside A and steviol, and at all concentrations of stevioside.

  - Treatment with the steviol glycosides tested over 24 hours resulted in minor increases in CYP3A29 mRNA expression (< 2.0-fold), while “no direct effect on CYP activity” was observed.
  - The authors concluded that rebaudioside A, stevioside, and steviol are unlikely to cause a food-drug interaction but noted that the study could not predict long term effects and effects *in vivo*.
- Zhao et al. (2020) studied the effect and mechanism of stevioside on preventive and therapeutic cardiac fibrosis caused by hyperglycemia in male C57BL/6 mice.
  - Stevioside supplementation reduced the expression of the cardiac fibrosis producing lysyl oxidase family (LOX) and weakened the collagen cross-linking lysyl oxidase-like 2 (LOXL2) caused by hyperglycemia.
  - Stevioside supplementation promoted the elimination of existing fibrosis via the regulation of matrix metalloproteinase (MMP 2/9) and tissue inhibitors of metalloproteinase (TIMP2/4).
  - No adverse effects were reported.

Part 2. Preparations That Are Primarily Rebaudioside A

A. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

Studies investigating the ADME of extracts from stevia are available on stevioside, rebaudioside A, and other steviol glycosides. Data evaluating the absorption and fate of these extracts from various animal species and humans indicate that one can extrapolate these results from rats to humans.

- Sloter (2008a) examined the potential of rebaudioside A toxicity in rats at up to 2,000 mg per kg bw per day.
  - Low levels of rebaudioside A were detected in the peripheral blood of rats post-administration of 2,000 mg per kg bw per day.
  - Estimates of absorbed dose for rebaudioside A of 0.6 µg per mL in plasma (corresponding to 0.02%) were based on amounts measured in urine collected over 24 hours in comparison to the daily administered dietary dose.
  - Mean fecal rebaudioside A and measured hydrolysis products, expressed as Total Rebaudioside A Equivalents, compared with daily administered dose results in an estimated dose recovery of approximately 84%.
- Zhou et al. (2019) investigated the interaction of organic anion transporter 3 (OAT3)-mediated update of the rebaudioside A metabolite, steviol acyl glucuronide, with selected prescription drugs.
  - The inhibitory potency of therapeutic drugs (those frequently prescribed for treating hyperglycemia, hyperlipidemia, and hyperuricemia, including probenecid and glimepiride) was examined against human renal excreter - organic anion transporter 3 (hOAT3) and rat organic anion transporter 3 (rOAT3) for uptake of steviol acyl glucuronide.

- OAT3-mediated update of steviol acyl glucuronide was examined *in vitro* using hOAT3 and rOAT3 transfected human embryonic kidney 293 (HEK203) cells. Both probenecid and glimepiride were potent inhibitors of hOAT3 and rOAT3, with no apparent species differences observed.
- Pharmacokinetic studies in male Sprague-Dawley rats revealed both probenecid and glimepiride significantly elevated plasma steviol acyl glucuronide concentrations, particularly between 6 and 8 hours after oral administration of rebaudioside A.
- The inhibition of OAT3 is a potential mechanism for the interaction between steviol acyl glucuronide and probenecid or glimepiride, which can alter pharmacokinetic and safety profiles of steviol acyl glucuronide and steviol glycosides—specifically rebaudioside A.
- The authors conclude that this interaction might be clinically relevant, and that care should be given to populations with concomitant use of stevia leaf extracts and probenecid or glimepiride.

B. Subchronic Toxicity Studies

- Curry and Roberts (2008) added up to 100,000 ppm of rebaudioside A (97%) to the diets of Wistar rats for 13 weeks and reported no treatment-related adverse effects. Hence, the NOAEL was reported to be 9,938 mg per kg for males and 11,728 mg per kg for females – the highest level of treatment.
- Rebaudioside A (99.25%) was added to the diets of CRL:CD(SD) rats for 90 days at target doses of 500, 1,000, and 2,000 mg per kg bw per day with no treatment-related effects. The NOAEL was determined to be ≥2,000 mg per kg (Eapen, 2007; Nikiforov and Eapen, 2008).
- Eapen (2008) added rebaudioside A (97.5%) to the diets of Beagle dogs for 6 months at target doses of 500, 1,000, and 2,000 mg per kg bw per day and reported no adverse effects. The NOAEL was determined to be >2,000 mg per kg bw per day.
- The oral administration of fermentative Reb A to Sprague-Dawley rats for 91 days did not lead to any adverse effects at consumption levels up to 2,057 mg per kg bw per day for males and 2,023 mg per kg bw per day for females, which were concluded to be the NOAELs (Rumelhard et al., 2016).

C. Genotoxicity Studies

- *In vitro* and *in vivo* genotoxicity assays covering mutation, chromosome damage, and deoxyribonucleic acid (DNA) strand breakage consistently and uniformly revealed negative results for rebaudioside A.
- Evaluation of fermentation-derived rebaudioside A demonstrated a similar safety profile to plant-derived rebaudioside A (Rumelhard et al., 2016).

The following key mutagenicity studies have been conducted on rebaudioside A and are negative for mutagenic responses:

- Bacterial mutagenicity studies negative for mutagenic response:
  - Wagner and Van Dyke (2006);
  - Williams and Burdock (2009); and
  - Rumelhard et al. (2016).
- Mouse lymphoma (L5178Y/TK+/) studies negative for mutagenic response:
  - Clarke (2006); and
  - Williams and Burdock (2009).
- Human lymphocyte study negative for mutagenic response: Rumelhard et al. (2016)
- Chromosome aberration studies negative for mutagenic response:
  - Chinese hamster lung fibroblasts – Nakajima (2000a); and
  - Human lymphocytes – Williams and Burdock (2009).
- Mouse micronucleus studies negative for mutagenic response:
  - Nakajima (2000b) (BDF1 mouse bone marrow);
  - Krsmanovic and Huston (2006);
  - Williams and Burdock (2009); and
  - Unscheduled DNA synthesis (UDS) study negative for mutagenic response - Williams and Burdock (2009)
- Bacterial forward mutation study negative for mutagenic response – Pezzuto et al. (1985)

D. Reproductive & Developmental Studies on Rebaudioside A

- Curry et al. (2008) conducted a two-generation reproductive toxicity study on rebaudioside A administered in the diet at 7,500, 12,500 and 25,000 ppm in Han Wistar rats. There were no signs of toxicity or adverse effects on body weights, body weight gain, or food consumption. Rebaudioside A did not affect reproductive performance parameters including mating performance, fertility, gestation lengths, estrous cycles, or sperm motility, concentration, or morphology in either the F_0 or F_1 generations. The NOAEL for reproductive effects was 25,000 ppm, and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm, or 2,048 to 2,273 mg per kg bw per day (the highest dose tested).
- An unpublished study on rebaudioside A was conducted on four groups of male and female Crl:CD(SD) rats (30 per sex per group) that were fed either a basal diet or the diet containing rebaudioside A (purity 95.7%) for at least 70 consecutive days prior to mating (Sloter, 2008a). The test diet was offered to the offspring selected to become the F_1 generation following weaning (beginning on postnatal day 21). The F_0 and F_1 males continued to receive rebaudioside A throughout mating, gestation, and lactation until the day of euthanasia. Both for parental systemic and reproductive toxicity, the NOAEL was ≥2,000 mg per kg bw per day (highest dose administered).

- In another unpublished study, the embryo/fetal developmental toxicity effects of rebaudioside A when administered via gavage were studied in rats (Sloter, 2008b). The NOAEL for maternal and embryo/fetal development was determined to be >2,000 mg per kg bw per day.
- Cho et al. (2018) investigated the impact of stevia and obesity on fertility and reproductive outcomes in Sprague-Dawley rats. Rats were administered 2-3 mg per kg bw per day rebaudioside A in drinking water starting two weeks prior to mating and throughout lactation. The authors reported that obese rats supplemented with rebaudioside A displayed a lower fertility index than untreated obese rats (53.3% vs. 85.7%, respectively); however, the rate of successful pregnancies was higher in obese rats supplemented with rebaudioside A than untreated obese rats (100% vs. 60.7%). No adverse effects or animal deaths were reported.
- Nettleton et al. (2020) investigated the impact of maternal low-dose rebaudioside A consumption on adiposity, glucose tolerance, gut microbiota, and the mesolimbic pathway in obese dams and their offspring. Pregnant obese rats and their offspring were fed high fat/sucrose diet plus 3 mg per kg bw per day rebaudioside A (Sigma-Aldrich) through 18 weeks postpartum. The authors noted that rebaudioside A consumption reduced the fertility of dams. The study supports findings that low-calorie sweeteners may not be metabolically inert.

E. Clinical Studies on Rebaudioside A

A summary of the clinical studies conducted on rebaudioside A is presented in Table 8.3.

Table 8.3. Human Studies with Rebaudioside A Preparations

Author/ Year	Substance Tested	Total Daily Dose	Population Characteristics	Study Design and Duration	Noted Effects Safety parameter Results
Maki et al. (2008a)	Rebaudioside A (97%)	Reb A: 1,000 mg Placebo: 0 (n=100) Age: 18-73 years	Primarily female patients with normal and low-normal systolic blood pressure (SBP) and diastolic blood pressure (DBP)	Randomized, double-blind, placebo-controlled trial for 4 weeks	The extract of *Stevia rebaudiana* increased glucose tolerance. The extract decreased plasma glucose levels during the test and after overnight fasting in all volunteers.
Maki et al. (2008b)	Rebaudioside A (97%)	Reb A: 1,000 mg (n=60) Placebo: 0 (n=62) Age: 33-75 years	Men and women with Type 2 diabetes	Randomized, double-blind, placebo-controlled trial for 16 weeks	No treatment related changes in blood pressure, body weight, and fasting lipids were noted. Rebaudioside A was well-tolerated, and records of hypoglycemic episodes showed no excess versus placebo.

DBP – diastolic blood pressure; mg – milligram; SBP – systolic blood pressure

F. Safety of Rebaudioside A

There have been a number of studies regarding the safety and toxicity of rebaudioside A:

- GRAS Notices submitted to FDA:
    - GRN 252: Merisant (2008) conducted studies that augmented genotoxicity data in three systems recognized by FDA as good predictors of carcinogenic potential. Two of these assays were conducted in mouse systems.
    - GRN 253: Cargill (2008) conducted studies that provided significant insight into the pharmacokinetics of rebaudioside A, while demonstrating clinical safety of rebaudioside A regarding lack of effects on blood pressure and glucose metabolism that could result from doses expected from use in food.
- JECFA concluded that all naturally occurring steviol glycosides are safe as long as there is a combined purity of not less than 95% and determined the ADI of the steviol glycosides applied to rebaudioside A because the pharmacokinetics are virtually the same (FAO, 2017).
- Carakostas et al. (2008) summarized the Cargill research program findings on rebaudioside A:
    - Steviol glycosides, rebaudioside A, and stevioside are not genotoxic *in vitro.*
    - In well-conducted *in vivo* assays, steviol glycosides, rebaudioside A, and stevioside have not been found to be genotoxic.
    - A report indicating that stevioside produces DNA breakage *in vivo* appears to be flawed (Nunes et al., 2007a) and was improperly interpreted as a positive response.
    - Steviol genotoxicity in mammalian cells is limited to *in vitro* tests that may be affected by excessive concentrations of the compound.
    - The primary evidence for steviol genotoxicity is derived from very specific bacterial tests or purified plasmid DNA that lack DNA repair capabilities.
    - Stevioside is not a carcinogen or cancer promoter in well-conducted rodent chronic bioassays.
    - While studies with rebaudioside A indicated minimal gastrointestinal (GI) absorption of the glycoside per se, the predominant metabolic pathway is comparable to that of stevioside. The use of the ADI established by JECFA, which was determined in studies employing stevioside as the main component, can be used as the ADI for rebaudioside A.
    - The dietary levels expected from consumption of rebaudioside A as a total replacement of sugar (Renwick, 2008) are lower than the ADI and, therefore, there is no safety concern for consumers.
- JECFA has evaluated the use of steviol glycosides in foods and agrees that, at the present time, the ADI for steviol glycosides of adequate purity, as defined by JECFA specifications, has been properly determined to be 4 mg per kg bw per person as steviol equivalents, which corresponds to 12 mg per kg bw per day for rebaudioside A, on a dry weight basis. Therefore, the JECFA-derived ADI was adopted as a safe exposure for rebaudioside A and

the corresponding food uses meeting the specifications within the limits determined by this esteemed international body of food safety experts can be considered to be GRAS.

- Williams and Burdock (2009) reviewed 3 *in vitro* and 2 *in vivo* genotoxicity and mutagenicity studies on rebaudioside A conducted according to Organisation for Economic Co-operation and Development (OECD) guidelines and found the studies revealed that rebaudioside A is:
    - non-mutagenic in an Ames test using *Salmonella typhimurium* and *Escherichia coli*;
    - non-mutagenic in a chromosomal aberration test using Chinese hamster V79 cells;
    - non-mutagenic in a mouse lymphoma assay using L5178Y+/- cells;
    - non-mutagenic a bone marrow micronucleus test in mice at doses up 750 mg per kg bw; and
    - non-mutagenic in an unscheduled DNA synthesis test in rats at 2,000 mg per kg bw.
    - The authors note that these studies provide additional evidence that rebaudioside A is not genotoxic at the doses tested and further support the GRAS determination of rebaudioside A.

Part 3. Studies on Other Steviol Glycosides Preparations

A. Absorption, Distribution, Metabolism & Excretion (ADME) Studies

- Koyama et al. (2003b) published an *in vitro* study where α-glucosylated steviol glycosides were degraded by fecal microflora to steviol glycosides. These glycosides are subsequently hydrolyzed to the aglycone, steviol, demonstrating that the metabolic fate of α-glucosylated steviol glycosides follows that of non-modified steviol glycosides. Due to the similarities in metabolic fate, the safety of α-glucosylated steviol glycosides can be established based on studies conducted with non-modified steviol glycosides.
- Purkayastha et al. (2014) compared the anaerobic *in vitro* metabolism of rebaudiosides A, B, D, and M with human fecal homogenates.
    - The rebaudiosides were hydrolyzed to steviol within 24 hours, with the majority of metabolism occurring within the first 8 hours.
    - Metabolism of rebaudiosides took longer at higher concentrations (2.0 mg per mL vs. 0.2 mg per mL).
    - There were no marked differences in rate or extent of hydrolysis observed between male and female fecal homogenates or individual rebaudiosides.
- Purkayastha et al. (2016) investigated the metabolic fate of steviolbioside, dulcoside A, and rebaudiosides A, B, C, D, E, F, and M in an *in vitro* study using pooled human fecal homogenates over the course of 24 to 48 hours.
    - The glycosidic side chains ---containing glucose, rhamnose, xylose, fructose, and those with deoxy-glucose including combinations of α(1-2), β-1, β(1-2), β(1-3), and β(1-6) linkages ---were mostly degraded to steviol within 24 hours.
    - The rate of metabolism was slower at higher concentrations (2.0 mg per mL vs. 0.2 mg per mL).

  - No appreciable differences in metabolism were observed between fecal homogenates obtained from males and females or those obtained from different ethnicities.
- Purkayastha and Kwok (2020) investigated the *in vitro* metabolic fate of steviol glycosides in fecal homogenates collected from adults and children.
  - Steviol glycosides obtained from stevia leaf extract (composed of more than 20 steviol glycosides, with Reb D and Reb M as the principal components), bioconversion reaction product (composed of Reb D and Reb M), minor steviol glycosides extracted from a stevia leaf extract (composed of Reb AM, Reb W2, Reb U2, Reb V, Reb N, and Reb O), enzyme modified steviol glycosides, and rebaudioside A standard were used as test samples.
  - All steviol glycosides preparations tested "shared qualitatively similar *in vitro* metabolic fates."
  - The authors concluded that "safety data for individual steviol glycosides can be used to support safety of all steviol glycosides produced by extraction and enzymatic conversion of stevia leaf extract."

B. Toxicity Studies

- One study showed a toxic response and was conducted by Nunes et al. (2007a). In the Nunes study, rats were dosed with 4 mg per mL steviol glycosides in drinking water (estimated 80 to 500 mg per kg bw per day) for 45 days. Positive findings were reported in the liver, brain, and spleen, but most notably the liver. This study is considered to be an outlier in critical reviews conducted by Geuns (2007), Williams (2007), and Brusick (2008). The authors responded to these critiques (Nunes et al., 2007b; Nunes et al., 2007c) and the consensus appears to be that Nunes et al. (2007a) used flawed methodology and improperly interpreted data as a positive response.
- Silva et al. (2018) addressed the genotoxic activity of stevia (Svetia™, purity not reported[11]).
  - Human lymphocytes were treated with 5% and 0.5% Svetia™ for 2 hours.
  - No statistically significant difference in genetic damage was observed in the 0.5% treatment concentration compared with the negative control, while the 5% treatment concentration resulted in a statistically significant difference ($P<0.0001$) compared with the control, with a decrease in migration average.
  - Human lymphocytes treated with 10% Svetia™ demonstrated significant ($P<0.0001$) genotoxic activity compared to the control; however, at treatment concentrations of 0.05%, 0.5%, and 5% Svetia™, a significant ($P<0.0001$) decrease in average migration of DNA was observed compared with the control.
  - The authors conclude that these results demonstrate the absence of genotoxicity at concentrations of up to 5% Svetia™ (Silva et al., 2018). It should be noted that these

[11] While the purity of the material used for the study was not reported by Silva et al. (2018), a search of the manufacturer's website (www.svetia.us) indicates that the trademarked material is a blend of cane sugar and 97% pure Reb A.

observations are inconsistent with data reported by Nunes et al. (2007a); however, as discussed above, the validity and importance of the Nunes et al. study has been discounted given the questions surrounding the methodology.

C. Other Studies

- Sánchez-Delgado et al. (2019) studied the effects of steviol glycosides in a seven-week study on healthy young adults aged 18-30 years old. Thirty-eight patients were assigned to one of three study groups and "washed out" for one week prior to study initiation.
    - For six weeks, study participants were administered one of the following dosage regimes:
        - Sucrose (8 X 5 g packets per day)
        - Sucralose (8 X 5 g packets per day)
        - Steviol glycosides (4 X 1 g packets per day)
    - Results were as follows:
        - Subjects in the sucrose treatment group showed increased triglycerides and cholesterol.
        - Subjects in the sucralose treatment group showed increased body weight.
        - Subjects in the steviol glycosides treatment group show decreased fat mass, decreased triglycerides, and decreased tumor necrosis factor-α.
    - The authors concluded that steviol glycosides may have positive effects on metabolic parameters.
- Halasa et al. (2020) published a case study vignette on the investigation of the presence of steviol glycosides metabolites in plasma, cerebrospinal fluid, amniotic fluid, and cord blood samples from as early as 2004. The end date was not provided.
    - Steviol glucuronide was detected primarily in the plasma.
    - Seven of the 38 adults (18%) had detectable steviol glucuronide concentrations, while two of 13 (15%) amniotic fluid samples and one of 15 (7%) cord blood samples were observed to contain steviol glucuronide.

Part 4. Studies on Crude Stevia Extracts

In several studies, pharmacological and biochemical effects of crude extracts of stevia leaves and purified steviol glycosides have been investigated.

- In experimental studies in rats, crude stevia leaf extract (5%) was administered to female rats at 0 or 5% for 12 days. The female rats were subsequently mated with untreated males for the last 6 days, making a total of 18 days of exposure for the females (Planas and Kuć, 1968). Fertility was reduced to 21% of the fertility of control rats and remained reduced during the 50- to 60-day recovery period. The study report did not discuss histological examinations, weights of organs, blood analysis, urine chemistry, and necropsy.

- The use of *S. rebaudiana* as an oral contraceptive has been reported by indigenous populations in Paraguay (Planas and Kuć, 1968; Schvartaman et al., 1977).
- In rat studies, dried stevia leaves were administered at 0.67 g per mL in 2 mL doses twice per day for 60 days (Oliveira-Filho et al., 1989). The only difference due to treatment was seminal vesicle weight, which fell to 60% compared with control. No treatment-related adverse effects were noted.
- Wang and Wu (2019) studied the angiotensin-converting enzyme (ACE) inhibiting activity of an ethanolic extract of stevia leaves and purified steviol glycosides from the ethanol extract.
    - Steviol glycosides were reported to have double the ACE inhibitory activity of the ethanolic extract from stevia leaves.
    - Sensory tests in decaffeinated coffee, decaffeinated tea, and peanut protein beverages prepared with steviol glycosides demonstrated the preparations were well-accepted.
    - Steviol glycosides had a significant antihypertensive effect in spontaneously hypertensive rats. The authors suggest that the effect was dosage-dependent.
    - No adverse effects were reported.
- Assi et al. (2020) studied the efficacy of stevia extract alone and in combination with the commonly used sulfonylurea, glimepiride, in a trial to introduce a new effective therapeutic regimen for type 2 diabetes mellitus.
    - Rats with type 2 diabetes were treated orally with 300 mg per kg per day stevia extract for 21 days.
    - Results indicated that treatment with stevia extract showed good control of blood glucose levels and that a significant elevation in insulin release to glimepiride was observed.
    - The authors reported that stevia extract reduced blood glucose, triglycerides, cholesterol, ALT, AST, urea, creatinine, tumor necrosis factor, and malondialdehyde levels, while improving insulin and adiponectin levels.
    - No adverse effects were reported.
- Ray et al. (2020) studied the effects of *Stevia rebaudiana* on glucose homeostasis, blood pressure, and inflammation.
    - No hypersensitivities or allergies were reported since 2008, and that the few prior reports were for “improperly filtered stevia extracts.”
    - No significant adverse effects were noted from any study included in the review.

Part 5. Studies on Principal Metabolite: Steviol

A. Acute Toxicity Studies

- Toskulkac et al. (1997) administered single doses of steviol (90%) to rats and hamsters:
    - Rat, oral LD_{50} >15 g per kg; and
    - Hamster, oral LD_{50} 5.2 g per kg bw in males and 6.1 g per kg bw in females.

- Histopathological examination of the kidneys of hamsters revealed severe degeneration of the proximal tubular cells, and these structural alterations were correlated with increased serum blood urea nitrogen and creatinine. The authors concluded that the cause of death was acute renal failure.

B. Developmental Toxicity Studies

- Wasuntarawat et al. (1998) treated groups of pregnant golden hamsters with steviol (90%) at doses of 0 mg (n not reported), 250 mg (n=20), 500 mg (n=20), or 1,000 mg (n=12) per kg bw per day by gavage in corn oil on days 6 -10 of gestation.
  - A significant decrease in body weight gain and increased mortality (1/20, 7/20, and 5/12) were observed at the three highest doses.
  - The number of live fetuses per litter and mean fetal weight decreased in parallel.
  - No dose-dependent teratogenic effects were seen.
  - The NOEL for both maternal and developmental toxicity was 250 mg per kg bw per day.

C. Mutagenicity & Genotoxicity Studies

The following key mutagenicity studies have been conducted on steviol and are negative for mutagenic responses:

- Bacterial mutagenicity studies negative for mutagenic response:
  - Compadre et al. (1988);
  - Procinska et al. (1991); and
  - Klongpanichpak et al. (1997).
- Chromosome aberration studies negative for mutagenic response:
  - Chinese hamster lung fibroblasts – Matsui et al. (1996)
- DNA damage (Comet assay)
  - Sekihashi et al. (2002)
- Mouse bone marrow/liver micronucleus studies negative for mutagenic response:
  - Oh et al. (1999)
- Micronucleus studies negative for mutagenic response:
  - Matsui et al. (1996) (mouse);
  - Temcharoen et al. (2000) (rat);
  - Temcharoen et al. (2000) (mouse); and
  - Temcharoen et al. (2000) (hamster).

The following key mutagenicity studies have been conducted on steviol and are positive or equivocal for mutagenic responses:

- Bacterial mutagenicity studies positive for mutagenic response:

  - Pezzuto et al. (1985) – Mutagenicity was dependent on pretreatment of rats with Arochlor and NADPH addition, as unmetabolized steviol was inactive. None of the other metabolites tested was mutagenic.
  - Compadre et al. (1988) – Mass spectral analysis of steviol and analogues under conditions known to produce a mutagenic response. 15-oxo-steviol, a product of the metabolite, 15-alpha-hydroxysteviol was found to be a direct-acting mutagen.
  - Matsui et al. (1996) – Steviol was equivocal for mutagenicity. Steviol was weakly positive in Umu chromotest, either with or without metabolic activation. Steviol was negative in the reverse mutation and other bacterial assays even in presence of S9 activation.
  - Temcharoen et al. (2000) – Mutagenic effects of steviol and/or metabolites found in *S. typhimurium* TM677 by tranversions, transitions, duplications, and deletions at the guanine phosphoribosyltransferase (gpt) gene.
  - Terai et al. (2002) – Steviol was found to be mutagenic in Arochlor-induced rat liver S9 fraction.
- Chinese hamster lung fibroblast study positive for mutagenic response:
  - Matsui et al. (1996) – Gene mutations found in Chinese hamster lung fibroblasts after metabolic activation of steviol. In hamsters, several metabolites of stevioside found that have not been found in rats or humans. Therefore, experimental relevance should be questioned when hamsters are used.

Each of the positive mutagenicity studies noted above had special circumstances or slightly different procedures. The positive mutagenicity studies were collectively not believed to present sufficient toxicological concern as determined by JECFA (WHO, 2006).

D. Endocrine Disruption Studies

- Shannon et al. (2016) investigated the endocrine disrupting potential of stevioside, rebaudioside A, and steviol in a series of *in vitro* bioassays and found that steviol:
  - antagonizes progesterone nuclear receptor transcriptional activity,
  - increases progesterone production, and
  - induces an agonistic response on the progesterone receptor of sperm cells (Catsper).
  - The authors conclude that steviol might not qualify as a safer alternative to sugar or synthetic sweeteners. However, one must consider the fact that it is difficult to translate *in vitro* concentrations to local concentrations *in vivo* at the receptor level and no adverse effects have been noted in any reproductive studies.

E. Other Studies

- Kurek et al. (2020) reported on the effect of steviol on cytotoxicity, adipogenesis, ROS concentration, and gene expression in the murine 3T3-L1 cell line.

- There was no observed effect on the proliferation of cells, lipid accumulation, or intracellular ROS generation at steviol concentrations up to 100 μM.
- Furthermore, it was reported that steviol reduced the expression of genes regulating the adipogenesis and lipogenesis process.

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Appendix 9 Summary of the Regulatory History of Steviol Glycosides

A. History of Traditional Medicinal and Human Food Use

- Stevia use as a sweetener and in traditional medicine by the Guarani tribes can be traced back for centuries (Esen, 2016; Gerwig et al., 2016; Brusick, 2008; Brandle et al., 1998).
- Stevia is commonly used to treat Type 2 diabetes in South America (Hawke, 2003). Doses in the range of 1 gram per person per day or more were reported to be necessary for therapeutic effects (Gregersen et al., 2004).
- Japan and Brazil approved stevia as a food additive in the 1980s (Raintree, 2012). Lester (1999) reported that 40% of the artificial sweetener market in Japan was stevia based.
- Use of steviol glycosides as a dietary supplement is presently permitted in the US, Canada, Australia, and New Zealand, and use as a natural health product is permitted in Canada.
- In 2005, it was estimated that sales of stevia in the US reached $45 million (Newsday, 2006).
- In 2010, Zenith International estimated stevia sales of 3,500 metric tons, which represents a 27% increase over 2009 figures. The market value is estimated to have increased to $285 million (Zenith, 2011).
- In 2013, worldwide sales of stevia were reported at 4,100 tons – representing a 6.5% increase over 2011 figures with an overall market value of $304 million (Zenith, 2013).
- In October 2014, it was reported that worldwide stevia sales increased 14% to 4,670 tons, with a market value of $336 million. It has been projected that the total market for stevia in 2017 would be 7,150 tons with an associated market value of $578 million (Zenith, 2014).
- NewHope360 reported that the global market for stevia in 2014 was $347 million, and that is expected to increase to $565.2 million by 2020. In addition, consumption is expected to increase from 2014 levels of 5,100.6 tons to 8,506.9 tons by 2020 (NewHope360, 2015).
- Nutritional Outlook reported that Mintel data indicated a 48% increase in stevia-containing products over the last five years (Decker and Prince, 2018).

B. Summary of Regulatory History of Enzyme Modified Steviol Glycosides

1. U.S. Regulatory History

To date, FDA has issued 64 “no questions” letters on GRAS Notices on rebaudioside A, rebaudioside D, rebaudioside M, or steviol glycosides, including those undergoing enzyme treatment (FDA, 2020).

In addition, the Flavor and Extract Manufacturers Association (FEMA) has included several steviol glycosides preparations that are used to formulate flavors on their GRAS lists as shown in Table 9.1.

Table 9.1. FEMA GRAS Status for Steviol Glycoside Preparations

STEVIOL GLYCOSIDES PREPARATION	FEMA NUMBER	REFERENCE
Rebaudioside A	4601	Smith et al. (2009)
Rebaudioside C; dulcoside B	4720	Leffingwell (2011)
Glucosyl steviol glycosides; enzymatically modified stevia extract	4728	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Stevioside	4763	Leffingwell and Leffingwell (2014); Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 60%	4771	Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside A 80%	4772	Marnett et al. (2013)
Steviol glycoside extract, *Stevia rebaudiana*, Rebaudioside C 30%	4796	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, *Stevia rebaudiana,* Rebaudioside A 22%	4805	Cohen et al. (2015a); Cohen et al. (2015b)
Steviol glycoside extract, *Stevia rebaudiana* Rebaudioside C 22%	4806	Cohen et al. (2015a); Cohen et al. (2015b)
Glucosylated stevia extract Steviol glycosides 80%	4845	Cohen et al. (2017)
Enzyme modified stevia, stevioside 20%	4876	Cohen et al. (2017)

2. Canadian Regulatory History

- On September 18, 2009, the Natural Health Products Directorate, Health Canada (Health Canada, 2009) adopted and revised the maximum limit for steviol glycosides in Natural Health products (NHPs) to be in accordance with the full ADI of 4 mg steviol per kg bw established by JECFA (WHO, 2008).
    - As a Medicinal Ingredient: The maximum daily limit without cautionary labelling and additional safety evidence was set at 4 mg per kg bw per day expressed as steviol content. This limit is equivalent to 10 mg per kg bw per day (i.e. ~ 710 mg per day for an adult) for stevioside or mixed steviol glycosides, 12 mg per kg bw per day (i.e. ~ 850 mg per day for an adult) for rebaudioside A, or 50 mg per kg bw per day (i.e. ~ 3,550 mg per day for an adult) of stevia leaf.
    - As a Non-Medicinal Ingredient: As a sweetener or flavor enhancer, the quantity used should be according to conditions of CGMP and should not exceed the amount required to accomplish the purpose for which that non-medicinal ingredient is permitted to be added. As a non-medicinal ingredient, it should not exceed 4 mg per kg bw per day expressed as steviol content.

- On November 30, 2012, Health Canada published its final clearance for use of steviol glycosides as a sweetener in foods (Health Canada, 2012).
- In March 2014, Health Canada updated the List of Permitted Sweeteners (Lists of Permitted Food Additives) to include steviol glycosides in applications as a table-top sweetener and as an ingredient in a variety of foods, beverages, baked goods, meal replacement bars, condiments, and confectionary and gums (Health Canada, 2014).
- On January 15, 2016, Health Canada approved the use of rebaudioside M for use as a high-intensity sweetener under the same conditions as the previously approved steviol glycosides (Health Canada, 2016).
- Health Canada (2017b) also modified the List of Permitted Sweeteners to include "all the steviol glycosides in the Stevia rebaudiana Bertoni plant (stevia plant)."
- On August 30, 2017, Health Canada's Food Directorate updated its List of Permitted Sweeteners to allow for the use of steviol glycosides as a sweetener in 'unstandardized snack bars,' including granola bars, cereal bars, fiber bars, and protein isolate-based bars (Health Canada, 2017b).
- On August 27, 2018, Health Canada's Food Directorate updated its List of Permitted Sweeteners to provide stakeholders with further information on the Lists of Permitted Food Additives as well as guidance on how to interpret and use these lists (Health Canada, 2018).
- On April 3, 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides from *Stevia rebaudiana* Bertoni in canned fruit products (Health Canada, 2019c).
- On May 14, 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides derived from *Saccharomyces cerevisiae* strains CD15380 and CD15407 at the same maximum levels of use as steviol glycosides derived from *Stevia rebaudiana* Bertoni (Health Canada, 2019b).
- Most recently, on June 27, 2019, Health Canada's Food Directorate modified the List of Permitted Sweeteners to allow for the use of steviol glycosides from various sources in "standardized flavoured milks" (Health Canada, 2019a).

3. European Regulatory History

- The Joint Expert Committee on Food Additives (JECFA) reviewed steviol glycosides at its 51st, 63rd, 68th and 73rd meetings and published its original review in 2000 (WHO, 2000).
- In 2006, JECFA established a temporary ADI (acceptable daily intake) of 0 - 2 mg per kg (on a steviol basis) at its 63rd meeting (WHO, 2006).
- In 2007, JECFA finalized food grade specifications (FAO, 2007b), although they were subsequently updated in 2008 (FAO, 2008) and 2010 (FAO, 2010).
- In 2008, Switzerland's Federal Office for Public Health approved the use of stevia as a sweetener citing the favorable actions of JECFA (Switzerland Federal Office of Public Health, 2008).

- In June 2008, the European Commission requested for EFSA to deliver a scientific opinion on the safety of steviol glycosides as a sweetener for use in the food categories specified in the dossiers from three petitioners.
    - EFSA reexamined the safety of steviol glycosides (EFSA, 2010) and the EFSA Panel established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per bw per day, which is similar to JECFA's determination.
    - On May 25, 2011, EFSA published the daily dietary intake for use of rebaudioside A as a flavoring substance in a variety of foods would be less than the ADI for steviol glycosides (EFSA, 2011a).
    - In 2014, EFSA evaluated extending the use of steviol glycosides as ingredients in food categories to include coffee, tea, and herbal and fruit infusions (assessed at 10 mg per L steviol glycosides) (EFSA, 2014).
    - In 2015, EFSA revised exposure estimates based on the EFSA Comprehensive European Food Consumption Database and the proposed extension of use for tea beverages and instant coffee and cappuccino products up to 29 mg per L of steviol equivalents, rather than 10 mg per L, as assessed in the previous 2014 EFSA opinion. EFSA noted that the mean exposure estimates remain below the ADI of 4 mg per kg bw per day for all population groups, with the exception of toddlers (in one country) at the upper range of the high-level exposure estimates (95th percentile: 4.3 mg per kg bw per day), which remains above the ADI. EFSA concluded that dietary exposure to steviol glycosides (E 960) is similar to the exposure estimated in 2014 and therefore does not change the outcome of the safety assessment (EFSA, 2015).
- In 2009, at the 69th meeting, the temporary status of the ADI was removed, and the ADI was raised to 0 – 4 mg per kg bw per day (on a steviol basis) as a result of the JECFA review of more recently completed clinical studies with steviol glycosides (WHO, 2008). In 2009, JECFA published a final monograph addendum on steviol glycosides (WHO, 2009).
- In 2009, several countries and the Calorie Control Council submitted a request to the Codex Committee on Food Additives to modify the JECFA specifications for steviol glycosides to include rebaudioside D and rebaudioside F as specifically named acceptable glycosides that would be considered as part of the minimum 95% steviol glycosides composition (CCFA, 2009). The proposal was discussed at the June, 2010 JECFA Meeting (FAO/WHO, 2009), and JECFA subsequently took final action in approving the modified steviol glycosides specifications to include rebaudioside D and rebaudioside F (FAO, 2010).
- In 2009, France published its approval for the food uses of rebaudioside A with a purity of 97% (AFSSA, 2009a; AFSSA, 2009b).
- On December 2, 2011, the EU approved steviol glycosides use as food additives (EU, 2011) based upon agreement between the JECFA and EFSA that steviol glycosides are safe for all populations to consume and are a suitable sweetening option for diabetics.
- On October 13, 2016, the EU updated regulation EU 2016/1814 to permit the following steviol glycosides in stevia blends: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside, and dulcoside (Searby, 2016).

- On November 3, 2016, the EU food additives regulation 231/2012 was amended to remove the previous requirement for stevia blends to contain at least 75% Reb A or stevioside.
- On January 31, 2018, the EFSA Panel of Food Additives and Nutrient Sources reviewed an application for glucosylated steviol glycoside preparations for use as a new food additive. The Panel concluded that the data supplied by the applicant were “insufficient to assess the safety” of the preparation. No safety concerns were raised by the EFSA Panel; however, their decision was based on the “limited” data provided in the dossier submitted by the applicant (EFSA, 2018).
- On September 24, 2019, the EFSA Panel on Food Additives and Flavourings concluded that there is no safety concern for Rebaudioside M produced via enzymatic bioconversion and recommended that the European Commission consider establishing specifications for the preparation (EFSA, 2019).
- On March 24, 2020, EFSA published a scientific opinion in response to a proposed amendment of the specifications for steviol glycosides, stating that all steviol glycosides share the same metabolic fate, and therefore the safety of 60 steviol glycosides identified in the leaves of *Stevia rebaudiana* Bertoni can be based on “read-across” from previously evaluated toxicological data. EFSA maintained that the ADI of 4 mg per kg bw applies to all 60 steviol glycosides. The EFSA Panel noted that the inclusion of more steviol glycosides, “whilst maintaining the assay value of not less than 95%, would allow less pure preparations” onto the market. The Panel stated that they “cannot conclude on the safety of the proposed amendment to the specifications of steviol glycosides (E 960) as [a] food additive if the purity assay value of not less than 95% for the total content of steviol glycosides is maintained.” Furthermore, the Panel noted that it is possible to manufacture steviol glycosides with a purity higher than 95% total steviol glycosides (EFSA, 2020).

4. Asian Regulatory History

- In May 2010, Hong Kong amended its food regulations to allow the use of steviol glycosides as a permitted sweetener in foods based upon the detailed safety evaluation and favorable findings as reported by JECFA (Hong Kong Centre for Food Safety, 2010).
- In July 2011, the Codex Alimentarius Commission adopted proposed maximum use levels for steviol glycosides in all major food and beverage categories which resulted in steviol glycoside approvals in Vietnam, the Philippines, Malaysia, Singapore and Thailand (Whitehead, 2013).
- The International Alliance of Dietary/Food Supplement Associations (IADSA) reported that the Codex Alimentarius Commission agreed to adopt the use of steviol glycosides for addition to chewable food supplements (NewHope360, 2011).
- On September 20, 2012, the Food Safety and Standards Authority of India (FSSAI) approved the use of steviol glycosides as a non-nutritive sweetener in a variety of foods using specifications and purity established by JECFA (FSSAI, 2012).

- Since December 10, 2012, over thirty registrations have been granted by FDA Philippines to stand-alone steviol glycosides sweeteners or foods containing steviol glycosides as ingredients (Philippines, 2014).
- Steviol glycosides are also listed under International Numbering System (INS) number 960 in the Food Additives Permitted Under the Singapore Food Regulations document prepared by the Agri-Food & Veterinary Authority (AVA) of Singapore (AVA, 2014)

5. Australia and New Zealand Regulation History

- In 2008, the Food Standards Australia New Zealand (FSANZ) completed its evaluation of an application for use of steviol glycosides in foods and recommended that the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) amend the Australia New Zealand Food Standards Code to allow the use of steviol glycosides in food (FSANZ, 2008).
- On May 13, 2011, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages, and flavored soy beverages to 200 mg per kg, and in plain soy beverages to 100 mg per kg (FSANZ, 2011).
- In 2015, FSANZ concluded that the use of Reb M does not pose any “public health and safety issues” (FSANZ, 2015).
- On January 14, 2016, Reb M was approved for use “as a food additive in accordance with the current permissions for steviol glycosides” (FSANZ, 2016a).
- In 2016, FSANZ called for submissions on permitting all minor steviol glycosides extracted from stevia leaf to be included in the definition of steviol glycosides in the Food Standards Code, noting that “[no] evidence was found to suggest that the proposed changes pose any public health and safety concerns” (FSANZ, 2016b).
- On February 8, 2017, FSANZ approved a draft variation of the definition of steviol glycosides to include all steviol glycosides present in the *Stevia rebaudiana* leaf (FSANZ, 2017).
- In 2018, FSANZ called for comments on the production of Reb M using enzymes derived from genetically modified yeast. The comment period closed on August 31, 2018 (FSANZ, 2018b). Subsequently, on October 31, 2018, FSANZ approved a draft variation to include a reference to the production method (FSANZ, 2018a).
- On May 14, 2020, FSANZ published an approval report for a draft variation to amend the specification for steviol glycosides from *Stevia rebaudiana* Bertoni in section S3—35 of the Australia New Zealand Food Standards Code to include rebaudioside E produced by enzymatic conversion from stevia leaf extract. The approved draft variation allows for the use of high purity rebaudioside E (≥85% rebaudioside E; ≥95% total steviol glycosides) within the existing permissions and limits for steviol glycosides (FSANZ, 2020a). Subsequently, on July 28, 2020, Amendment No. 193 was published to include rebaudioside E produced by enzymatic conversion from stevia leaf extract (FSANZ, 2020b).

6. South Africa

- On September 10, 2012, the South African Department of Health promulgated a new sweetener regulation: Regulation R733 (Regulations Relating to the Use of Sweeteners in Foodstuffs), allowed for the use of extracts of stevia rebaudiana, in composition and quantities in line with Codex standards, in food and beverages. Steviol glycosides can be used to a maximum level of 330 mg per kg (Food Stuff South Africa, 2012).

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Appendix 10 Summary of Published Safety Reviews

A. Summary of JECFA Reviews

- 51st Meeting (WHO, 2000) – Stevioside evaluation determined that there was insufficient and inconsistent information on the stevioside or steviol. No human metabolism data or mutagenicity data were available. JECFA determined that the ADI could not be determined without further data.
- 63rd Meeting (WHO, 2006) – More data were submitted; however, the data were inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g., those with hypotension or diabetes). The Committee allocated a temporary ADI, pending submission of further data on the pharmacological effects of steviol glycosides in humans. A temporary ADI of 0–2 mg per kg bw was established for steviol glycosides, expressed as steviol, based on a NOEL for stevioside of 970 mg per kg bw per day (or 383 mg per kg bw per day, expressed as steviol) in the 2-year study in rats and a safety factor of 200.
- 68th Meeting (WHO, 2007) – Further data were submitted showing the purity at 95% and that all steviol glycosides hydrolyze to steviol upon ingestion. JECFA determined that it was unnecessary to maintain a limit for the sum of stevioside and rebaudioside content that could include product that was at least 95% stevioside or at least 95% rebaudioside A. The Chemical and Technical Assessment report, written after the 2007 meeting, explained the Committee's thinking, which resulted in flexibility in the identity specifications (FAO, 2007a; FAO, 2007b).
- 69th Meeting (WHO, 2008) – Based on additional clinical studies, JECFA finalized the evaluation of steviol glycosides and raised the ADI to 0 - 4 mg per kg bw per day and removed the "temporary" designation. A summary of the Committee's key conclusions was published in the final toxicology monograph addendum (WHO, 2009).

B. Summary of FSANZ Review of Steviol Glycosides

- In 2008, FSANZ reviewed the safety of steviol glycosides and concluded that they are well-tolerated and unlikely to have adverse effects on blood pressure, blood glucose, or other parameters in normal, hypotensive, or diabetic subjects at doses of up to 11 mg per kg bw per day. FSANZ agreed with JECFA in setting an ADI of 4 mg steviol equivalents per kg bw per day (FSANZ, 2008).
- On May 13, 2011, FSANZ approved an increase in the maximum permitted level (MPL) of steviol glycosides (expressed as steviol equivalents) in ice cream, water based beverages, brewed soft drinks, formulated beverages and flavored soy beverages to 200 mg per kg and in plain soy beverages to 100 mg per kg (FSANZ, 2011).
- On January 16, 2016, FSANZ approved the addition of rebaudioside M as a steviol glycoside intense sweetener (FSANZ, 2016a).

- On February 20, 2017, FSANZ broadened the definition and, hence, specification for steviol glycosides preparations to include any mixture of individual steviol glycosides extracted from the stevia leaf.

C. Summary of EFSA Review of Steviol Glycosides

- On March 10, 2010, EFSA adopted a scientific opinion on the safety of steviol glycosides (mixtures that comprise not less than 95% of stevioside and/or rebaudioside A) as a food additive based upon JECFA's 2008 findings and in response to the European Commission's request to reevaluate the safety of steviol glycosides as a sweetener (EFSA, 2010).
    - EFSA agreed that the results of toxicology studies on either stevioside or rebaudioside A are applicable for the safety assessment of steviol glycosides.
    - EFSA established an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg per kg bw per day primarily based on the application of a 100-fold uncertainty factor to the NOAEL in the two-year carcinogenicity study in the rat when administering 2.5% stevioside in the diet (Toyoda et al., 1997).
- On January 11, 2011, EFSA revised the exposure assessment of steviol glycosides from its use as a food additive, for children and adults, based on the revised proposed uses presented.
    - EFSA reduced usage levels in 16 foods by a factor of 1.5 to 3, with no changes for 12 food groups.
    - The mean estimated exposure to steviol glycosides (equivalents) in European children (aged 1-14 years) ranged from 0.4 to 6.4 mg per kg bw per day and from 1.7 to 16.3 mg per kg bw per day at the 95th percentile.
    - A correction was considered to be necessary for the consumption of non-alcoholic flavored drinks (soft drinks) by children, and the corrected exposure estimate at the 95th percentile for children ranged from 1.0 to 12.7 mg per kg bw per day.
    - For adults, the mean and 97.5th percentile intakes were estimated to range from 1.9 to 2.3 and 5.6 to 6.8 mg per kg bw per day, respectively.
    - These revised exposure estimates to steviol glycosides remain above the established ADI of 4 mg per kg bw (steviol equivalent).

D. Other Published Reviews

- Stevia and steviol glycosides have been extensively investigated for their biological, toxicological, and clinical effects (Carakostas et al., 2008; Geuns, 2003; Huxtable, 2002).
- Four additional reviews have appeared on the toxicology and biological activity of stevia extracts and steviol glycosides (Yadav and Guleria, 2012; Brown and Rother, 2012; Brahmachari et al., 2011; Chatsudthipong and Muanprasat, 2009). The studies are not always closely comparable because:
    - These reviews do not clearly differentiate between studies on crude stevia extract and purified steviol glycosides.

  - Studies on biological activity used routes of administration other than oral.
  - Some studies may have used doses that are much higher than anticipated human use levels.
  - Roberts and Munro (2009) criticized the Chatsudthipong and Muanprasat (2009) review with points that are applicable – in general – to all the reviews:
    - Lack of purity of the material,
    - Route of exposure in relation to metabolism and safety assessment - *in vitro* and intravenous, intraperitoneal, or subcutaneous dosing studies are not relevant to the safety of steviol glycosides consumed orally.
    - Paucity of discussion of worldwide regulatory authorities affirming the safety of purified forms of stevioside and rebaudioside A as a food ingredient.

- In 2015, Urban et al. reviewed the potential allergenicity of steviol glycosides. The authors noted that: “hypersensitivity reactions to stevia in any form are rare” and concluded that current data do not support claims that steviol glycosides are allergenic. In addition, the authors stated that there is “little substantiated scientific evidence” to warrant consumer warning statements to consumers about allergy to highly purified stevia extracts.
- The effects of non-nutritive low-calorie sweeteners on gut microbiota were reviewed by Plaza-Diaz et al. (2020). It was noted that there have been no reports of negative interactions between steviol glycosides and colonic microbiota; however, it is possible that steviol glycosides modify the gut microbiota. The authors note that further studies are necessary to “clarify its specific effects.”
- A recent review by Ray et al. (2020) focused on the effects of *Stevia rebaudiana* on glucose homeostasis, blood pressure, and inflammation. The authors reported that no hypersensitivities or allergies were reported since 2008, and that the few prior reports were for “improperly filtered stevia extracts.” Furthermore, Ray et al. notes that additional randomized controlled trials are needed to confirm the beneficial effects of stevia. No significant adverse effects were noted from any study included in the review.

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Appendix 11 GRAS Associates Expert Panel Report

GRAS ASSOCIATES, LLC

11810 Grand Park Ave
Suite 500
North Bethesda, MD 20852

THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF THE PROPOSED USES OF SHANDONG SHENGXIANGYUAN BIOTECHNOLOGY'S PROSTEVIA HIGH PURITY GLUCOSYLATED STEVIOL GLYCOSIDES

September 11, 2020

Foreword

An independent panel of experts ("Expert Panel") was convened by GRAS Associates, LLC on behalf of their client, Shandong Shengxiangyuan Biotechnology Co., Ltd. ("Shandong"), to evaluate the safety and Generally Recognized as Safe (GRAS) status of Shandong's proposed uses of Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) in conventional foods. The members of this Expert Panel† are qualified to serve in this capacity by qualification of scientific training and experience in the safety of food and food ingredients.

Discussion

A significant amount of safety information related to the consumption of steviol glycosides is generally available, and has been discussed in Part 6, as well as in Appendices 7-10, of Shandong's Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) GRAS dossier. First, there is a history of safe consumption of steviol glycosides when used as an ingredient in food products in the U.S., Canada, South America, Europe, Asia, and Australia and New Zealand. Second, a number of experimental studies have investigated the safety of steviol glycosides, included those derived from enzymatic glycosylation processes. The composite evidence from historical safe consumption and experimental studies collectively demonstrate the safety of Prostevia high purity glucosylated steviol glycosides for human food consumption.

The majority of the studies reviewed on steviol glycosides, steviol, and enzyme modified steviol glycosides have been discussed in detail in previous GRAS Notices (GRNs), including GRN 337, GRN 662, GRN 821, and GRN 858, as well as GRN 733 previously submitted by Shandong.

With regard to the safety documentation, the key pharmacokinetic data establish that steviol glycosides are not absorbed through the gastrointestinal (GI) tract, *per se*; they are converted to steviol by bacteria normally present in the large intestine, and the steviol is absorbed but rapidly

† Dr. Emmel, Chair of the Expert Panel, is a chemist with substantial food safety experience in addressing steviol glycosides and other food ingredients. Dr. Kapp is a toxicologist with over 35 years of experience. He is a Fellow of the Academy of Toxicological Sciences, a Fellow of the Royal Society of Biology, and a European Registered Toxicologist. Dr. Lewis is a biologist with more than 10 years of experience preparing GRAS dossiers. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in deliberations of GRAS Expert Panels.

metabolized to steviol glucuronide and excreted. It has been well-established experimentally from various published studies that the steviol glycoside molecules are not absorbed from the GI tract (Gardana et al., 2003; Koyama et al., 2003b). The action of bacteria in the large intestine is directly supported by the published study that showed that steviol glycosides can be converted to steviol in the large intestine by normal anaerobic GI flora as demonstrated by an *in vitro* study in fecal homogenates (Koyama et al., 2003a; Renwick and Tarka, 2008). Purkayastha and Kwok (2020) concluded that samples of stevia leaf extract, bioconverted steviol glycosides, a preparation of minor steviol glycosides, and enzyme modified steviol glycosides shared qualitatively similar *in vitro* metabolic fates as rebaudioside A in pooled human fecal homogenate samples, leading the authors to conclude that "safety data for individual steviol glycosides can be used to support safety of all steviol glycosides produced by extraction and enzymatic conversion of stevia leaf extract."

The Expert Panel reviewed the recent publications on steviol glycosides including those by Abolhasani et al. (2020), Afonso et al. (2020), Assi et al. (2020), Halasa et al. (2020), Kurek et al. (2020), Nettleton et al. (2020), Plaza-Diaz et al. (2020), Ray et al. (2020), and Zhao et al. (2020), and did not identify any that raise safety concerns with regard to the use of steviol glycosides in conventional foods.

The acceptable daily intake (ADI) for steviol glycosides has been set largely based on a published chronic study in rats (Toyoda et al., 1997) and several published studies that show there are no pharmacological effects in humans at doses several fold higher than the ADI (Barriocanal et al., 2006; Barriocanal et al., 2008; Wheeler et al., 2008). Recently, Roberts et al. (2016) noted in a persuasive argument using a chemical-specific adjustment factor (CSAF) that the ADI could be higher. The toxicity of the metabolite, steviol, has been well reviewed in the published literature (Geuns, 2003; WHO, 2006; Urban et al., 2013). In addition, FDA has issued "no questions" letters in response to 64 GRN submissions for steviol glycosides preparations.

The Expert Panel notes that Shandong's manufacturing process for Prostevia high purity glucosylated steviol glycosides is similar to the processes described for other GRAS enzyme modified steviol glycosides materials, as described in GRN 337, GRN 375, GRN 448, GRN 452, GRN 607, GRN 656, GRN 662, GRN 821, and GRN 858.

The GRAS Associates Expert Panel convened on behalf of Shandong has reviewed the proposed uses for Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin). The highest 90th percentile consumption by any populations subgroup of Prostevia high purity glucosylated steviol glycosides was calculated to be approximately 4.95 mg per kg body weight (bw) per day, which is equivalent to 1.66 mg per kg bw per day steviol equivalents (calculated by a weighted-sum estimate) for any population group, on a worst-case scenario basis. This estimated intake value is well below the JECFA ADI of 4 mg per kg bw per day expressed as steviol equivalents. Therefore, Prostevia high purity glucosylated steviol glycosides is expected to be safe within established allowable limits.

A compelling case can be made that scientific consensus exists regarding the safety of steviol glycosides when of sufficiently high purity. The central role of conversion to steviol and subsequent elimination with these naturally occurring steviol glycosides extends to the manner in which the various steviol glycosides molecules are metabolized and eliminated from the body. While the

scientific conclusions are not unanimous regarding the safe human food uses of steviol glycosides, the Panel believes that a wide consensus does exist in the scientific community to support a GRAS conclusion as evidenced by several in-depth review publications (Geuns, 2007; Williams, 2007; Brusick, 2008; Waddell, 2011; Carakostas, 2012; Urban et al., 2013) that refute safety concerns expressed by a minority of scientists. In addition, Roberts et al. (2016) suggest that the ADI for steviol glycosides could be as high as 6 to 16 mg per kg bw per day, which is higher than has been previously accepted by the scientific community, providing the potential for an even more robust safety profile.

In summary, sufficient qualitative and quantitative scientific evidence in the composite is available to support the safety-in-use of Shandong's Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) preparation given the following conditions:

- Shandong's Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) continues to meet the designated specifications;
- The minimum sweetness intensity for Prostevia high purity glucosylated steviol glycosides remains unchanged; and
- Prostevia high purity glucosylated steviol glycosides is produced in accordance with Current Good Manufacturing Practices (CGMPs).

Conclusion

The Expert Panel critically reviewed the data provided by Shandong for their Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) preparation, as well as publicly available published information obtained from peer-reviewed journals and other safety assessments prepared by other Expert Panels and well-respected international regulatory bodies.

The ingestion of Shandong's Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) from the intended uses results in intakes that are safe within the limits of established historical use and published safety studies and the widely accepted ADI of 4 mg per kg bw per day steviol equivalents.

The Expert Panel unanimously concluded that the proposed uses of Shandong's Prostevia high purity glucosylated steviol glycosides preparation, manufactured as described in Part 2.b. of Shandong's GRAS dossier, and declared within the subject notification meets the FDA definition of safety in that there is "reasonable certainty of no harm under the intended conditions of use" as described herein, and Shandong's Prostevia high purity glucosylated steviol glycosides (≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin) preparation is generally recognized as safe (GRAS).

GRAS ASSOCIATES, LLC

Robert W. Kapp, Jr., Ph.D.
Fellow ATS, FRSB, & ERT (UK)

Kara Lewis, Ph.D.

Katrina V. Emmel, Ph.D.
Panel Chair

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END

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

Form Approved: OMB No. ; Expiration Date:
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* N/A

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position
Rose Qiao	Global Sales Manager

Company *(if applicable)*: Shandong Shengxiangyuan Biotehnology Co., Ltd.

Mailing Address *(number and street)*: No. 58 East Haiguan Rd.

City	State or Province	Zip Code/Postal Code	Country
Qufu	Shandong	273100	China

Telephone Number	Fax Number	E-Mail Address
86 13854704977	8605374400999	rosestevia_1@126.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position
William J. Rowe	President

Company *(if applicable)*: GRAS Associates

Mailing Address *(number and street)*: 11810 Grand Park Ave., Suite 500

City	State or Province	Zip Code/Postal Code	Country
North Bethesda	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
519-341-3367	888-531-3466	wrowe@nutrasource.ca

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

High purity glucosylated steviol glycosides; Prostevia; enzyme modified steviol glycosides

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media with paper signature page

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

Intended to be used as a table top sweetener and as a general purpose non-nutritive sweetener for incorporation into foods in general, other than infant formulas and meat and poultry products, at per serving levels reflecting good manufacturing practices and principles, in that the quantity added to foods should not exceed the amount reasonably required to accomplish its intended technical effect.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	
1	High purity glucosylated steviol glycosides	N/A	N/A	Enzyme modified *Stevia rebaudiana* Bertoni leaf extract	
2	Maltodextrin	CAS	9050-36-6		
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s)),* and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

Prostevia high purity glucosylated steviol glycosides is composed of ≥80% glucosylated steviol glycosides, <15% unreacted steviol glycosides, and ≤5% maltodextrin.

Prostevia high purity glucosylated steviol glycosides is prepared from the enzymatic glucosylaton of a purified *Stevia rebaudiana* Bertoni leaf extract using a cyclodextrin glucanotransferase enzyme produced by a genetically modified strain of *Bacillus licheniformis* and maltodextrin as the glucose source.

There are no known toxicants.

3. Synonyms

Provide as available or relevant:

1	Enzyme modified steviol glycosides; glucosylated steviol glycosides; Prostevia
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☒ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Shandong Shengxiangyan Biotechnology Co., Ltd. *(name of notifier)* has concluded that the intended use(s) of High purity glucosylated steviol glycosides; Prostevia; enzyme modified steviol glycosides *(name of notified substance)* described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Shandong Shengxiangyan Biotechnology Co., Ltd. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Shandong Shengxiangyan Biotechnology Co., L *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

No. 58 East Haiguan Rd., Qufu, Jining, Shandong Province, The People's Republic of China
(address of notifier or other location)

Shandong Shengxiangyan Biotechnology Co., Ltd. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. ---------- *(GRAS Affirmation Petition No.)*

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Katrina Emmel Digitally signed by Katrina Emmel Date: 2020.09.14 10:54:26 -07'00'	Katrina Emmel on behalf of William J. Rowe, President	09/14/2020

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Appendices 1-11 in the body of the dossier	