Application to Amend the Specifications for Steviol Glycosides, Under Australia and New Zealand Food Standards Code – Standard 1.3.1 – Food Additives, to Include Rebaudioside D Manufactured by Enzymatic Bioconversion of Stevia Leaf Extract

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Application to Amend the Specifications for Steviol Glycosides, Under Australia and New Zealand Food Standards Code – Standard 1.3.1 – Food Additives, to Include Rebaudioside D Manufactured by Bioconversion of Stevia Leaf Extract

A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the Food Standards Australia New Zealand (FSANZ) *Application Handbook* (FSANZ, 2016) the following general information must be provided:

1. Format of the application;
2. Applicant details;
3. Purpose of the application;
4. Justification for the application;
5. Information to support the application;
6. Assessment procedure;
7. Confidential commercial information;
8. Other Confidential information;
9. Exclusive capturable commercial benefit;
10. International and other national standards;
11. Statutory declaration; and,
12. Checklist.

Each point is addressed in turn in Section A that follows.

A.1 Format of the Application

1. Information Related to Changes to Standard 1.3.1 – Food Additives

This application for an amendment to Standard 1.3.1 and related Schedules is prepared pursuant to Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2016) which requires the following structured format to assess an application for a new food additive:

A. General information on the application;

B. Technical information on the food additive;

C. Information on the safety of the food additive; and,

D. Information on dietary exposure to the food additive.

The application is presented in this format. At the start of each section (A to D) the information that must be addressed therein is specified in more detail. Additionally, an executive summary for the application is provided as a separate electronic document to this application. The application has been prepared in English and submitted electronically, as required by the FSANZ *Application Handbook* (FSANZ, 2016).

A.2 Applicant Details

SweeGen, Inc. (SweeGen) is a science-based developer, producer, and distributer of non-caloric, non-GMO, high-quality sweeteners for the food, flavour, and beverage industries.

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In addition, the Food & Nutrition Group at Intertek Scientific & Regulatory Consultancy is involved in the preparation, submission, and stewardship of this application. His contact details are listed below:

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A.3 Purpose of the Application

SweeGen is submitting this application to FSANZ concerning rebaudioside D that is produced using a new methodology and is therefore seeking the amendment of Standard 1.3.1 and related Schedules for steviol glycosides. SweeGen uses a novel multi-step biosynthesis pathway process to manufacture high purity rebaudioside D (≥95%) using enzymes uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase that facilitate the transfer of glucose to purified stevia leaf extract *via* glycosidic bonds. These enzymes are made by a strain of *Pichia pastoris* (*P. pastoris*).

Schedule 3 of the *Australia New Zealand Food Standards Code* (The Code) contains specifications for "steviol glycosides from *Stevia rebaudiana* Bertoni" (S3—35), which includes rebaudioside D. This specification "relates to a steviol glycosides preparation obtained from the leaves of the *Stevia rebaudiana* Bertoni plant." Although rebaudioside D produced by enzymatic bioconversion is chemically identical to rebaudioside D extracted from the leaves of *Stevia rebaudiana* (*S. rebaudiana*) Bertoni, the rebaudioside D for which this application is being made by SweeGen does not comply with specification S3—35 based on the source (*i.e.,* it is produced by enzymatic bioconversion *versus* extraction from the leaf).

This application, therefore, aims to amend The Code to encompass the acceptability and permissibility of SweeGen's new manufacturing methodology as another means to safely and effectively produce rebaudioside D. This application does not intend to change the purity specification (≥95% steviol glycosides) or propose an extension for the use of rebaudioside D in additional food products nor does it propose to increase the permitted quantities of rebaudioside D in permitted food products.

A.4 Justification of the Application

A.4.1 Technological Function for the Food Additive

Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni, including rebaudioside D, are already permitted for use as high-intensity sweeteners in Australia and New Zealand for the replacement of sucrose in reduced-calorie or no-sugar-added products. The primary reason for developing alternative methods to the traditional extraction methods for steviol glycosides is that not all glycosides are naturally produced to the same degree in the leaves of *S. rebaudiana* Bertoni. For example, stevioside is a major glycoside present in the leaves of the plant, constituting about 5 to 10% in dry leaves (JECFA, 1999), whereas rebaudioside D is a minor glycoside that is present at much lower levels. Some of the minor glycosides, such as rebaudioside D, have more favourable sensory characteristics when compared to the major glycosides (*i.e.,* stevioside, rebaudioside A) and have taste profiles that are more reflective of sucrose, and hence the development of the new technology to produce a glycoside with preferential sensory characteristics for product development.

A.4.2 Costs and Benefits for Industry, Consumers, and Government Associated with Use of the Food Additive

Since rebaudioside D provides improved sensory characteristics over the major steviol glycosides (*i.e.,* stevioside, rebaudioside A), but is naturally present in much lower quantities within the *S. rebaudiana* Bertoni leaf, it is in the interest of industry to develop alternative production methods that yield higher quantities of rebaudioside D than traditional leaf extraction. SweeGen's new manufacturing methodology uses UDP-glucosyltransferase and sucrose synthase enzymes to bioconvert purified stevia leaf extract to rebaudioside D and yields a final product of no less than 95% purity. Therefore, it is expected that SweeGen's high purity rebaudioside D will present an attractive alternative as a sweetener for food manufacturers. SweeGen anticipates that food manufacturers may incorporate their rebaudioside D into products after importation into Australia and New Zealand. In addition,

globally-positioned companies may also import their own finished products containing SweeGen's rebaudioside D.

The benefits to the consumer would mirror those for other steviol glycosides currently permitted for use in Australia and New Zealand. SweeGen's rebaudioside D, like other steviol glycosides, would be used in foods and beverages to replace sugar, which will benefit consumers seeking products that have reduced caloric content. In addition, this would also include consumers with specific medical conditions that require reduced sugar intake, such as those with diabetes, as the consumption of steviol glycosides does not interfere with glucose homeostasis (EFSA, 2010).

Since SweeGen does not intend to propose an extension for the use of rebaudioside D in additional food products nor do they wish to propose to increase the permitted quantities of rebaudioside D in permitted food products, there is no perceived benefit or added cost to the government.

A.5 Information to Support the Application

Technical information specific to SweeGen's new manufacturing methodology for rebaudioside D is presented in detail in Section B, including information regarding the production of UDP-glucosyltransferase and sucrose synthase enzymes from strains of *P. pastoris* and their use as processing aids. Since these enzymes are not approved processing aids in Australia and New Zealand, information regarding their manufacture and safety, including the source microorganism utilised to produce them, are presented pursuant to Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook*. FSANZ reviewed an application to expand the definition of steviol glycosides to include all steviol glycosides present in the *S. rebaudiana* Bertoni leaf in 2016, and as such reviewed the safety of steviol glycosides (FSANZ, 2017a). Since the safety of rebaudioside D and steviol glycosides in general have been previously reviewed and established by FSANZ, Section C provides a short summary of steviol glycoside safety and focuses on presenting: a) new safety publications present in the scientific literature which have not previously been evaluated by FSANZ; and b) recent opinions released by regulatory agencies and/or scientific bodies (*i.e.,* Joint FAO/WHO Expert Committee on Food Additives [JECFA]).

A.6 Assessment Procedure

SweeGen considers the most appropriate procedure to be adopted in assessing the application to be the General Procedure – Level 2. It is anticipated that this application will involve amending *Standard 1.3.1 – Food Additives* of The Code to modify the specifications outlined in Schedule 3 for steviol glycosides from *S. rebaudiana* Bertoni (section S3–35). SweeGen also requests that the evaluation be expedited.

A.7 Confidential Commercial Information (CCI)

SweeGen requests that certain proprietary information required for Section B.5 (Manufacturing Process) be considered confidential commercial information (CCI). Non-confidential general summaries of proprietary manufacturing information are provided within this application, and all details considered CCI have been removed and are presented in Appendix A. SweeGen requests that all information presented in Appendix A remain confidential as it holds significant commercial value to the company, including proprietary details on the manufacture of the production strains, enzymes, and the final rebaudioside D product, as well as unpublished amino acid sequences of the enzymes.

A.8 Other Confidential Information

SweeGen requests that the identity of the companies that perform analysis testing (*i.e.,* stability, residue, *etc.*) are to remain confidential. More specifically, SweeGen wishes not to disclose the

companies by name but is amicable with the general disclosure of the companies' location (*i.e.,* "a lab in Europe").

A.9 Exclusive Capturable Commercial Benefit (ECCB)

SweeGen is currently not the only manufacturer of rebaudioside D. Therefore, as there are other manufacturers of rebaudioside D, the application would not confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the FSANZ Act, as there are other companies who would likely benefit from approval of this application.

A.10 International and Other National Standards

A.10.1 The Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The JECFA Committee recently re-evaluated the safety, dietary intake, and specifications for steviol glycosides at its 82nd meeting in 2016. The safety of steviol glycosides as well as the acceptable daily intake (ADI) of 0 to 4 mg/kg body weight, expressed as steviol, were confirmed. Details of a new manufacturing process for rebaudioside A utilising a strain of *Yarrowia lipolytica* (*Y. lipolytica*) that was genetically modified to overexpress the steviol glycoside biosynthetic pathway were submitted to and reviewed by the Committee. As a result, the Committee issued a new specification monograph for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (JECFA, 2016). The purity of rebaudioside A from genetically modified *Y. lipolytica* must be no less than 95% total steviol glycosides on the dried basis. The Committee also reviewed data demonstrating the shared metabolism of all steviol glycosides and issued new specifications for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a), expanding the definition of steviol glycosides to "a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni". The purity of steviol glycosides from *S. rebaudiana* Bertoni must be no less than 95% total steviol glycosides on the dried basis.

A.10.2 United States

In the United States (U.S.), steviol glycosides are Generally Recognised as Safe (GRAS) for use as general purpose sweeteners in foods, and to date, over 50 GRAS notices have been submitted to the U.S. Food and Drug Administration (FDA) for review. These notices include submissions for purified individual steviol glycosides, mixtures of steviol glycosides, and glucosylated steviol glycosides, all with a total steviol glycoside content of no less than 95%. With the exception of the most recent GRAS notifications currently pending review, the U.S. FDA has raised no objections to the GRAS status of steviol glycoside products for use as general purpose sweeteners in foods. Of particular relevance to this submission, GRN No. 715 was submitted by Blue California[1] for rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract, which is the same product that is the subject of this application (Blue California, 2017; U.S. FDA, 2017a). The U.S. FDA responded with no questions to the GRAS status of Blue California's rebaudioside D produced *via* enzymatic bioconversion for use as a table top sweetener and as a general purpose non-nutritive sweetener in foods (U.S. FDA, 2017a).

A.10.3 Other Jurisdictions

Steviol glycosides are approved for use in a number of other jurisdictions, including the European Union, Canada, Asia, Central/South America, Africa, and the Middle East (PureCircle Stevia Institute, 2018). In the European Union, commercially available steviol glycoside products must comply with the

[1] All rights of Blue California have been granted to SweeGen, Inc. in regard to steviol glycosides. See Appendix I.

specifications for steviol glycosides (E 960) adopted by the European Commission in 2012 and recently updated in 2016 (EU, 2012, 2016). Presently, the specifications stipulate that steviol glycoside products must contain no less than 95% of 11 named steviol glycosides: stevioside, rebaudiosides A, B, C, D, E, F and M, steviolbioside, rubusoside, and dulcoside. Health Canada has approved steviol glycosides for use as food additives in Canada, and recently expanded the definition in the list of permitted sweeteners to include all the steviol glycosides in the *S. rebaudiana* Bertoni plant (Health Canada, 2017a).
In several Asian countries including Japan, China, Hong Kong, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Singapore, Taiwan, Thailand, and Vietnam, steviol glycosides are approved food additives/sweetening agents. For example, the Ministry of Health and Welfare in Japan has authorised the use of 3 types of stevia extracts, including α-glucosyltransferase-treated stevia, powdered stevia, and stevia extract (Japan Food Chemical Research Foundation, 2014). Purified stevioside (crude extract, 50% purity, and ≥90% purity) and *S. rebaudiana* leaf extracts are also accepted for general use as sweeteners in foods and beverages in Japan (Marie, 1991; Das *et al.*, 1992; Ferlow, 2005). The Food Safety and Standards Authority of India (FSSAI) has approved the use of steviol glycosides as a non-nutritive sweetener in a variety of food and beverage categories (FSSAI, 2015; MOHFW, 2016). In several Central/South American countries (*e.g.,* Argentina, Bolivia, Brazil, Chile, Columbia, Costa Rica, Ecuador, El Salvador, Guatemala, Mexico, Paraguay, Peru, Uruguay, Venezuela, Honduras) stevioside, *S. rebaudiana* leaves, and highly refined stevia extracts are permitted for use as low-calorie sweeteners. Steviol glycosides are also approved as food additives in the Middle East (Belarus, Bahrain, Iran, Jordan, Kazakhstan, Kuwait, Lebanon, Omar, Qatar, Saudi Arabia, Turkey, Uzbekistan, Yemen), Africa (Algeria, Cape Verde, Egypt, Equatorial Guinea, Gambia, Ghana, Guinea Bissau, Guinea Conakry, Kenya, Liberia, Libya, Morocco, Mauritania, Nigeria, Sierra Leone, South Africa, Tunisia), Switzerland, and Russia.

A.11 Statutory Declaration

Signed Statutory Declarations for Australia and New Zealand are provided in Appendix B.

A.12 Checklist

Completed checklists relating to the information required for submission with this application based on the relevant guidelines in the FSANZ *Application Handbook* are provided in Appendix C.

B. TECHNICAL INFORMATION ON THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2016) the following technical information must be provided:

1. Nature and technological purpose of the food additive;
2. Information to enable identification of the additive;
3. Information on the chemical and physical properties of the additive;
4. Information on the impurity profile;
5. Manufacturing process;
6. Specifications for identity and purity;
7. Information for food labelling;
8. Analytical method for detection; and,
9. Potential additional purposes of the food additive when added to food.

These points are addressed in the Section that follows. In addition, to fulfil the requirements outlined in Guideline 3.3.2 – Processing Aids of the FSANZ *Application Handbook*, the following information on the enzymatic processing aids, including the production microorganisms, are presented:

1. Technical information on the processing aid;
2. Information related to the safety of an enzyme processing aid;
3. Additional information related to the safety of an enzyme processing aid derived from a microorganism; and,
4. Additional information related the safety of an enzyme processing aid derived from a genetically-modified microorganism.

B.1 Nature and Technological Purpose of Rebaudioside D

B.1.1 Technological Purpose

SweeGen's rebaudioside D is produced by enzymatic bioconversion of purified stevia leaf extract and the final product is a highly purified preparation containing no less than 95% rebaudioside D. As per the technological purposes listed in Schedule 14 – Technological purposes performed by substances used as food additives, SweeGen's rebaudioside D fulfils the function as an intense sweetener and a flavour enhancer, consistent with rebaudioside D and steviol glycoside preparations already approved for use in Australia and New Zealand. SweeGen does not intend for this application to extend the use of rebaudioside D or steviol glycosides in general to foods for which its use levels have not already been permitted; SweeGen intends to use their rebaudioside D in the current food categories and at use levels currently permitted for steviol glycosides. Likewise, SweeGen does not intend to propose additional or different food matrices to which the addition of steviol glycosides has not already been approved.

B.1.2 Sweetness Potency

The sweetness equivalency to sucrose of SweeGen's rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract was evaluated by a sensory panel. Serial dilutions of sucrose (1.0, 2.5, and 5.0%) were prepared in bottled water at room temperature. The rebaudioside D solution at a concentration of 300 ppm was prepared in bottled water at room temperature. Participants (n=13) consumed the rebaudioside D solution and results were evaluated against the serially diluted sucrose samples starting with the lowest to the highest concentration. Results were averaged and converted to sweetness equivalency compared to sucrose. The results were consistent among all participants. Based on the results, rebaudioside D was determined to be 202 times sweeter than sucrose (full study report provided in Appendix D).

B.1.3 Stability

Extensive stability testing has been conducted on steviol glycosides and at the 68th meeting of the JECFA Committee it was concluded that "steviol glycosides are thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage" (JECFA, 2007). At the 82nd meeting in 2016, the Committee reviewed additional stability data and concluded, "the stability of steviol glycosides extract preparations established by JECFA at the 68th meeting can be extended to include steviol glycosides extract preparations containing higher levels of new glycosides added to the definition appearing in commercial products, mainly rebaudioside D and rebaudioside M" (FAO, 2016). Oehme *et al.* (2017) evaluated the structural stability of 3 commercial batches each of the dried stevia leaves, the first aqueous infusion of the ground stevia, and a high-purity stevia leaf extract (≥95% steviol glycosides), to determine whether the manufacturing process adversely impacts steviol glycoside composition. Changes in steviol glycosides were analysed by high-performance liquid chromatography (HPLC)-UV and HPLC-ESI-MS/MS. The authors reported that all 9 steviol glycosides defined by JECFA were detected in all samples, demonstrating that processing does not chemically alter or modify the steviol glycoside content.

To confirm that these conclusions apply to SweeGen's rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract, a 6-month accelerated stability study was conducted on 5 representative batches of the final rebaudioside D product (Lot No. D195-160113, D195-160126, D195-160265, D195-160324, D195-160425). The samples were stored at 40±2°C at a relative humidity of 75±5%. Rebaudioside D was observed to be stable over the course of the accelerated stability study, based on appearance, moisture content, and percent rebaudioside D content measured by HPLC compared to baseline (Table B.1.3-1).

Table B.1.3-1 Accelerated Storage Stability Data for SweeGen's Rebaudioside D

Duration (months)	Appearance	Manufacturing Lot No.									
		D195-160113		D195-160126		D195-160265		D195-160324		D195-160425	
		Moisture (%)	Reb D (HPLC, %)	Moisture (%)	Reb D (HPLC, %)	Moisture (%)	Reb D (HPLC, %)	Moisture (%)	Reb D (HPLC, %)	Moisture (%)	Reb D (HPLC, %)
0	White powder	1.03	97.4	1.82	96.2	2.15	96.1	1.56	97.2	1.36	96.8
1	White powder	1.15	96.9	1.63	96.7	2.03	96.6	1.72	97.5	1.53	96.6
2	White powder	1.48	97.6	1.95	96.4	2.22	96.5	1.78	97.5	1.49	96.6
3	White powder	1.32	97.2	2.02	96.5	2.14	96.4	1.73	97.4	1.62	96.5
4	White powder	1.26	97.4	2.15	96.6	2.21	96.4	1.77	97.4	1.70	96.4
5	White powder	1.30	97.3	2.09	96.5	2.18	96.5	1.70	97.3	1.68	96.5
6	White powder	1.41	96.4	2.20	96.4	2.31	96.4	1.77	97.4	1.77	96.8

HPLC = high-performance liquid chromatography; Reb D = rebaudioside D

B.2 Information to Enable Identification of Rebaudioside D

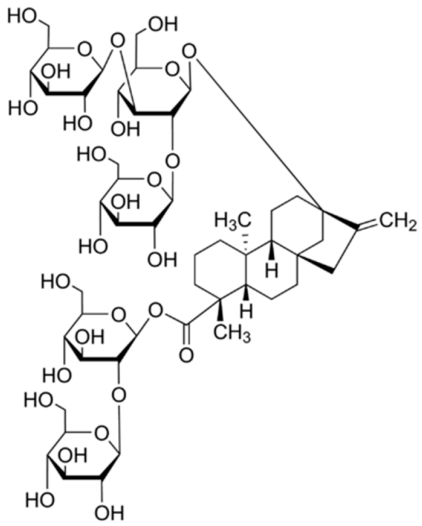
Information to enable the identification of rebaudioside D, including the chemical structure, the chemical name, the molecular weight and formula, and the common name, are presented below.

B.2.1 Identity of Substance

SweeGen's rebaudioside D is produced by enzymatic bioconversion of purified stevia leaf extract and the final product is a high purity preparation containing no less than 95% rebaudioside D. Rebaudioside D is a minor naturally occurring steviol glycoside that is present in the leaves of *S. rebaudiana* Bertoni. Rebaudioside D is an ent-kaurane diterpene glycoside with a steviol backbone conjugated to 5 glucose units, an ether at position C-13, and an ester at position C-19 (see Figure B.2.1-1).

Chemical name:	13-[(O-β-D-Glucopyranosyl-3-O-β-D-glucosylpyranosyl-β-D-glucosylpyranosyl)oxy]-kaur-16-en-18-oic acid, 2-O-β-D-glucosylpyranosyl-β-D-glucosylpyranosyl ester
Common name:	Rebaudioside D
Synonyms:	Reb D
Chemical formula:	$C_{50}H_{80}O_{28}$
Molecular weight:	1129.15 Daltons
CAS Number:	63279-13-0

Figure B.2.1-1 Chemical Structure of Rebaudioside D



From Sigma-Aldrich: www.sigmaaldrich.com

B.3 Information on the Chemical and Physical Properties of Rebaudioside D

SweeGen's rebaudioside D is a white to off-white powder that is slightly soluble in water with a slight characteristic odour, consistent with rebaudioside D extracted from the leaves of *S. rebaudiana* Bertoni. Steviol glycosides are a group of compounds that share a similar molecular structure, where different sugar moieties are attached to the aglycone steviol (an *ent*-kaurene-type diterpene). Steviol glycosides include any compound containing a steviol backbone conjugated to any number or combination of the

principal sugar moieties, including glucose, rhamnose, xylose, fructose, deoxyglucose, galactose, and arabinose (JECFA, 2017a). Based on the similar chemical structure, all steviol glycosides including rebaudioside D share a common metabolic fate following consumption (Purkayastha *et al.,* 2016). Specifically, steviol glycosides are hydrolysed to steviol in the large intestine, which is subsequently absorbed and conjugated with glucuronic acid to form steviol glucuronide. The glucuronide metabolite is then excreted primarily *via* the urine in humans [Kraemer and Maurer, 1994; Koyama *et al*., 2003a,b; Geuns and Pietta, 2004 (unpublished); Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008; Roberts *et al.,* 2016].

B.4 Information on the Impurity Profile

SweeGen's rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract consists of ≥95% rebaudioside D. As described in Section B.6.1, SweeGen has established product specifications for rebaudioside D that are consistent with the specifications in Schedule 3 of The Code for "steviol glycosides from *Stevia rebaudiana* Bertoni" (S3—35) and comply with the assay and impurity specifications in FAO JECFA Monograph 20 for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). In addition to the chemical and microbiological specifications, since the starting steviol glycoside material (≥95%) is extracted from the leaves of *S. rebaudiana* Bertoni, pesticide residue analyses were conducted on 5 representative batches of the final rebaudioside D product (Lot No. D195-160113, D195-160126, D195-160265, D195-160324, D195-160425). The results of the analyses provided in Appendix E demonstrate the absence of any residual pesticides in the product. The final rebaudioside D product has also been tested for residual protein to ensure that the processing enzymes have been effectively removed from the finished product. Analysis of the same 5 batches of final rebaudioside D product (Lot No. D195-160113, D195-160126, D195-160265, D195-160324, D195-160425) using the bicinchoninic acid (BCA) assay with a limit of detection of 5 ppm confirms the absence of protein residues in the final product (results provided in Appendix F).

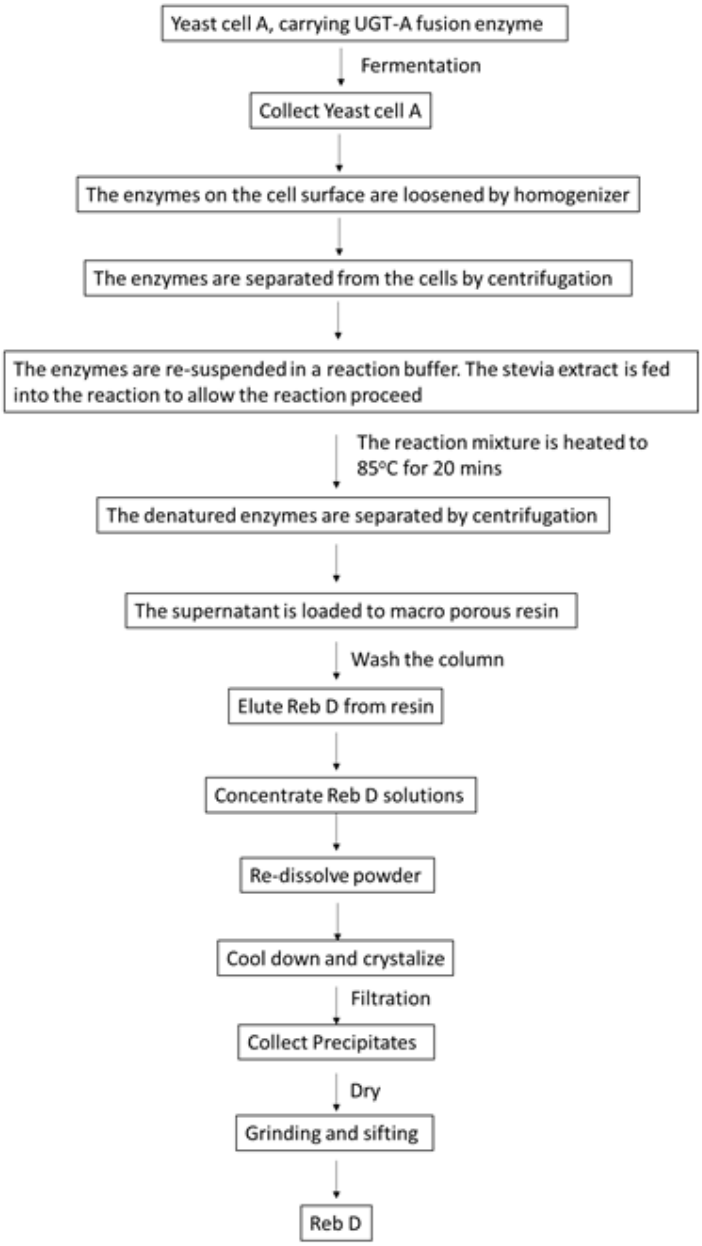
B.5 Manufacturing Process

B.5.1 Overview

SweeGen uses a novel multi-step biosynthesis pathway process to manufacture high purity rebaudioside D (≥95%) using enzymes that facilitate the transfer of glucose to purified stevia leaf extract *via* glycosidic bonds (*e.g.,* UDP-glucosyltransferase, sucrose synthase). The enzymes are produced by strains of *P. pastoris*. SweeGen's rebaudioside D is manufactured in compliance with current Good Manufacturing Practices (cGMP). The manufacturing process can be broadly divided into 2 stages. In the first stage, a strain of *P. pastoris* undergoes fermentation to generate the UDP-glucosyltransferase and sucrose synthase enzymes required for the bioconversion. Following the fermentation step, the enzymes are isolated from the source microorganisms. In the second stage, the enzymes are mixed with stevia extract (≥95% steviol glycosides, extracted from the leaves of *S. rebaudiana* Bertoni) to generate rebaudioside D. The resulting rebaudioside D undergoes a series of purification and isolation steps to generate the final high-purity rebaudioside D (≥95%). A schematic overview and detailed description of the production process is presented in Figure B.5.1-1 and Section B.5.3, respectively.

It should be noted that SweeGen's rebaudioside D is currently manufactured outside of Australia/New Zealand. Since the preparation will not be manufactured in Australia or New Zealand, the fermentation substrates, production organisms, and all processing aids used in the manufacturing process will not enter the territory.

Figure B.5.1-1 Schematic Overview of Rebaudioside D Production Process



B.5.2 Identity of Raw Materials and Processing Aids

All materials and processing aids utilised in the manufacture of SweeGen's rebaudioside D are food-grade and comply with relevant Food Chemical Codex (FCC) or other internationally-recognised standards. A list of all raw materials, processing aids, and filtration aids used in Stage 1 of the manufacturing process to generate the enzymes is provided in Appendix A.

Stage 2 of the production process (production of rebaudioside D) requires the use of various processing and filtration aids that are already recognised for use in the manufacture of steviol glycoside preparations, in addition to the raw materials purified stevia leaf extract (≥95% steviol glycosides) and sucrose (Table B.5.2-1). A certificate of analysis for a typical batch of purified stevia leaf extract, the starting raw material, is provided in Appendix G.

Table B.5.2-1 Raw Materials and Processing Aids Used in Stage 2 of the Manufacturing Process

Material	Function
Raw Material Substrates	
Stevia leaf extract (≥95% steviol glycosides)	Starting raw material
Sucrose	Substrate
UDP-glucose	Substrate
Processing Aids	
Potassium monophosphate	Buffer solution
Potassium biphosphate	Buffer solution
UDP-glucosyltransferase and sucrose synthase	Catalysts/enzymes
Water	Solvent
Ethanol	Solvent
Activated charcoal	Decolourant
Filtration Aids	
Nylon membrane cloth	Filtration aid
Macroporous resin column	Filtration aid
Filter paper	Filtration aid

UDP = uridine diphosphate

B.5.3 Details of the Manufacturing Process

B.5.3.1 Stage 1 – Enzyme Production

The first stage of the manufacturing process involves preparation of the enzymes that are utilised as processing aids in Stage 2. The enzymes are generated by a strain of *P. pastoris* that expresses the UDP-glucosyltransferase and sucrose synthase enzymes necessary to convert purified stevia leaf extract to rebaudioside D. The strain is designated Yeast A and carries the uridine'5 diphospho-glucuronosyl transferase (UGT)-A fusion enzyme (*i.e.,* glucosyltransferase fused with sucrose synthase). A comprehensive description of the methods used in the genetic modification and steps taken to construct the source organism is provided in Appendix A according to Section 3.3.2 – Processing Aids, subsection E, of the *Application Handbook* (FSANZ, 2016).

The glycerol stock of Yeast A is removed from the -70°C freezer, thawed to room temperature, and grown in 50 mL yeast culture seed media. After 12 hours, the growing seed culture 1 is transferred to 2 L yeast culture seed media as seed culture 2. When the cells read OD_{600} = 10, they are transferred to 500 L fermenters. This seed culture 3 is then transferred to a 60 tonne production fermenter. The yeast cells are cultured, according to Blue California's[2] published patents, for 48 hours. After confirming their

[2] All rights of Blue California have been granted to SweeGen, Inc. in regard to steviol glycosides. See Appendix I.

catalytic activity in a small shaking flask, Yeast A is harvested by centrifugation and re-suspended in a reaction buffer. Yeast A is passed through a homogeniser operated at minimum pressure to release the enzymes present on the cell surface without lysing the cells. The enzymes are separated from the yeast cells *via* centrifugation, and the supernatant containing the UGT-A fusion enzyme is collected and used in the bioconversion.

B.5.3.2 Stage 2 – Rebaudioside D Production

A) Bioconversion

For the catalytic reaction needed to convert purified stevia leaf extract to rebaudioside D, the UGT-A fusion enzyme is mixed together in a 60 tonne reaction tank with slow agitation. Purified stevia leaf extract (≥95% steviol glycosides) is fed into the tank to allow the reaction to proceed. The reaction mixture containing rebaudioside D is collected in a storage tank and is heated to 85°C for 20 minutes to denature the enzymes. The mixture is filtered to remove the denatured enzymes.

B) Extraction and Purification

The remaining steps employed to purify rebaudioside D are consistent with the purification procedures described for steviol glycosides in the most recent JECFA Chemical and Technical Assessment (FAO, 2016). The supernatant is loaded onto large columns containing a macroporous resin. The supernatant flows through the column by gravity and is bound to the resin. The column is rinsed with a series of buffer solutions and rebaudioside D is eluted with food-grade ethanol numerous times. The eluent is collected and condensed in a wipe-film evaporator. The condensate is chilled to allow rebaudioside D to crystallise and precipitate from the solution. The wet crystals are collected, washed, and dissolved in ethanol. The re-dissolved rebaudioside D is treated with activated charcoal to remove remaining impurities, re-crystallised, dried, and processed to the final high-purity rebaudioside D product (≥95%).

B.5.4 Additional Information Regarding the Source Microorganisms and Enzymes Utilised as Processing Aids

As described in Section B.5.3 above, UDP-glucosyltransferase and sucrose synthase enzymes are used as processing aids to produce rebaudioside D, and these enzymes are produced using a strain of *P. pastoris*. Therefore, additional information is provided according to Section 3.3.2 – Processing Aids, subsections A, C, D & E of the *Application Handbook* (FSANZ, 2016).

UDP-glucosyltransferase (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13) are food enzymes used in the processing of the raw material, and specifically perform the function of converting purified stevia leaf extract to rebaudioside D.

B.5.4.1 Information on the Identity of the Enzymes

B.5.4.1.1 UDP-glucosyltransferase

Source (strain):	*Pichia pastoris* containing DNA sequences encoding UGT and sucrose synthase enzymes
Common/Accepted Name:	Glucosyltransferase
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB]:	2.4.1.17

Chemical/Systematic Name:	UDP-glucose β-D-glucosyltransferase
Chemical Abstracts Service (CAS) Number:	9030-08-4

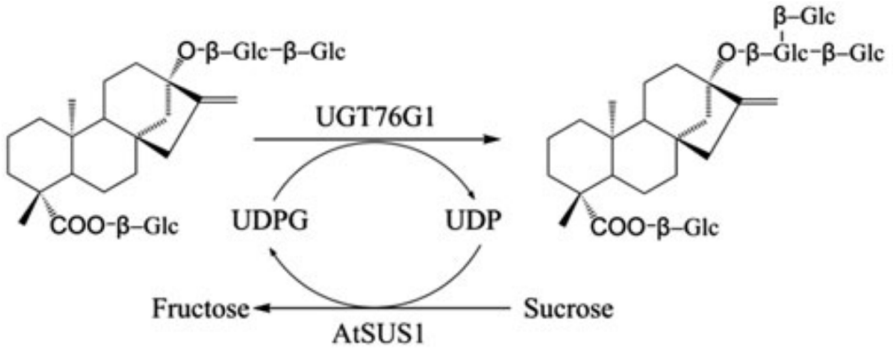
B.5.4.1.2 Sucrose Synthase

Source (strain):	*Pichia pastoris* containing DNA sequences encoding UGT and sucrose synthase enzymes
Common/Accepted Name:	Sucrose synthase
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB]:	2.4.1.13
Chemical/Systematic Name:	NDP-glucose:D-fructose 2-α-D-glucosyltransferase
Chemical Abstracts Service (CAS) Number:	9030-05-1

B.5.4.2 Information on the Chemical and Physical Properties of the Enzymes

The family of UDP-glycosyltransferases are involved in the transfer of a sugar residue from an activated donor molecule (*e.g.,* UDP-glucose) to an acceptor molecule (Richman *et al.,* 2005). Steviol glycoside synthesis in the *S. rebaudiana* Bertoni plant involves successive glucosylation steps starting with steviol to form steviolmonoside, followed by steviobioside, and then stevioside *etc.* For example, UDP-glucosyltransferase UGT76G1 catalyses the reaction of stevioside to form rebaudioside A by glucosylation at the C-4 carboxyl group (Richman *et al.,* 2005; Humphrey *et al.,* 2006). In this reaction, the activated sugar donor (UDP-glucose) and fructose are formed from UDP and sucrose, the reaction of which is catalysed by sucrose synthase (Humphrey *et al.,* 2006; Wang *et al.,* 2015). The reaction mechanism of UDP-glucosyltransferase and sucrose synthase to form rebaudioside A from stevioside is shown in Figure B.5.4.2-1 below. Thus, the coupled activities of UDP-glucosyltransferase and sucrose synthase were adapted by SweeGen to the efficient production of rebaudioside D from stevia extract (Mao *et al*., 2016a,b).

Figure B.5.4.2-1 Mechanism of Formation of Rebaudioside A from Stevioside with UDP-Glucosyltransferase and Sucrose Synthase (adapted from Wang *et al.,* 2015)



Glc = glaucoma; UDP = uridine 5'-diphosphate.

B.5.4.3 General Information on the Use of the Enzymes as a Food Processing Aid in Other Countries

The UDP-glucosyltransferase and sucrose synthase enzymes described in this application are only used as processing aids by SweeGen to produce rebaudioside D and rebaudioside M from purified stevia leaf extract. SweeGen's rebaudioside D is currently manufactured outside of Australia/New Zealand and is GRAS for use in the U.S. as a table top sweetener and as a general purpose non-nutritive sweetener in foods (U.S. FDA, 2017a). Health Canada has no objections to the use of SweeGen's high-purity rebaudioside D manufactured using genetically modified yeast, provided that the product is used in accordance with the permitted uses of steviol glycosides as set out in Item S.1.2 of the *List of Permitted Sweeteners* and meets the specifications for steviol glycosides from *S. rebaudiana* Bertoni set by JECFA (JECFA, 2017a) (see Appendix A).

B.5.4.4 Information on the Potential Toxicity of the Enzymes

The individual UDP-glucosyltransferase and sucrose synthase enzymes that are present in the UGT-A fusion enzyme are derived from plants and are not associated with any known toxicity. Furthermore, several steps are undertaken in the manufacturing process to inactivate and remove the enzyme system, including heating, treatment with activated carbon, resin purification, and filtration. The final rebaudioside D product was tested for residual protein to ensure that the processing enzymes were effectively removed and no protein was detected (see Section B.4).

To confirm that the UGT-A fusion enzyme does not harbour any toxic potential, the Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information (NCBI) was used to conduct a sequence alignment query of the UGT-A fusion enzyme FASTA protein sequence against downloaded protein sequences obtained from a curated database of venom proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot[3]). BLAST searches also were conducted against a curated database of virulence proteins and toxins maintained by UniProt (UniProtKB/Swiss-Prot/TrEMBL[4]). A sequence alignment of ≥35% identity was used as a threshold for identification as a positive alignment (Codex Alimentarius, 2003; Goodman *et al.,* 2008; Goodman and Tetteh, 2011). The BLAST search results are summarised in Table B.5.4.4-1 below, and only results in which a ≥35% identity match was identified are presented. Full results of these searches are provided in Appendix A. The UGT-A fusion enzyme did not have any sequence matches with ≥35% identity to known animal proteins and toxins. The UGT-A fusion enzyme shared 37% identity with 2 virulence proteins, however, the sequence matches had low query coverage (3%) paired with high E-values (0.54). Based on these searches the UGT-A fusion enzyme was not considered to share homology or structural similarity with any known animal venom proteins and toxins or virulence factors (Pearson, 2000; Bushey *et al.,* 2014).

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

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

Table B.5.4.4-1 Full FASTA Search of UniProtKB/Swiss-Prot Tox-Prot and UniProtKB/Swiss-Prot/TrEMBL Databases with UGT-A Fusion Enzyme (Summary of Alignments with ≥35% Identity)

Query	Description	Length	Query Cover	E-value	% Identity
Toxin					
No sequence homology ≥35% identity[a]					
Virulence					
93156	MGTA_MYCTU GDP-mannose-dependent alpha-mannosyltransferase	378	3%	0.54	37% (16/43)
91993	MGTA_MYCTO GDP-mannose-dependent alpha-mannosyltransferase	378	3%	0.54	37% (16/43)

UGT = uridine'5 diphospho-glucuronosyl transferase.
[a] Sequence homology with <35% identity was identified in the search, however, the results are not presented in this table as they did not meet the identity threshold of ≥35%.

B.5.4.5 Information on the Potential Allergenicity of the Enzymes

A sequence homology search was conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001) and the Codex Alimentarius (2009) using the AllergenOnline Database version 18B (available at http://www.allergenonline.org; updated March 23, 2018) maintained by the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska (FARRP, 2018). This was done to determine whether the UGT-A fusion enzyme contains amino acid sequences similar to other known allergens that might produce an allergenic response. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. The sequence homology search using full-length FASTA alignment was performed on April 19, 2018 and the full reports are provided in Appendix A. As shown in Table B.5.4.5-1 below, 2 sequence alignments with E-values of 0.76 and 0.81 were identified. E-values larger than $1x10^{-7}$, however, are unlikely to identify proteins that may share immunologic or allergic cross-reactivity to known allergens (Hileman *et al.*, 2002). Additionally, the UGT-A fusion enzyme did not share greater than 50% identity with the identified allergens, indicating the unlikely potential for cross-reactivity.

Table B.5.4.5-1 Full FASTA Search of AllergenOnline Database Version 18B with UGT-A Fusion Enzyme

Sequence G.I. #	Organism	Description	Length	E-value	% Identity	Amino acid alignment
262272875	*Blattella germ*	allergen Bla g 3 isoform 1 precursor	657	0.76	23.1%	121
2398759	*Phleum pratense*	pollen allergen PhlpVb	284	0.81	22.6%	234

UGT = uridine'5 diphospho-glucuronosyl transferase.

In addition to the full-length FASTA search, and in accordance with the FAO/WHO guideline, the AllergenOnline database was also searched using a sliding window of 80-amino acid sequences derived from the full-length amino acid sequences. The 80-amino acid alignment search was conducted using default settings (E-value cut-off = 1 and maximum alignments of 20). According to the approach adopted by the Codex Alimentarius Commission, significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen must be considered a possibility. Using this search strategy, no identity matches of greater than 35% were identified with the UGT-A fusion enzyme amino acid sequence.

B.5.4.6 Origins and History of Use of the Source Microorganism

A wild-type *P. pastoris* was used as the parental microorganism to construct the UDP-glucosyltransferase and sucrose synthase producing strain. Further details regarding the origins of the source microorganism are provided in Appendix A. *P. pastoris* belongs to the Saccharomycetaceae family and the taxonomic identity of *P. pastoris* is presented in Table B.5.4.6-1.

Table B.5.4.6-1 Taxonomic Identity of *Pichia pastoris*

Kingdom	Fungi
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Komagataella*
Species	*Pichia pastoris*

P. pastoris was first introduced for use in commercial production of a single cell protein as an animal feed additive over 40 years ago (Ahmad *et al.,* 2014). Since then, *P. pastoris* has been extensively used in food production, such as cheese and wine, and human pharmaceuticals, such as integral membrane proteins and affinity-tagged membrane proteins (Ahmad *et al.,* 2014). The use of *P. pastoris* as an expression host was reportedly 17% of the total recombinant genes in 2009 (Sørensen, 2010). *P. pastoris* has been granted qualified presumption of safety (QPS) status in the EU for use in enzyme production (EFSA, 2017) and therefore is considered safe for the derivation of genetically-modified strain lineages intended for use in the production of food enzymes. In the U.S., dried *P. pastoris* is an approved food additive for use as a source of protein in feed formulations for broiler chickens (21 CFR 573.750) (U.S. FDA, 2017b). *P. pastoris* is utilised as a source organism for the production of a phospholipase C enzyme preparation and the U.S. FDA responded with no questions regarding its GRAS status for use as an enzyme in degumming vegetable oils for food use (GRN 204 - U.S. FDA, 2006). This same phospholipase C enzyme preparation from *P. pastoris* has also undergone a safety review by JECFA and no safety concerns were expressed (JECFA, 2009a).

B.5.4.7 Pathogenicity/Toxicogenicity of the Source Microorganism

P. pastoris is a non-pathogenic and non-toxicogenic microorganism and has been granted QPS status by the European Food Safety Authority (EFSA) for use in enzyme production (EFSA, 2017). In addition, the DNA insert encodes only for the enzymes of interest and does not have any sequence similarity to other principal bacterially-produced toxins.

B.5.4.8 Genetic Stability of the Source Microorganism

The genetic traits of *P. pastoris* have been reported to be stable by a number of investigators (Cereghino and Cregg, 2000; Lim *et al.,* 2002; Daly and Hearn, 2005; Macauley-Patrick *et al*., 2005; Jin *et al*., 2006; Gasser *et al*., 2013). These investigators have evaluated the genetic stability of wildtype *P. pastoris* and following recombinant engineering. In addition, several steps are undertaken in the manufacturing process to inactivate and remove the enzymes from the final product, including heating and filtration steps. Furthermore, batch analyses demonstrate that the final product is of high-purity (*i.e.,* contains ≥95% rebaudioside D) and is absent of protein.

B.5.4.9 Information on the Methods Used in the Genetic Modification of the Source Microorganism

A comprehensive description of the methods used in the genetic modification of the source organism to generate Yeast strain A, which carries the UGT-A fusion enzyme, and the steps taken to construct the enzyme production strain are provided in Appendix A.

B.6 Specification for Identity and Purity of Rebaudioside D

B.6.1 Product Specifications for Rebaudioside D

SweeGen has established food-grade specifications for rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract. As shown in Table B.6.1-1, the product specifications are consistent with the specifications in Schedule 3 of The Code for "steviol glycosides from *Stevia rebaudiana* Bertoni" (S3—35) and comply with the assay and impurity specifications in the FAO JECFA Monograph 20 for "steviol glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). All methods of analysis are internationally-recognised methods.

Table B.6.1-1 Product Specifications for Rebaudioside D

Specification Parameters	SweeGen (Rebaudioside D)	JECFA (Steviol glycosides)	Method of Analysis
Physical parameters			
Appearance	Powder	Powder	Visual
Colour	Off-white to white	White to light yellow	Visual
Solubility	Soluble in water	Freely soluble in a mixture of ethanol and water (50:50)	
Purity	≥95% (Reb D)	≥95%total steviol glycosides	HPLC
Chemical parameters			
Residual ethanol	<1,000 ppm	≤5,000 ppm	USP 34
Residual methanol	<200 ppm	≤200 ppm	USP 34
Loss on drying	≤6%	≤6%	USP 34
pH (1% solution)	4.5 to 7	4.5 to 7.0	USP 34
Total ash	≤1%	≤1%	USP 34
Arsenic	<0.5 ppm	≤1 ppm	ICP-MS (AOAC 993.14)
Lead	<0.5 ppm	≤1 ppm	ICP-MS (AOAC 993.14)
Mercury	<0.5 ppm	Not specified	ICP-MS (AOAC 993.14)
Cadmium	<0.5 ppm	Not specified	ICP-MS (AOAC 993.14)
Microbiological parameters			
Total plate count	<1,000 CFU/g	≤1,000 CFU/g	AOAC 990.12
Total coliforms	<10 CFU/g	Not specified	AOAC 991.14
Yeast and mould	<100 CFU/g	≤200 CFU/g	AOAC 997.02
Salmonella spp.	Negative	Negative in 25 g	AOAC-RI 100201
Escherichia coli	Negative	Negative in 1 g	AOAC 991.14

AOAC = Association of Analytical Communities; CFU = colony forming units; HPLC = high performance liquid chromatography; ICP-MS = Inductively coupled plasma mass spectrometry; JECFA = Joint FAO/WHO Expert Committee on Food Additives; ppm = parts-per-million; USP = United States Pharmacopeia.

B.6.2 Product Analysis

B.6.2.1 Batch Analyses

Five non-consecutive batches of SweeGen's rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract were analysed and the results in Table B.6.2.1-1 demonstrate compliance with the defined product specifications. The certificates of analyses are provided in Appendix G.

Table B.6.2.1-1 Analytical Results for 5 Non-Consecutive Batches of Rebaudioside D

Parameter	Specification	Manufacturing Lot No.				
		D195-160113	D195-160126	D195-160265	D195-160324	D195-160425
Physical parameters						
Appearance	Powder	Pass	Pass	Pass	Pass	Pass
Colour	Off-white to white	Pass	Pass	Pass	Pass	Pass
Solubility	Soluble in water	Very slightly soluble	Very slightly soluble	Very slightly soluble	Very slightly soluble	Very slightly soluble
Purity (% Reb D)	≥95	97.4	96.2	96.1	97.2	96.8
Chemical parameters						
Residual ethanol (ppm)	<1,000	<20	<20	<20	<20	<20
Residual methanol (ppm)	<200	<50	<100	<100	<50	<80
Loss on drying (%)	≤6	0.53	0.85	0.86	0.55	0.70
pH (1% solution)	4.5 to 7	6.05	5.95	5.95	5.95	6.05
Total ash (%)	≤1	0.12	0.132	0.157	0.114	0.071
Arsenic (ppm)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Lead (ppm)	<0.5	<0.25	<0.25	<0.25	<0.25	<0.25
Mercury (ppm)	<0.5	<0.1	<0.1	<0.1	<0.1	<0.1
Cadmium (ppm)	<0.5	<0.25	<0.25	<0.25	<0.25	<0.25
Microbiological parameters						
Total plate count (CFU/g)	<1,000	<1,000	<1,000	<1,000	<1,000	<1,000
Total coliforms (CFU/g)	<10	<3	<3	<3	<3	<3
Yeast and mould (CFU/g)	<100	<50	<50	<50	<50	<50
Salmonella spp.	Negative	ND	ND	ND	ND	ND
Escherichia coli	Negative	ND	ND	ND	ND	ND

CFU = colony forming units; ND = not detected; ppm = parts-per-million.

B.7 Information for Food Labelling

Rebaudioside D is classified as a steviol glycoside under Schedule 3 and as such, it would follow similar food labelling as for current steviol glycoside preparations. Steviol glycosides are considered intense sweeteners and flavour enhancers when added to various food products. Steviol glycosides have been assigned the INS number of 960. Rebaudioside D will be labelled under the functional class, sweetener, either as sweetener (960) or sweetener (steviol glycosides).

B.8 Analytical Method for Detection

The analytical methods used to confirm that SweeGen's rebaudioside D meets the established chemical and microbial specifications (Section B.6.1) are internationally recognised (*e.g.*, Association of Analytical Communities [AOAC], U.S. Pharmacopeia [USP], JECFA). The rebaudioside D content in the final product is quantified according to the JECFA HPLC method for steviol glycosides described in FAO JECFA Monograph 20 for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a). Details of the HPLC method and chromatographic data are available in Appendix H.

B.9 Potential Additional Purposes of the Food Additive when Added to Food

As an intense sweetener, SweeGen's rebaudioside D can be added to foods to replace the sweetness provided by sugars without significantly contributing to available energy. As such, rebaudioside D can be used by consumers to control caloric intake. Consumers following a weight-loss program looking to restrict their refined sugar intake or individuals with diabetes avoiding sugar consumption may also use rebaudioside D for these purposes.

C. INFORMATION RELATED TO THE SAFETY OF THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2016) the safety information outlined must be provided to extend the use of a currently permitted food additive.

1. Information on the toxicokinetics and metabolism of the food additive and, if necessary, it's degradation products and/or major metabolites; and,

2. Information on the toxicity of the food additive and, if necessary, its degradation products and major metabolites.

These points need only include reports of studies conducted since the last safety evaluation by FSANZ, and are addressed in the Section that follows.

Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2016) states that if available, safety assessment reports prepared by international agencies of other national government agencies should be provided. A summary of the safety assessment reports prepared by international agencies that have been published since the last safety evaluation by FSANZ are outlined in the following section.

C.1 Introduction

The safety conclusions for steviol glycosides in general, including rebaudioside D, are based on the fact that all steviol glycosides share a common metabolic fate following ingestion. Steviol glycosides are hydrolysed to steviol in the large intestine, which is subsequently absorbed and conjugated with glucuronic acid to form steviol glucuronide that is excreted primarily *via* the urine in humans. On this basis, safety studies conducted on specific steviol glycosides can be used as surrogates for other individual steviol glycosides, including rebaudioside D, due to the shared metabolic fate.

In 2016, FSANZ received an application to expand the definition of steviol glycosides to include all steviol glycosides present in the *S. rebaudiana* leaf. The safety of all steviol glycosides was reviewed by FSANZ at this time and an approval report was issued February 20, 2017 to expand the steviol glycoside definition (FSANZ, 2017a). Therefore, for this application for a specification amendment, only safety studies conducted with steviol glycosides that were published in 2016 through 2018 were reviewed and summarised in the sections that follow. To identify scientific publications relevant to the safety of steviol glycosides and rebaudioside D, a comprehensive and detailed search of the published scientific literature was conducted up to May 2018. The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®.

C.2 Information on the Toxicokinetics & Metabolism of Steviol Glycosides

To support the conclusions regarding the shared metabolic fate for all steviol glycosides, Purkayastha *et al.* (2016) conducted *in vitro* experiments to compare the metabolism of several related steviol glycosides ("major" and "minor") in the presence of human faecal homogenates. For this collection of experiments, pooled faecal homogenates were prepared from samples obtained from 12 healthy volunteers (6/sex) and incubated under anaerobic conditions with individual steviol glycosides at concentrations ranging from 0.2 to 2.0 mg/mL (depending on solubility) for up to 48 hours at 37°C (see Table C.2-1). Rebaudioside A was used as a control in each experiment to allow for the comparison of results between experiments. The amount of steviol produced was measured by liquid chromatography-mass spectrometry (LC-MS). The data presented by Purkayastha *et al.* (2016) demonstrate that steviol glycosides containing different numbers and types of sugar moieties (*e.g.,* glucose, rhamnose, and xylose are represented in these experiments) are hydrolysed to steviol at rates that are generally similar. The authors noted that since "*none of the glycosides show a rate of hydrolysis that is significantly different from the others, it follows that there is no concern that any of the steviol glycosides would result in rapid absorption of steviol in humans*". These data support the conclusion that all steviol glycosides share a common metabolic fate and that safety data generated with an individual steviol glycoside may be applied to support the safety of all purified steviol glycosides in general.

Table C.2-1 Summary of *In Vitro* Experiments Conducted by Purkayastha *et al.* (2016)

Study	Steviol glycosides	Concentration(s)	Incubation times	% Converted to steviol at 24h*
1	Rebaudioside A Rebaudioside B Rebaudioside D	0.2 and 2.0 mg/mL	4, 8, 24, and 48h	47.3-55.9 40.4-64.8 63.8-102.0
2	Rebaudioside A Rebaudioside C	2.0 mg/mL	4, 8, 24, and 48h	29.6-52.4 22.9-40.3
3	Rebaudioside A Rebaudioside M	0.2 mg/mL	8, 16, and 24h	97.0-103.7 (0.2 mg/mL) 107.2-115.1 (0.2 mg/mL)

Table C.2-1 Summary of *In Vitro* Experiments Conducted by Purkayastha *et al.* (2016)

Study	Steviol glycosides	Concentration(s)	Incubation times	% Converted to steviol at 24h*
4	Rebaudioside A	0.2 and 2.0 mg/mL	4, 8, 16, and 24h	34.8-78.9
	Rebaudioside E			54.2-67.8
	Steviolbioside			50.4-77.9
5	Rebaudioside A	0.2 and 2.0 mg/mL	8, 16, 24, and 48h	74.8-101.5 (0.2 mg/mL)
				12.2-32.5
	Rebaudioside F			15.9-41.2 (0.2 mg/mL)
				2.9-6.6
	Dulcoside A			42.8-60.3 (0.2 mg/mL)
				29.5-43.2
	Rebaudioside M			75.5-96.6 (0.2 mg/mL)

* At the 2.0 mg/mL test concentration unless otherwise stated.

Roberts *et al.* (2016) conducted pharmacokinetic studies with stevioside in rats and humans in order to derive chemical-specific adjustment factors (CSAF) for extrapolating toxicokinetic data derived in rats to humans. In deriving the ADI for steviol glycosides (0 to 4 mg steviol equivalents/kg body weight) a 100-fold uncertainty factor was applied to the no-observed-adverse-effect level (NOAEL) in the chronic study in rats conducted by Toyoda *et al.,* 1997. In this study, Roberts *et al.* (2016) set out to determine if the default uncertainty factor of 10 for interspecies differences (4 for toxicokinetic, 2.5 for toxicodynamic differences) could be decreased. Male Sprague-Dawley rats (n=6) were administered a single gavage dose of stevioside dissolved in water at 40 mg/kg body weight (steviol equivalents of 16 mg/kg body weight). Clinical signs were observed at the time of dosing as well as approximately 2 hours afterward, and mortality/moribundity was evaluated twice daily for the study duration. Blood samples were collected at numerous time points between 0.5 to 72 hours following stevioside administration and steviol and steviol glucuronide plasma concentrations were measured using LC-MS/MS. The clinical arm of the study was an open-label single-dose design, where healthy male subjects (n=10) consumed a single dose of stevioside dissolved in water (40 mg/kg body weight) and blood samples were collected between 0.5 to 72 hours post-dose for quantification of plasma levels of steviol and steviol glucuronide using LC-MS/MS. The occurrence of any adverse events was monitored throughout the study and no compound-related adverse events were reported. Pharmacokinetic parameters were calculated independently from both the rat and human data and then compared. Peak plasma concentrations (C_{max}) of steviol were comparable between rats (76 ng/mL) and humans (72 to 77 ng/mL), but occurred earlier in the rats (4 *vs.* 19 hours). Steviol glucuronide concentrations peaked at 4,400 ng/mL at about 22 hours in humans, whereas in rats the steviol glucuronide C_{max} occurred at 6 hours and was approximately 25-fold lower (160 ng/mL). To evaluate overall systemic exposure, the area-under-the-curve (AUC) was calculated from the concentration *vs.* time data. Steviol exposure was reported to be 2.8-fold higher in humans than rats (~1,650 ng*h/mL *vs.* 590 ng*h/mL) and steviol glucuronide exposure was 57-fold higher (~136,000 ng*h/mL *vs.* 2,400 ng*h/mL). Roberts *et al.* (2016) used these data to propose a decrease in the 100-fold uncertainty factor applied to the NOAEL of 383 mg steviol/kg body weight/day from the Toyoda *et al.* (1997) study. The authors suggest that the default 10-fold uncertainty factor that accounts for interspecies differences, made up a factor of 4 for toxicokinetic and a factor of 2.5 for toxicodynamic differences, may be decreased to as low as 2.5. This is based on using a factor of 1 instead of 4 for toxicokinetic differences since the C_{max} values measured in this study for steviol were comparable between rats and humans. However, since the AUC values for steviol were 2.8-fold higher in humans than in rats, these data suggest that a factor of 2.8 for toxicokinetic differences should also be considered, and that the uncertainty factor for interspecies differences could be up to 7 (2.5 x 2.8). Utilising this range of uncertainty factors, Roberts *et al.* (2016) re-calculate the ADI and propose a higher ADI for steviol glycosides of 6 to 16 mg/kg body weight as steviol equivalents.

C.3 Information on the Toxicity of Steviol Glycosides

C.3.1 Toxicological Studies

C.3.1.1 Repeat-Dose Toxicity

Rumelhard *et al.* (2016) evaluated the subchronic toxicity of rebaudioside A produced *via* fermentation using a strain of *Y. lipolytica* genetically modified to express the *S. rebaudiana* metabolic pathway. Sprague-Dawley rats (n=20/sex/group) were provided rebaudioside A (>95% purity) in the diet at doses of 0, 500, 1,000 or 2,000 mg/kg body weight/day. Throughout the course of the study no deaths or clinical signs of toxicity were reported. Males in the high dose group had significantly lower body weights, body weight gain, and cumulative body weight gain compared to the control group and these changes were not associated with decreased food consumption. The authors suggested that the changes in body weight were related to the decreased caloric value of the rebaudioside A diet compared to the basal diet and did not consider them to be adverse. No effects related to consumption of rebaudioside A were reported in the haematology, coagulation, serum chemistry, and urinalysis parameters measured nor in the results of the gross pathological and histopathological examinations. Based on these data, the authors proposed a no-adverse-effect level (NOEL) for "fermentative" rebaudioside A of 2,057 or 2,023 mg/kg body weight/day for males and females, respectively, the highest doses tested in this study, and equivalent to 679 or 668 mg steviol/kg body weight/day. The results of these toxicological studies with "fermentative" rebaudioside A corroborate the results of similar studies previously conducted with other individual steviol glycosides and further support the lack of toxicity associated with this family of compounds.

C.3.1.2 Genotoxicity

Rumelhard *et al.* (2016) also evaluated the potential for genotoxicity of rebaudioside A produced *via* fermentation using a strain of *Y. lipolytica* genetically modified to express the *S. rebaudiana* metabolic pathway. Mutagenicity of the purified rebaudioside A (>95% purity) was assessed using the bacterial reverse mutation test (*E. coli* strain WP_2 *uvrA* and *S. typhimurium* strains TA1535, 1537, 98, and 100) at concentrations of up to 5,000 µg/plate, both in the presence and absence of metabolic activation with rat S9-mixture. An *in vitro* micronucleus test was also conducted using cultured peripheral human lymphocytes and rebaudioside A was tested at up to 5,000 µg/plate for 3 and/or 24 hours with and without metabolic activation. No mutagenic or cytotoxic effects were reported in either of the assays under all conditions tested.

Sharif *et al.* (2017) investigated the anticancer potential of stevioside by assessing the cytotoxicity and genotoxicity of stevioside (purity not reported) at concentrations up to 200 µM on CCD18Co myofibroblast cells (non-target cell) and human colon derived cancer cells HCT 116 (target cell). Cell viability was measured *via* a MTT assay as an indicator of cytotoxicity, which involved cultured cells that were dosed with 0, 12.5, 25, 50, 100, and 200 µM stevioside for 24 hours, followed by addition of MTT solution, a 4-hour incubation, addition of dimethylsulfoxide, and an absorbance reading using a microplate reader. An alkaline comet assay, a measure of genotoxicity, was used to detect DNA strand breaks by treating seeded cells with 200 µM stevioside for 24 hours and quantifying DNA tail intensity and tail moment using CometScore software program. While cell viability decreased gradually from the lowest to highest concentrations of stevioside for both CCD18Co and HCT 116 cells, the relative decrease between the cell types was not significantly different. At an exposure of 200 µM stevioside, no change in the DNA tail intensity was reported for both CCD18Co and HCT 116 cells compared to the respective control, whereas DNA tail moment was unchanged in the CCD18Co cells but significantly increased in the HCT 116 cells. The authors concluded that although stevioside is not cytotoxic or genotoxic in the non-target CCD18Co myofibroblast cells, it does not appear to hold potent anticancer potential towards the target HCT 116 cells.

The potential genotoxicity of stevia was investigated in *in vitro* micronucleus and chromosomal aberration tests in human lymphocytes (Uçar *et al.,* 2018). Human lymphocytes were collected from healthy non-smoking volunteers (male and female) and used in both tests. Cells were incubated for 24 and 48 hours, and stevia (steviol glycoside purity = 99%) was tested in duplicate at concentrations of 0 (negative control), 1, 2, 4, 8, and 16 µg/mL. Mitomycin C was used as a positive control in both tests. In the chromosome aberration test, 0.06 µg/mL colchicine was added at 70 hours, and at 72 hours cells were collected and prepared for analysis. A total of 400 metaphases/concentration were analysed for chromosome aberrations. In the micronucleus test, cells were harvested at 72 hours and prepared for analysis. A total of 4,000 binucleated cells/concentration were analysed. No significant increases in the number of chromosome aberrations and binucleated cells were observed in either test, indicating the lack of genotoxic potential of stevia.

C.3.1.3 Long-term Toxicity and Carcinogenicity

The chronic toxicity and carcinogenicity of steviol glycosides have been previously addressed in the safety evaluations by the scientific bodies and regulatory agencies described in Section C.4. No new data were identified in relation to this endpoint.

C.3.1.4 Reproductive and Developmental Toxicity

The reproductive and developmental toxicity of steviol glycosides have been previously addressed in the safety evaluations by the scientific bodies and regulatory agencies described in Section C.4. No new data were identified in relation to this endpoint.

C.3.1.5 Immune Function/Immunotoxicity

Noosud *et al.* (2017) examined the effects of stevioside (>95% purity) on rat plasma levels of tumour necrosis factor alpha (TNF-α) and IL-1β *in vivo* and the release of these pro-inflammatory cytokines *in vitro* from rat peripheral blood mononuclear cells (PBMCs) isolated from rats exposed to stevioside. Male Wistar rats (170 to 220 g in weight; n=6/group) were administered stevioside by gavage at doses of 0, 500, and 1,000 mg/kg body weight/day for 6 weeks. Blood samples were collected at the end of the exposure period and plasma and PBMCs were isolated. To induce cytokine production, PBMCs were stimulated with and without lipopolysaccharide (LPS) *in vitro* for 24 hours and supernatant fluids were collected. Rat enzyme-linked immunosorbent assay (ELISA) kits were used to measure TNF-α and IL-1β concentrations in plasma and their release from PBMCs. Oral intake of stevioside was not found to be toxic to PBMCs as evidenced by similar cell viability in the stevioside and control groups. TNF-α and IL-1β were not detected in the plasma for both control and treatment groups. Following LPS stimulation of PBMCs *in vitro,* TNF-α and IL-1β released from stevioside exposed cells (both doses) were significantly decreased compared to the control group, demonstrating an inhibitory effect on cytokine release. The authors propose that stevioside may be able to inhibit the release of proinflammatory cytokines such as TNF-α and IL-1β *in vivo* and that further studies should be conducted.

C.3.1.6 Antidiabetic Effects

Aranda-González *et al.* (2016) conducted a similar study in rats to evaluate the antidiabetic effects of purified steviol glycosides. Diabetes was induced in Male Wistar rats by injecting 65 mg/kg streptozotocin (except in the normoglycemic control group). Rats were provided daily with prepared food pellets containing the following for 28 days: glibenclamide as a positive control at a dose of 5mg/kg body weight/day (n=12); rebaudioside B, rebaudioside C, rebaudioside D, dulcoside A or steviolbioside at 20 mg/kg body weight/day (n=4/group). The non-diabetic and diabetic control groups (n=4/group) were provided with standard pellets. An intraperitoneal glucose tolerance test (IPGTT) was conducted following 28 days of steviol glycoside consumption and no difference was seen in the percentage change of glucose compared with the diabetic control. Likewise, when a similar experiment was conducted with

normoglycemic rats, steviol glycosides were not reported to have any effect on the IPGTT compared to control. The authors concluded that 28 days of exposure to rebaudioside B, rebaudioside C, rebaudioside D, dulcoside A, and steviolbioside at 20 mg/kg body weight/day had no antihyperglycemic effects in normoglycemic or induced-diabetic rats.

Ilić *et al.* (2017) investigated the antidiabetic effects of stevioside hydrate (>95% purity) in male *NMRI Haan* mice *via* the oral glucose tolerance test (OGTT), the adrenaline test, and following alloxan-induced diabetes. Mice were orally administered aqueous stevioside solution at a dose of 20 mg/kg body weight/day or saline as control for 10 days and then subjected to the OGTT, adrenaline, or alloxan tests (both control and stevioside mice, n=6/treatment/group). A 4th group was co-administered alloxan (150 mg/kg body weight/day) with stevioside (20 mg/kg body weight/day) or saline for 10 days, and then subjected to the alloxan test. On day 10 after baseline glucose levels were measured, 500 mg/kg body weight glucose was orally administered for the OGTT, 0.2 mg/kg body weight adrenaline was subcutaneously administered for the adrenaline test, and 150 mg/kg body weight alloxan was administered intraperitoneally for the alloxan test. Glucose levels were measured in capillary blood 30 minutes after glucose administration, 45 minutes after adrenaline administration, and 48 hours after alloxan exposure. Pancreatic tissue was collected after euthanasia for histological analysis. Results of the OGTT indicated that blood glucose was significantly increased in the saline-treated group but not in the stevioside treated group. Following adrenaline administration, a rise in blood glucose, although not statistically significant, was observed in both the saline and stevioside groups. Treatment with stevioside prior to alloxan exposure preserved a higher number of pancreatic β-cells as well as the morphology of the pancreatic islets compared to all the other alloxan exposed groups. The increase in blood glucose was lower for the stevioside/alloxan co-treated group compared to the saline/alloxan group, and this difference was statistically significant for rats treated with stevioside before alloxan exposure *versus* after. The authors concluded that a low dose of stevioside may have antidiabetic effects and that further investigation is necessary.

The effects of steviol and steviol glycosides on pancreatic β-cell function and taste preferences of *Trpm5*$^{-/-}$ mice were investigated in i*n vitro* and *in vivo* studies (Philippaert *et al*., 2017). Taste preferences were investigated using TRPM5, an ion channel present in pancreatic β-cells and type II taste receptors that is associated with sweet, bitter, and umami taste perception. The results of these studies demonstrate that the sweeteners (stevioside, reb A, and steviol) potentiate the activity of TRPM5, which facilitates insulin release from islet cells. The effect of steviol glycosides on TRPM5 activity modulates and intensifies taste responses, specifically bitter, sweet, and umami. In addition, the glucose lowering effect of stevioside is dependent on TRPM5 expression in pancreatic islets.

Philippaert *et al*. (2017) also investigated the effect of chronic stevioside exposure on the development of diabetes induced by a high-fat diet (HFD) in male C57BL6/J wildtype or *Trpm5*$^{-/-}$ mice (N=8/group). Stevioside was provided in the drinking water at a concentration of 0.1%, providing a dose of 25 mg/kg/day. Animals were given the control diet (HFD) or treatment diet (HFD plus stevioside) for 20 weeks. At the end of the study period, the authors observed a time-dependent development of glucose intolerance in the wildtype control group using an intraperitoneal glucose tolerance test. Conversely wildtype mice consuming the treatment diet had normal glycaemic profiles after 20 weeks. Similar effects were not observed in *Trpm5*$^{-/-}$ mice.

Philippaert *et al*. (2017) also investigated the reversal of glucose homeostasis by stevioside withdrawal in male C57BI6/J mice (N=8 to 10/group). Animals were given a HFD with stevioside (124 µM stevioside in drinking water; mg/kg dose not reported) for 15 weeks, HFD with stevioside for 10 weeks followed by 5 weeks HFD without stevioside, and a control group consuming a HFD for 15 weeks. The authors observed an improved glucose tolerance in animals consuming a HFD with stevioside. Deteriorated glucose tolerance was observed in animals consuming the HFD with stevioside for 10 weeks followed by a 5 week period in which stevioside was removed; the levels were similar to that of untreated animals. Overall, the authors concluded that targeting TRPM5 may have the potential to prevent and treat type 2

diabetes. It was also suggested that other modulators of TRPM5 including, stevioside, rebaudioside A, and steviol may play a role in the future development of TRPM5-targetted antidiabetic drugs.

Reynolds *et al.* (2017) evaluated the effects of chronic rebaudioside A exposure on circadian rhythms, insulin action *in vivo*, and susceptibility to diet-induced obesity. Male C57BL6/J mice (10/group) were provided with normal drinking water or drinking water containing 0.1% rebaudioside A (116 to 207 mg/kg body weight/day) for approximately 7 months. On the first day of rebaudioside A exposure, both groups of mice were placed in cages with running wheels for 32 days, and wheel running activity was monitored under both a regular 12-hour light-dark cycle as well as complete darkness. Mice were then returned to standard cages for a 3-month recovery period, after which glucose, pyruvate, and insulin tolerance testing (*i.e., in vivo* insulin action) was performed with 7- to 10-day recovery periods between each test. Susceptibility to obesity was then assessed by providing mice with a high-fat diet for 2-months. No changes in circadian wheel running activity nor body weight during this portion of the study were reported. The results of the glucose, insulin, and pyruvate tolerance tests were all similar between the 2 groups, and likewise, rebaudioside A exposure did not alter the susceptibility to diet-induced obesity.

C.3.2 Human Studies

Ritu and Nandini (2016) studied the hypoglycaemic and hypolipidemic effects of stevia leaf powder (steviol glycoside purity not reported) in 20 human volunteers with type 2 diabetes mellitus. Half of the subjects were given 1 g of stevia leaf powder (purity not reported), assumed to be consumed daily, for 60 days. A 3-day dietary evaluation indicated that subjects in the stevia group had a lower mean caloric intake than the control group and consumed more protein and fewer carbohydrates. Biochemical parameters, including blood glucose, cholesterol, and triglycerides were assessed after a period of 30 and 60 days. No differences were reported after 30 days, whereas after 60 days fasting and post-prandial blood glucose levels in the stevia leaf group were reported to be significantly lower than baseline. Although serum cholesterol, triglycerides, and very low-density lipoprotein cholesterol (VLDL-C) were significantly decreased in the stevia group, significant decreases were also reported in the control group and lipid profiles measured at baseline were not equivalent between the 2 groups. The significance of the findings reported in this study appear to be unclear, particularly considering the differences noted in dietary intake between the 2 groups.

Shin *et al.* (2016) evaluated the glycaemic effects of a mixture of rebaudioside A and erythritol in a population of pre-diabetic adult subjects with glucose intolerance. The study was a single, open, clinical trial in 25 male and female subjects who were instructed to consume 2 packets of sweetener dissolved in water, twice a day (after breakfast and dinner) for 2 weeks. Each sweetener packet contained 16 mg rebaudioside A and 986 mg of erythritol. Based on the reported average body weight of the study subjects the approximate exposure to rebaudioside A was 1 mg/kg body weight. Subjects were instructed to maintain a regular diet throughout the study period, which was confirmed by daily diet records filled out by the participants. The primary outcome of the study was defined as the change in fructosamine for blood glucose monitoring from baseline to the end of the 2-week study period. Secondary outcomes included fasting plasma glucose, 2-hour glucose, C-peptide, and insulin levels. Fructosamine levels did not change significantly between baseline and the end of the study, and no significant differences were reported in any of the secondary outcomes evaluated. Two adverse events involving abdominal discomfort were reported during the study and both resolved spontaneously. The authors concluded that the consumption of the mixed sweetener product containing rebaudioside A and erythritol did not alter glucose homeostasis in individuals with glucose intolerance.

Al-Dujaili *et al*. (2017) investigated the potential effects of a natural stevia preparation (obtained from Boots Ltd.) on blood pressure, stress hormone levels, and anthropometric parameters in a randomised, crossover placebo-controlled study in healthy humans. The study subjects included healthy volunteers (males and females; 8/group; mean age 27.75±13.75 years; body mass index [BMI] 26.33±5.26 kg/m^2)

consuming either a placebo containing 5 g sucrose or stevia dissolved in a hot drink (0.2 g; ~2.7 mg stevia/kg body weight) 3 times per day for 7 days. All subjects were subjected to a 3-day washout period before and after each treatment period, and were instructed to refrain from consuming other sweeteners or sugars throughout the study period. A 24-hour urine sample and saliva samples were collected at baseline and after each treatment period and analysed for cortisol and cortisone concentrations. In addition, the following parameters were measured at baseline and after each treatment: blood pressure, weight, height, and BMI. No significant effect on weight or BMI was observed following stevia consumption. The authors observed a significant increase in systolic and diastolic blood pressure compared to baseline following stevia consumption; however, the values were within the expected reference range. Salivary cortisol levels in the stevia group were reported to slightly but significantly increase in the morning compared to baseline. This effect was not sustained through the midday or afternoon time points. Levels of free urinary cortisol and cortisone were reported to significantly increase and decrease, respectively, compared to baseline following stevia consumption. No significant changes in any parameter compared to baseline were observed following consumption of the placebo. The authors concluded that consumption of stevia for a short period resulted in a small but significant increase in blood pressure, and the effect may have been associated with the increase in cortisol levels. The authors noted several limitations in their study, including the population size and indicated that further research is required to determine the significance of their findings.

C.4 Safety Assessment Reports Prepared by International or National Agencies

The safety of steviol glycosides has been reviewed by several scientific bodies and regulatory agencies, such as FSANZ, the U.S. FDA, JECFA, EFSA, European Commission's Scientific Committee on Food (SCF), and Health Canada. The large consumer and industry interest into the use of steviol glycosides as sweeteners has prompted extensive safety testing of these compounds and, as a result, a large safety database exists. This database includes a thorough evaluation of the metabolic fate and pharmacokinetics of various steviol glycosides in experimental animals and humans, acute toxicity studies, short-term and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicity studies, *in vitro* and *in vivo* mutagenicity and genotoxicity studies, and clinical studies. Many of the early toxicology studies examined the safety of stevioside due to its predominance in *S. rebaudiana* leaves (Aze *et al*., 1991; Toyoda *et al.,* 1997). However, due to the shared metabolic fate of steviol glycosides (*i.e.,* hydrolysis into steviol), regulatory agencies and authoritative bodies have expanded their safety opinions to encompass the safety of all steviol glycosides rather than individual glycosides. The recent opinions/reports issued since the last steviol glycoside safety evaluation by FSANZ are summarised below.

C.4.1 Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The safety of steviol glycosides has been extensively reviewed by JECFA at their 51st, 63rd, 68th, 69th, and 82nd meetings in 1998, 2004, 2007, 2008, and 2016 respectively (JECFA, 1999, 2006, 2007, 2009b, 2017b). Initially, the Committee established a temporary ADI for steviol glycosides of 0 to 2 mg/kg body weight, expressed as steviol, based on a NOAEL of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and application of a safety factor of 200 (JECFA, 2006). In 2008, following review of additional animal and human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose, the Committee concluded that the results from these studies were sufficient to remove the additional safety factor of 2, and established a full ADI of 0 to 4 mg/kg body weight (expressed as steviol) for steviol glycosides.

The JECFA Committee recently re-evaluated the safety, dietary intake, and specifications for steviol glycosides at its 82nd meeting in 2016. The safety of steviol glycosides as well as the ADI of 0 to 4 mg/kg body weight, expressed as steviol, were confirmed. Details of a new manufacturing process for

rebaudioside A utilising a strain of *Y. lipolytica* that was genetically modified to overexpress the steviol glycoside biosynthetic pathway were submitted to and reviewed by the Committee. As a result, the Committee issued a new specification monograph for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (JECFA, 2016). The Committee also reviewed data demonstrating the shared metabolism of all steviol glycosides and issued new 'tentative' specifications[5] for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a), expanding the definition of steviol glycosides to "a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni". The purity of steviol glycosides from *S. rebaudiana* Bertoni must be no less than 95% total steviol glycosides on the dried basis.

C.4.2 U.S. Food and Drug Administration (FDA)

Since the beginning of 2016, 17 GRAS notices regarding purified steviol glycosides (≥95% purity), including stevia leaf extract, glucosylated steviol glycosides, steviol glycosides manufactured using genetically modified yeast, and steviol glycosides manufactured *via* enzymatic bioconversion have been submitted to the U.S. FDA. A summary of the steviol glycoside GRAS notices submitted to the U.S. FDA since the beginning of 2016 and the agency's corresponding response, where available, is presented in Table C.4.2-1. With the exception of the most recent GRAS notifications currently pending review, the U.S. FDA has raised no objections to the GRAS status of these steviol glycoside products for use as general purpose sweeteners in foods. Of particular relevance to this submission, GRN No. 715 was submitted by Blue California[6] for rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract, which is the same product that is the subject of this application (Blue California, 2017; U.S. FDA, 2017a). The U.S. FDA responded with no questions to the GRAS status of this rebaudioside D preparation for use as a table top sweetener and as a general purpose non-nutritive sweetener incorporated for use in foods.

Table C.4.2-1 Summary of GRAS Notices Submitted to the U.S. FDA for Steviol Glycosides in 2016, 2017, and 2018

Company	Substance	FDA Response	GRAS Notice No.
PureCircle, Ltd.	Glucosylated steviol glycosides (minimum 80% purity)	No questions	GRN 000607 (U.S. FDA, 2016a)
PureCircle, Ltd.	Purified steviol glycosides	No questions	GRN 000619 (U.S. FDA, 2016b)
Cargill, Inc.	Steviol glycosides produced in *Saccharomyces cerevisiae*	No questions	GRN 000626 (U.S. FDA, 2016c)
DSM Nutritional Products, LLC	Rebaudioside A from *Yarrowia lipolytica*	No questions	GRN 000632 (U.S. FDA, 2016d)
Hunan Huacheng Biotech Inc.	High purity steviol glycosides (minimum purity 97%) consisting primarily of rebaudioside A	No questions	GRN 000638 (U.S. FDA, 2016e)
GLG Life Tech Corporation	Enzyme-modified steviol glycosides	No questions	GRN 000656 (U.S. FDA, 2016f)
PureCircle USA	Glucosylated steviol glycosides (minimum purity 95%)	No questions	GRN 000662 (U.S. FDA, 2016g)
Blue California[a]	Rebaudioside M	No questions	GRN 000667 (U.S. FDA, 2017c)

[5] The tentative status was removed at the 84th meeting and full specifications are to be published that include the additional sugar moieties arabinose and galactose.
[6] All rights of Blue California have been granted to SweeGen, Inc. in regard to steviol glycosides. See Appendix I.

Table C.4.2-1 Summary of GRAS Notices Submitted to the U.S. FDA for Steviol Glycosides in 2016, 2017, and 2018

Company	Substance	FDA Response	GRAS Notice No.
Xinghua GL Stevia Co., Ltd.	Purified steviol glycosides	No questions	GRN 000702 (U.S. FDA, 2017d)
Blue California[a]	Rebaudioside D	No questions	GRN 000715 (U.S. FDA, 2017a)
Shangdong Shengxiangyuan Biotechnology	Purified steviol glycosides	No questions	GRN 000733 (U.S. FDA, 20178a)
PureCircle Limited	Steviol glycosides consisting primarily of rebaudioside M	No questions	GRN 000744 (U.S. FDA, 2017b)
PureCircle Limited	Steviol glycosides consisting primarily of rebaudioside M	No questions	GRN 000745 (U.S. FDA, 2017c)
DSM Food Specialties	Steviol glycosides consisting primarily of rebaudioside M produced in *Yarrowia lipolytica*	No questions	GRN 000759 (U.S. FDA, 2018d)
Sichuan Ingia Biosynthetic Co., Ltd.	Rebaudioside D	No questions	GRN 000764 (U.S. FDA, 2018e)
Cargill, Inc.	Stevia leaf extract	Pending	GRN 000768 (U.S. FDA, 2018f)
Tate and Lyle	Rebaudioside M	No questions	GRN 000780 (U.S. FDA, 2018g)
GLG Life Tech Corporation	Steviol glycosides (minimum purity 95%)	Pending	GRN 000790 (U.S. FDA, 2018h)
Steviana Bioscience (Suzhou) Inc.	Purified steviol glycosides	Pending	GRN 000795 (U.S. FDA, 2018i)
Sichuan Ingia Biosynthetic Co., Ltd.	Rebaudioside M	Pending	GRN 000799 (U.S. FDA, 2018j)

FDA = Food and Drug Administration; GRAS = Generally Recognized as Safe; U.S. = United States.
[a] All rights of Blue California have been granted to SweeGen, Inc. in regard to steviol glycosides. See Appendix I.

C.4.3 Health Canada

In 2016, Health Canada expanded the definition of steviol glycosides from stevioside, rebaudioside A, B, C, D, F, M, dulcoside A, rubusoside, and steviolbioside to include rebaudioside M, supporting the conclusion that the various individual steviol glycosides share a common metabolic fate of hydrolysis to steviol, conjugation with glucuronic acid, and elimination *via* the urine in humans (Health Canada, 2016). In 2017, Health Canada expanded the definition further to include all the steviol glycosides in the *S. rebaudiana* Bertoni plant (Health Canada, 2017b). Detailed safety assessments were conducted by Health Canada in both cases and the agency concluded that the expanded definitions of steviol glycosides raised no safety concerns. Expansion of the definition confirms that the safety data generated from 1 specific steviol glycoside can be used to support safety of another steviol glycoside. In line with this, Health Canada specifically has no objections to the use of SweeGen's high-purity rebaudioside D manufactured using genetically modified yeast, provided that the product is used in accordance with the permitted uses of steviol glycosides as set out in Item S.1.2 of the *List of Permitted Sweeteners* and meets the specifications for steviol glycosides from *S. rebaudiana* Bertoni set by JECFA (JECFA, 2017a) (see Appendix A).

C.4.4 European Food Safety Authority (EFSA)

In a recent evaluation in response to a proposed amendment of the specifications of steviol glycosides, EFSA did not agree to expand the definition of steviol glycosides to include all individual steviol glycosides, due to uncertainties on the rate and extent of the metabolism of the different steviol glycosides to steviol (EFSA, 2018a). Likewise, in a recent evaluation of glucosylated steviol glycosides,

EFSA concluded that the data provided was not sufficient to assess the safety of glucosylated steviol glycosides due to the limited evidence on the complete hydrolysis of glucosylated steviol glycosides, metabolic fate data for steviol glycosides cannot be used in a read-across approach (EFSA, 2018b).

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2016) the following dietary exposure information must be provided:

1. A list of the foods or food groups proposed to contain the food additive.

2. The maximum proposed level and/or concentration range of the food additive for each food group or food.

3. For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption (Not applicable).

Each point is addressed in turn in the Section that follows.

D.1 Proposed Food Uses and Use-Levels of Rebaudioside D

The currently approved food uses and use-levels for steviol glycosides in Australia and New Zealand are presented in Table D.1-1 below (FSANZ, 2017b). SweeGen intends to market rebaudioside D for use as an intense sweetener under the same conditions of use as those presently authorised for steviol glycosides.

Table D.1-1 Summary of Currently Permitted Food Uses and Use-Levels for Steviol Glycosides in Australia and New Zealand

Category No	Food Description	Steviol Glycoside Concentration (mg/kg) as Steviol Equivalents
1.1.2	Liquid milk products and flavoured milk	115
1.2.2	Fermented milk products and rennetted milk products	175
3	Ice cream and edible ices	200
4.3.2	Fruits and vegetables in vinegar, oil, brine, or alcohol	160
4.3.4.1	Low joule chutneys, low joule jams, and low joule spreads	450
4.3.6	Fruit and vegetable preparations including pulp	210
5.1	Chocolate and cocoa products	550
5.2	Sugar confectionary	1100
6.3	Processed cereal and meal products	250
7.1.1	Fancy breads	160
7.2	Biscuits, cakes, and pastries	160
11.4	Tabletop sweeteners	GMP
13.3	Formula meal replacements and formulated supplementary foods	175
13.4	Formulated supplementary sports foods	175
14.1.2.1	Fruit and vegetable juices	50
14.1.2.2.2	Low joule fruit and vegetable juice products	125
14.1.2.2.3	Soybean beverage (plain)	100 (plain)
	Soybean beverage (flavoured)	200 (flavoured)
14.1.3	Water based flavoured drinks	200
14.1.4	Formulated beverages	200
14.1.5	Coffee, coffee substitutes, tea, herbal infusions, and similar products	100
20.2.0.1	Custard mix, custard powder, and blancmange powder	80
20.2.0.2	Jelly	260
20.2.0.3	Dairy and fat based desserts, dips, and snacks	150 (only dairy and fat based dessert products)
20.2.0.4	Sauces and toppings (including mayonnaises and salad dressings)	320

GMP = good manufacturing practice

D.2 Exposure Data

As previously noted, rebaudioside D is intended for use as an intense sweetener under the same conditions of use as those presently authorised for steviol glycosides. Therefore, intakes of rebaudioside D will be the same as for steviol glycosides currently on the Australian/New Zealand marketplace as it is intended to be a direct replacement for other steviol glycosides. As such, a separate intake assessment for rebaudioside D was not performed for the purpose of this application. Furthermore, it should be noted that use-levels for steviol glycosides are expressed as steviol equivalents, and as such, are not specified for any specific steviol glycoside; rather, the use-levels are based on the total content of the aglycone, steviol, in the final food product resulting from the addition of any steviol glycoside product meeting the appropriate specifications.

D.3 Use of the Food Additive in Other Countries

In the U.S., SweeGen's rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract has GRAS status for use as a table top sweetener and a general purpose non-nutritive sweetener in foods (GRN 715 – U.S. FDA, 2017a). GRAS Notice GRN 715 was filed with the U.S. FDA on the same substance, rebaudioside D produced *via* enzymatic bioconversion of purified stevia leaf extract, which is the subject of this application.

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CALIFORNIA JURAT WITH AFFIANT STATEMENT **GOVERNMENT CODE § 8202**

☒ See Attached Document (Notary to cross out lines 1–6 below)
☐ See Statement Below (Lines 1–6 to be completed only by document signer[s], *not* Notary)

1
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Signature of Document Signer No. 1 *Signature of Document Signer No. 2 (if any)*

A notary public or other officer completing this certificate verifies only the identity of the individual who signed the document to which this certificate is attached, and not the truthfulness, accuracy, or validity of that document.

State of California
County of Orange

Subscribed and sworn to (or affirmed) before me on this 3rd day of October, 2018,
Date *Month* *Year*
by
(1)___
(and (2)_____),
Name(s) of Signer(s)

proved to me on the basis of satisfactory evidence to be the person(s) who appeared before me.

Signature ____

Place Notary Seal Above

OPTIONAL

Though this section is optional, completing this information can deter alteration of the document or fraudulent reattachment of this form to an unintended document.

Description of Attached Document

Title or Type of Document: Statutory Declaration - Australia **Document Date:** 10/3/2018

Number of Pages: 1 **Signer(s) Other Than Named Above:** _____

Statutory Declaration – Australia

STATUTORY DECLARATION

Statutory Declarations Act 1959 [1]

I,
USA as Manager- Technical and Regulatory Affairs, SweeGen Inc,

make the following declaration under the *Statutory Declarations Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief.

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declarations Act 1959*, and I believe that the statements in this declaration are true in every particular.

Declared at 30321 Esperanza Avenue, Rancho Santa Margarita, California on 3 of October, 2018

Before me,

[*Signature of person before whom the declaration is made*][2]
[*Full name, qualification and address of person before whom the declaration is made (in printed letters)*]

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[1] http://www.comlaw.gov.au/Series/C1959A00052.

[2] A statutory declaration must be made before a prescribed person under the *Statutory Declarations Act 1959*. The list of prescribed persons is available in the Statutory Declarations Regulations 1993 at http://www.comlaw.gov.au/Series/F1996B00198.

CALIFORNIA JURAT WITH AFFIANT STATEMENT **GOVERNMENT CODE § 8202**

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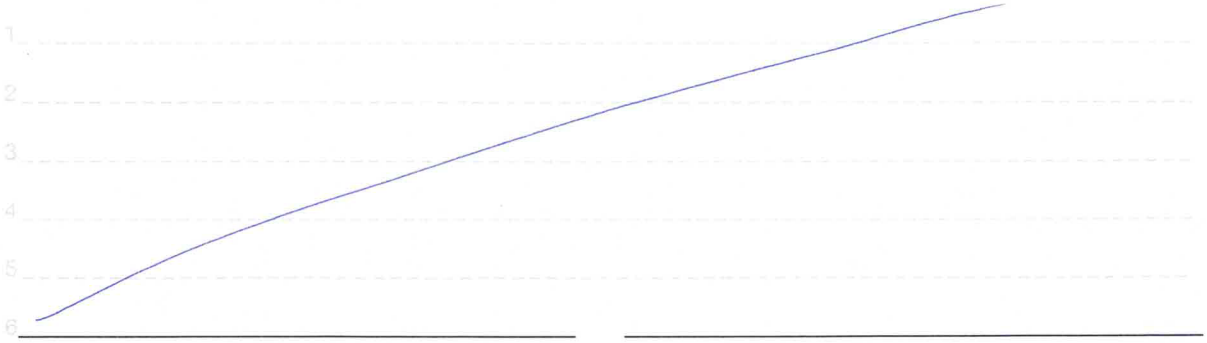

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State of California
County of Orange

Subscribed and sworn to (or affirmed) before me on this 3rd day of October, 2018,
Date *Month* *Year*
by
(1)_____ _____
(and (2)_____),
Name(s) of Signer(s)

proved to me on the basis of satisfactory evidence to be the person(s) who appeared before me.

Signature _____

Place Notary Seal Above

OPTIONAL

Though this section is optional, completing this information can deter alteration of the document or fraudulent reattachment of this form to an unintended document.

Description of Attached Document

Title or Type of Document: Statutory Declaration - New Zealand **Document Date:** 10/3/2018

Number of Pages: 1 **Signer(s) Other Than Named Above:** _____

Statutory Declaration – New Zealand

STATUTORY DECLARATION

Oaths and Declarations Act 1957[3]

I,
USA as Manager- Technical and Regulatory Affairs, SweeGen Inc, solemnly and sincerely declare that:

1. the information provided in this application fully sets out the matters required; and
2. the information is true to the best of my knowledge and belief; and
3. no information has been withheld which might prejudice this application to the best of my knowledge and belief.

And I make this solemn declaration conscientiously believing the same to be true and by virtue of the *Oaths and Declarations Act 1957*.

Declared at 30321 Esperanza Avenue, Rancho Santa Margarita, California this 3 of October,

Declared before me

[*Signature of person before whom the declaration is made*][4]

[3] http://www.legislation.govt.nz/act/public/1957/0088/latest/DLM314553.html.

[4] A statutory declaration must be made before a person authorised to take a statutory declaration under section 9 of the *Oaths and Declarations Act 1957*.

Checklist for General Requirements (3.1.1)

Check	Page No.	Mandatory Requirements
✓	6	A. Form of application • Application in English • Executive Summary (separated from main application electronically) • Relevant sections of Part 3 clearly identified • Pages sequentially numbered • Electronic copy (searchable) • All references provided
✓	6	B. Applicant Details
✓	7	C. Purpose of the Application
✓	7 - 8	D. Justification for the application • Regulatory impact information • Impact on international trade
✓	8	E. Information to support the application • Data requirements
✓	8	F. Assessment procedure • General
✓	8; Appendix A	G. Confidential commercial information • CCI material separated from other application material • Formal request including reasons • Non-confidential summary provided
✓	8 - 9	H. Other confidential information • Confidential material separated from other application material • Formal request including reasons
✓	9	I. Exclusive capturable commercial benefit • Justification provided
✓	9 - 10	J. International and other national standards • International standards • Other national standards
✓	10; Appendix B	K. Statutory Declaration
✓	Appendix C	L. Checklists provided with application • 3.1.1 Checklist • All page number references from application included • Any other relevant checklists for Chapters 3.2-3.7

Checklist for Food Additives (3.3.1)

Check	Page No.	Mandatory Requirements
✓	12 - 13	A.1 Nature and technological purpose information
✓	14	A.2 Identification information
✓	14 - 15	A.3 Chemical and physical properties
✓	15	A.4 Impurity profile
✓	15 - 23; Appendix A (A-7)	A.5 Manufacturing process
✓	23 - 24	A.6 Specifications
✓	24	A.7 Food Labelling
✓	25	A.8 Analytical detection method
✓	25	A.9 Additional functions
✓	27 - 28	B.1 Toxicokinetics and metabolism information
✓	29 - 33	B.2 Toxicity information
✓	33 - 36	B.3 Safety assessments from international agencies
✓	38	C.1 List of foods likely to contain the food additive
✓	38	C.2 Proposed use levels
✓	39	C.3 Likely level of consumption
N/A		C.4 Percentage of food group to contain the food additive
✓	39	C.5 Use in other countries (if applicable)
N/A		C.6 Where consumption has changed, information on likely consumption

N/A, not applicable

Checklist for Processing Aids (3.3.2)

Check	Page No.	Mandatory Requirements
✓	18	A.1 Type of processing aid
✓	18 - 19	A.2 Identification information
✓	19	A.3 Chemical and physical properties
✓	17 - 18; Appendix A (A-5 to A-6)	A.4 Manufacturing process
✓	Appendix A (A-6 to A-7)	A.5 Specification information
N/A		A.6 Analytical method for detection
✓	20; Appendix A (A-7 to A-9)	C.1 Information on enzyme use in other countries
✓	20 - 21; Appendix A (A-15)	C.2 Toxicity information of enzyme
✓	21; Appendix A (A-10 to A-14)	C.3 Allergenicity information of enzyme
✓	22; Appendix A (A-2)	D.1 Information on source organism
✓	22	D.2 Pathogenicity and toxicity of source microorganism
✓	22	D.3 Genetic stability of source organism
✓	23; Appendix A (A-1 to A-5)	E.1 Nature of genetic modification of source organism

N/A, not applicable

SWEETNESS EQUIVALENCY OF BESTEVIA REB-D

INTRODUCTION:

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. Bestevia-D, a trademarked product produced by Blue California, is made from isolating the sweetest compound of fermentation, Rebaudioside D, in order to create a non-caloric sweetener that can be used in similar applications to sucrose.

PURPOSE:

To determine the sweetness equivalence of Bestevia-D (Rebaudioside D) produced by Blue California in comparison to sucrose.

TEST SAMPLES:

Samples of BESTEVIA-D and Sucrose were prepared in water at room temperature respectively for comparison.

EQUIPMENT & MATERIALS:

Bestevia-D

Sucrose

Purified water

Analytical Scale

100ml beakers

Glass stirrers

Plastic cups

PROCEDURE:

1. 13 participants were pre-screened for taste acuity prior to completing the taste panel
2. Sensory evaluation of Reb D was performed using sucrose as a control. The sucrose sample purchased from Sigma-Aldrich and prepared control samples at three different concentrations of 1.0%, 2.5%, and 5.0% sucrose in bottled water (w/v) at room temperature.
3. The steviol glycoside Reb D at 300ppm for sensory evaluation was prepared by adding corresponding mass into a 1000 mL of bottled water.
4. The mixture was stirred at room temperature until complete dissolved.
5. The steviol glycoside sample was evaluated against several control sucrose samples at 1.0%, 2.5%, and 5.0% by a panel of thirteen volunteers.

RESULTS:

All the value from tasters were averaged and converted to the sweetness equivalency comparing to sucrose. The blind results showed consistent results among majority of thirteen volunteers. The result indicates that the rebaudioside D is 202 times sweeter to sucrose.

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On August 16, 2017, Blue California granted to SweeGen a license to use the regulatory approvals and independent verifications obtained by Blue California, including without limitation:

a.) The FDA No Objection Letter Re: GRAS Notice No. GRN 000667 on Reb M;
b.) Bureau of Chemical Safety | Food Additives section, Health Canada, Government of Canada's approval on Reb M.
c.) Non GMO Project Verified on Reb M.

For sake of clarification, SweeGen's products utilize the process(es) provided in the regulatory approvals described above.