# APPLICATION TO AMEND THE SPECIFICATIONS FOR STEVIOL GLYCOSIDES, UNDER AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE – STANDARD 1.3.1 – FOOD ADDITIVES, TO INCLUDE A NEW MANUFACTURING METHOD FOR SELECTED STEVIOL GLYCOSIDES FROM STEVIA LEAF EXTRACTS WITH HIGHLY PURIFIED STEVIOSIDE AND REBAUDIOSIDE

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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 a New Manufacturing Method for Selected Steviol Glycosides from Stevia Leaf Extracts with Highly Purified Stevioside and Rebaudioside

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# A. GENERAL REQUIREMENTS

In accordance with *Section 3.1.1 – General Requirements* of the Food Standards Australia New Zealand (FSANZ) *Application Handbook* (FSANZ, 2016a), the following general information is 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 declarations; and
12. Submission checklists.

## 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 *Application Handbook* (FSANZ, 2016a), 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–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 within the *Application Handbook* (FSANZ, 2016a).

## A.2 Applicant Details

PureCircle Limited (PureCircle) is the world's leading producer of high purity steviol glycoside ingredients for the global food and beverage industry. The contact details for the person responsible for this application are listed below.

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## A.3 Purpose of the Application

PureCircle is submitting this application to FSANZ concerning selected steviol glycosides that are produced using a new manufacturing method and is therefore seeking to amend Standard 1.3.1 and related Schedules for steviol glycosides. Enzymes derived from genetically modified strains of *Escherichia coli* (*E. coli*) K-12[1], namely uridine diphosphate (UDP)-glucosyltransferases (EC 2.4.1.X) and sucrose synthase (EC 2.4.1.13), are utilised to convert highly purified steviol glycosides rebaudioside A (reb A) (>95%) and/or stevioside (>95%) extracted from the leaves of *Stevia rebaudiana* (*S. rebaudiana*) Bertoni to rebaudioside M (reb M) and rebaudioside D (reb D) and/or to rebaudioside AM (reb AM; an isomer of reb D), respectively. The resulting blends of steviol glycosides may contain small amounts of the starting glycosides reb A and/or stevioside but will primarily contain the intermediate glycoside reb D and the final glycosides reb M and/or reb AM (hereinafter referred to as "steviol glycosides with a high reb M and/or reb D content" and "steviol glycosides with a high reb AM content"). The final purified products contain ≥95% steviol glycosides and meet the current purity requirements in Australia and New Zealand for steviol glycosides and the definition established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Currently, *Schedule 3 – Identity and Purity* of the *Australia New Zealand Food Standards Code* (*The Code*) contains a "Specification for steviol glycosides from *Stevia rebaudiana* Bertoni" under S3-35 (FSANZ, 2019a). The current specification indicates that steviol glycosides must be obtained from leaves of the *S. rebaudiana* Bertoni plant through (a) extraction with hot water; or (b) enzymatic conversion of purified stevia leaf extract to produce reb M using UDP-glucosyltransferase and sucrose synthase sourced from strains of *Pichia pastoris* (*P. pastoris*). Although PureCircle's steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are chemically identical to steviol glycosides from *S. rebaudiana* Bertoni, they do not comply with the manufacturing requirements laid out in S3-35. Therefore, PureCircle seeks to amend *The Code* in order to include a new manufacturing method for selected steviol glycosides as a permissible method for producing steviol glycosides from *S. rebaudiana* Bertoni (*i.e.,* the enzymatic conversion of highly purified stevia leaf extract using UDP-glucosyltransferases and sucrose synthase derived from strains of *E. coli* K-12). It is not the intent of this application to amend the use limits, permitted uses, or purity specifications (≥95% steviol glycosides) for steviol glycosides.

## A.4 Justification of the Application

### A.4.1 Technological Function for the Food Additive

Selected steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, would be used as high-intensity sweeteners for the replacement of sucrose in reduced-calorie or no-sugar-added products. It is well-recognised that the usefulness of high-intensity sweeteners extends beyond its ability to provide sweetness, but also needs to take into account other taste attributes (*i.e.*, the taste quality). Minor steviol glycosides, such as reb D, reb M, and reb AM, have been shown in sensory evaluations to provide favourable taste characterisation compared to preparations containing major individual steviol glycosides alone. With the continued strive to limit unwanted taste characteristics that are often associated with the use of high-intensity sweeteners in foods and beverages, the specific flavour profiles imparted by high-intensity sweeteners are critical determinants for their practical use as sugar replacement in food.

[1] *Escherichia coli* K-12 is a non-pathogenic and non-toxicogenic strain of *Escherichia coli* that is standardly used in the food industry.

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

Amending the specification for steviol glycosides from *S. rebaudiana* Bertoni to include an alternative manufacturing method for producing minor steviol glycosides is of clear interest to the food industry in Australia and New Zealand since minor glycosides provide improved sensory characteristics compared to major individual steviol glycosides. This will allow for the replacement of greater amounts of sugar in food and beverage products at the presently permitted use-levels for steviol glycosides. Furthermore, the use of alternative manufacturing methods to produce minor steviol glycosides provides cost benefits to industry. Minor glycosides are present in low amounts in the leaves of *S. rebaudiana* Bertoni; therefore, standard extraction processes typically yield very low quantities of highly purified minor steviol glycosides.

It is expected that steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract will present an attractive alternative as a sweetener for food manufacturers in Australia and New Zealand. It is anticipated that steviol glycosides produced by enzymatic conversion of stevia leaf extract may be imported into Australia and New Zealand that manufacturers would then incorporate into their products and in addition, global companies may also import their finished products. As a high-intensity sweetener, steviol glycosides produced by enzymatic conversion of stevia leaf extract will be used to replace sugar in foods and the group of consumers to whom this would be beneficial would be any individuals that are seeking foods and beverages with reduced calories from sugar for the purposes of maintaining a reduced-calorie diet. This would also include individuals with specific medical conditions that require reduced sugar intakes, such as diabetics, as steviol glycosides do not interfere with glucose homeostasis (EFSA, 2010). While a wide array of high-intensity sweeteners is already available for use in Australia and New Zealand, including notably other steviol glycoside preparations, selected steviol glycosides produced by enzymatic conversion of stevia leaf extract also provide the advantage of providing improved flavour profiles compared to preparations containing major individual steviol glycosides alone.

Steviol glycosides are already approved for many food uses at specified use-levels within Australia and New Zealand, and since PureCircle intends to market steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract in the same approved food-categories and at the same use-levels, there is no perceived benefit or added cost to government.

## A.5 Information to Support the Application

Technical information specific to PureCircle's alternative manufacturing method for selected steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract is presented in detail in Section B. This includes information regarding the production of the UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of *E. coli* K-12 and their use as processing aids. Since UDP-glucosyltransferase and sucrose synthase produced by *E. coli* K-12 are not permitted processing aids in Australia and New Zealand, information regarding their manufacture and safety, including the source microorganisms utilised to produce them, is presented pursuant to *Section 3.3.2 – Processing Aids* of the FSANZ *Application Handbook*. In 2018, FSANZ evaluated a similar application to include an alternative manufacturing process for reb M produced by enzymatic bioconversion of stevia leaf extract using UDP-glucosyltransferase and sucrose synthase derived from strains of *P. pastoris*, and at this time, reviewed the safety of steviol glycosides (A1157 - FSANZ, 2018). Since the safety of steviol glycosides in general has been previously reviewed and established by FSANZ, Section C provides a short summary of steviol glycoside safety and focuses on presenting (i) new safety publications available in the scientific literature which have not previously been evaluated by FSANZ; and (ii) recent opinions released by regulatory agencies and/or scientific bodies (*i.e.,* JECFA).

## A.6 Assessment Procedure

PureCircle considers the most appropriate assessment procedure for the application herein is related to an amendment to *Standard 1.3.1 – Food Additives* of *The Code* to modify the specification outlined in *Schedule 3* for "Steviol glycosides from *Stevia rebaudiana* Bertoni" (S3–35) to include the option of manufacturing selected steviol glycosides *via* enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract using enzymes derived from strains of *E. coli* K-12. This revision is expected to fall under the General Procedure (Subdivision D of the FSANZ Act), Cost Category Level 2. PureCircle also requests that the application undergo the expedited procedure.

## A.7 Confidential Commercial Information (CCI)

PureCircle requests the information contained within Appendix A be considered confidential commercial information (CCI). Non-confidential general summaries of proprietary information are provided within this application, and all details considered CCI have been removed and are presented in Appendix A. PureCircle 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 and enzymes, the unpublished sequences of the enzymes, details of sensory investigations, and reports on *in vitro* microbial metabolism.

## A.8 Other Confidential Information

PureCircle has no other confidential information to declare.

## A.9 Exclusive Capturable Commercial Benefit (ECCB)

Steviol glycoside preparations are currently produced by other manufacturing companies, aside from PureCircle. However, selected steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are only produced by PureCircle using a specific manufacturing process. On this basis, PureCircle anticipates that this application would confer Exclusive Capturable Commercial benefit (ECCB) in accordance with Section 8 of the FSANZ Act which states:

> "*An **exclusive, capturable commercial benefit** is conferred upon a person who applies for the development of a food regulatory measure or the variation of a food regulatory measure under Section 22 if:*
>
> *(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard or draft variation of the standard that would be prepared in relation to the application; and*
>
> *(b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application*".

As such, PureCircle is expecting to pay the full costs of processing this application.

# A.10 International and Other National Standards

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

The safety, dietary intake, and specifications for steviol glycosides were recently re-evaluated by the JECFA Committee in 2016 at the 82nd meeting. The Committee confirmed the safety and acceptable daily intake (ADI) of 0 to 4 mg/kg body weight (expressed as steviol). The Committee also reviewed a new manufacturing process for reb A, which was described to utilise a genetically modified strain of *Yarrowia lipolytica* (*Y. lipolytica*) that overexpresses the steviol glycoside biosynthetic pathway. This resulted in a new specification monograph for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (JECFA, 2016) with a defined purity of no less than 95% reb A on the dried basis. An amended specification for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" was issued after reviewing the data on the shared metabolism of steviol glycosides and specifically, the definition of steviol glycosides was expanded to include "*a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni*" (JECFA, 2017a). The purity of steviol glycosides from *S. rebaudiana* Bertoni was established at no less than 95% steviol glycosides on the dried basis.

## A.10.2 United States

Over 50 Generally Recognized as Safe (GRAS) Notices (GRN No. 252, 253, 275, 278, 282, 287, 303, 304, 318, 323, 329, 337, 348, 349, 354, 365, 367, 369, 375, 380, 388, 389, 393, 395, 418, 448, 452, 456, 461, 467, 473, 493, 512, 516, 536, 548, 555, 607, 619, 626, 632, 638, 656, 662, 667, 702, 715, 733, 744, 745, 759, 764, 768, 780, 790, 795, 799, 812, 821, 823) for major individual steviol glycosides (stevioside and reb A, C, D, and X/M), mixtures of steviol glycosides (*e.g.,* reb A and stevioside as the principal components), or glucosylated steviol glycosides have been submitted to the United States (U.S.) Food and Drug Administration (FDA) for review since 2008. With the exception of the most recent GRAS notifications that have not yet been reviewed, the FDA has raised no objections regarding the petitioners' conclusions of the GRAS status of steviol glycoside products for use as general purpose sweeteners in foods (U.S. FDA, 2019). Of note, GRN 744 was submitted by PureCircle to the FDA (PureCircle Limited, 2017) and considered the safety of steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract, equivalent to the steviol glycoside preparation presented herein. The FDA raised no objections regarding the GRAS status of PureCircle's steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract for use as general purpose sweetener in foods, excluding meat and poultry products and infant formula, at levels in accordance with current Good Manufacturing Practices (cGMP) (U.S. FDA, 2018).

### A.10.3 European Union

The European Commission has permitted the use of steviol glycosides as a sweetening agent under *Commission Regulation* (EU) No 1131/2011 and as subsequently amended by *Commission Regulation* (EU) No 913/2013, *Commission Regulation* (EU) 2016/441, and *Commission Regulation* (EU) 2016/479 (EU, 2011, 2013, 2016a,b). Steviol glycosides are permitted for use in a variety of food and beverage categories in the EU at levels of up to 3,300 mg/kg (as steviol equivalents) in chewing gum. The most recent specifications were published in 2016, which were updated to include reb M in the list of steviol glycosides (stevioside, reb A, B, C, D, E, and F, steviolbioside, rubusoside, and dulcoside) that comprised the assay value of no less than 95% total steviol glycosides, and also removed the requirement of a steviol glycoside preparation "comprising mainly (at least 75%) of stevioside and/or rebaudioside A". These changes were justified based on the fact that all steviol glycosides are converted to steviol in the intestine and therefore "the specific steviol glycosides (E 960) composition would not be of a safety concern".

### A.10.4 Canada

Health Canada established an ADI of 4 mg steviol equivalents/kg body weight in July 2012 and recommended that steviol glycosides be approved for use as a sweetening agent (Health Canada, 2012). Health Canada currently defines steviol glycosides in the list of permitted sweeteners as "Steviol glycosides from *Stevia rebaudiana* Bertoni", and the total steviol glycoside content must be no less than 95% (Health Canada, 2018). Steviol glycosides are permitted for use as sweeteners in a variety of food and beverage products, at a maximum use-level of up to 0.35% as steviol equivalents in breath fresheners products and chewing gums.

### A.10.5 Asia

In Japan, the Ministry of Health and Welfare approved 3 types of stevia extracts: α-glucosyltransferase-treated stevia, powdered stevia, and stevia extract (Japan Food Chemical Research Foundation, 2014). In addition, purified stevioside (as a crude extract, 50% pure extract, and ≥90% pure extract) and *S. rebaudiana* leaf extracts also are accepted for general use as sweeteners in a variety of foods and beverages including pickling gum, pickles, dried seafood, meat, fish, soy sauce, bean pastes, sugarless chewing gums, juices, cola, table-top sweeteners, and ice cream in Japan (Marie, 1991; Das *et al.*, 1992; Ferlow, 2005). In India, the Food Safety and Standards Authority of India has approved the use of steviol glycosides in a number of food and beverage categories as per the official gazette notification (FSSAI, 2015; MOHFW, 2016). Steviol glycosides also are approved for use as a food additive (sweetening agent) in several other Asian countries such as China, Hong Kong, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Singapore, Taiwan, Thailand, and Vietnam (PureCircle Stevia Institute, 2018).

### A.10.6 Other Jurisdictions

Stevioside, *S. rebaudiana* leaves, and highly refined extracts are permitted for use as low-calorie sweeteners in several South/Central American countries, including Argentina, Bolivia, Brazil, Chile, Colombia, Cost Rica, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Paraguay, Peru, Uruguay, and Venezuela (PureCircle Stevia Institute, 2018). Likewise, steviol glycosides are approved for use as food additives in various countries in the Middle East, Eastern Europe, and Africa (PureCircle Stevia Institute, 2018). For example, in Nigeria the National Agency for Food and Drug Administration and Control permitted reb M for use in foods and beverages in 2014. The use-levels of reb M must be in accordance with maximum levels established for steviol glycosides under Codex Alimentarius Commission's General Standards for Food Additives (Codex, 2018).

## A.11 Statutory Declarations

Signed statutory declarations for Australia and New Zealand are provided as Appendix B.

## A.12 Submission Checklists

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, 2016a) the following technical information is 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.

In addition, to fulfil the requirements outlined in *Section 3.3.2 – Processing Aids* of the FSANZ *Application Handbook*, the following information on the enzymatic processing aids, including the production microorganisms, is provided:

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 Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside from Stevia Leaf Extract

## B.1.1 Technological Purpose

PureCircle's selected steviol glycosides, primarily reb M, reb D and reb AM, are produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract and the final product contains no less than 95% total steviol glycosides. Consistent with steviol glycoside preparations already approved for use in Australia and New Zealand, PureCircle's steviol glycosides produced by enzymatic conversion of stevia leaf extract functions as an intense sweetener and a flavour enhancer in accordance with the technological purposes listed in *Schedule 14 – Technological purposes performed by substances used as food additives* (FSANZ, 2016b). PureCircle intends to market steviol glycosides produced by enzymatic conversion of stevia leaf extract in the current food categories and at the current use-levels permitted for steviol glycosides, and does not intend for this application to (i) extend the use of steviol glycosides to additional foods or at different use-levels from what is currently permitted; and (ii) propose additional food matrices to which the addition of steviol glycosides has not already been approved.

## B.1.2 Sweetness Potency

The sweetness intensities of individual steviol glycosides vary, for example, reb A is often quoted as 200 to 300 times sweeter than sucrose (DuBois *et al.,* 1991), whereas reb M has been shown to be up to 350 times as sweet as sugar (Prakash *et al.*, 2014). Overall, the sweetness potencies of steviol glycosides range from 200 to 350 times that of sucrose.

The sweetness potency of a preparation of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A from stevia leaf extract was determined by a sensory panel (n=30 participants). Isosweet tests were conducted where panellists compared 2 samples at a time, one being the control 5% sucrose sample, and the other being the steviol glycoside test sample at different concentrations in each pairing. The panellists were instructed to select the sweeter sample in each pair. Tests were conducted with randomised serving orders and were balanced within each pair and sample set. Breaks between sampling were 90 seconds, and filtered room temperature water and unsalted crackers were used for palate cleansing. Steviol glycosides with a high reb M content produced by enzymatic conversion of reb A from stevia leaf extract was determined to be 200 times sweeter than sucrose at 5%, which is consistent with the sweetness potency of reb M extracted from *S. rebaudiana* Bertoni. The full study report is considered CCI and is provided in Appendix A.

The sweetness potency of a preparation of steviol glycosides with a high reb AM content produced by enzymatic conversion of stevioside from stevia leaf extract was determined by a third-party research lab using a sensory panel consisting of 50 discrimination panellists. Isosweet tests were conducted where panellists compared 2 samples at a time, one being the control 5% sucrose sample, and the other being the steviol glycoside test sample at different concentrations in each pairing. The panellists were instructed to select the sweeter sample in each pair. Tests were conducted with randomised serving orders and were balanced within each pair and sample set. Breaks between sampling were 90 seconds, and filtered room temperature water and unsalted crackers were used for palate cleansing. Steviol glycosides with a high reb AM content produced by enzymatic conversion of stevioside from stevia leaf extract was determined to be 152 times sweeter than sucrose at 5%. The full study report is considered CCI and is provided in Appendix A. To include Reb AM produced by enzymatic conversion of stevioside from stevia leaf extract under S3-35 "Specification for steviol glycosides from *Stevia rebaudiana* Bertoni", it is proposed that the sweetness potency range be widened from "approximately 200 to 300 times sweeter than sucrose" to "approximately 150 to 300 times sweeter than sucrose".

The sensory characteristics of a preparation of steviol glycosides with a high reb AM content produced by enzymatic conversion of stevioside from stevia leaf extract were also assessed. The sensory effects of reb AM on the flavour profile of 3 different types of beverages was evaluated, including a commercial raspberry watermelon coconut water, a stevia (reb A) sweetened no-sugar-added chocolate flavoured dairy protein shake, and a no-calorie lemon-lime carbonated soft drink. The full study report is considered CCI and is provided in Appendix A, and overall, the results of these sensory evaluations demonstrate that when compared with reb A and other steviol glycosides, reb AM was associated with decreased bitterness lingering and aftertaste and higher overall liking.

## B.1.3 Stability

The stability of steviol glycosides has been previously reviewed by a number of the scientific advisory bodies involved in the evaluation of steviol glycoside safety, including FSANZ, as well as JECFA and the European Food Safety Authority (EFSA). Stability is also discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999). Specifically, JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in foods at the 68$^{th}$ meeting (JECFA, 2007b). The Committee noted that steviol glycosides do not undergo browning or caramelisation when heated and are reasonably stable under elevated temperatures used in food processing. Under acidic conditions (pH 2 to 4), steviol glycosides (approximately 90 to 94% purity), are stable for at least 180 days when stored at temperatures up to 24°C. When exposed to elevated temperatures (80°C, in water, 8 hours), however, 4% and 8% decomposition was observed in solutions of steviol glycosides at pH 4.0 and 3.0, respectively, indicating that the stability of steviol glycosides is pH and temperature dependent. When the temperature was increased to 100°C, expectedly higher rates of steviol glycoside decomposition (10% and 40% at pH 4.0 and 3.0, respectively) were observed. Based on the above findings, as well as additional publicly available stability studies, JECFA concluded that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions.

Although the stability of each individual steviol glycoside was not specifically addressed during any of the previous evaluations, it is expected that the stability of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract would be similar to that of the individual steviol glycosides given the similarities in structure. To confirm this viewpoint, additional stability studies with steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract were conducted to determine bulk stability under normal and/or accelerated storage conditions, as well as stability in solution at various pH levels and temperatures. These studies are summarised in the sections that follow and demonstrate that the stability of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract is similar to individual steviol glycosides from stevia leaf extract, consistent with previous stability conclusions made by JECFA.

### *B.1.3.1 Storage Stability*

The storage stability of steviol glycosides with a high reb M and/or reb D content produced by enzymatic conversion of reb A from stevia leaf extract was assessed (Lot No. LB110117; see Appendix D for stability study report). Steviol glycosides with a high reb M and/or reb D content powder samples were stored in glass containers for up to 12 weeks at (i) 25°C, 60% relative humidity; and (ii) 40°C, 75% relative humidity. To assess storage stability, samples were tested by high-performance liquid chromatography (HPLC) at baseline and at various time points thereafter, based upon measured values of individual steviol glycosides, as well as total steviol glycosides. As reported in Table B.1.3.1-1, steviol glycosides with a high reb M and/or reb D content powder stored under both conditions for 12 weeks was stable in its individual steviol glycoside content as well as total steviol glycoside content (<1% degradation measured). A similar test for the storage stability of a powder preparation of steviol glycosides with a

high reb AM content from stevia leaf extract has been conducted over 8 weeks. The results presented in Table B.1.3.1-2 demonstrate that steviol glycosides with a high reb AM content was also stable in its individual steviol glycoside content as well as total steviol glycoside content (<1% degradation measured) over 8 weeks.

**Table B.1.3.1-1 Storage Stability of Steviol Glycosides with a High Reb M and/or Reb D Content Produced by Enzymatic Conversion of Reb A from Stevia Leaf Extract (Lot No. LB110117), as percent (%) dry basis**

| Week | 0 | 4 | 8 | 12 |
|---|---|---|---|---|
| **25°C, 60% relative humidity** | | | | |
| Reb D | 0.67 | 0.69 | 0.72 | 0.72 |
| Reb M | 96.72 | 96.59 | 96.47 | 96.06 |
| Reb A | ND | ND | ND | ND |
| **Total steviol glycosides** | **97.39** | **97.28** | **97.19** | **96.78** |
| **40°C, 75% relative humidity** | | | | |
| Reb D | 0.67 | 0.70 | 0.73 | 0.75 |
| Reb M | 96.72 | 96.72 | 96.15 | 96.08 |
| Reb A | ND | ND | ND | ND |
| **Total steviol glycosides** | **97.39** | **97.42** | **96.88** | **96.83** |

ND = not detected; Reb = rebaudioside.

**Table B.1.3.1-2 Storage Stability of Steviol Glycosides with a High Reb AM Content Produced by Enzymatic Conversion of Stevioside from Stevia Leaf Extract, as percent (%) dry basis**

| Week | 0 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|---|
| **25°C, 60% relative humidity** | | | | | |
| Reb AM | 98.68 | 98.63 | 98.73 | 98.62 | 98.66 |
| Stevioside | ND | ND | ND | ND | ND |
| Reb E | ND | ND | ND | ND | ND |
| **40°C, 75% relative humidity** | | | | | |
| Reb AM | 98.68 | 98.41 | 98.48 | 98.64 | 98.63 |
| Stevioside | ND | ND | ND | ND | ND |
| Reb E | ND | ND | ND | ND | ND |

ND = not detected; Reb = rebaudioside.

### *B.1.3.2 pH Stability*

The general stability of steviol glycosides with a high reb M and/or reb D content produced by enzymatic conversion of reb A from stevia leaf extract (Lot No. LB110117) was assessed over a pH range of 2.0 to 8.0 for a total of 12 weeks at 4 different temperatures: 4, 25, 37, and 56°C (see Appendix D for stability study report). Samples were prepared at concentrations of approximately 1,000 mg/L in 500 mL of buffer solution and stored in amber glass vials. Buffer was prepared by mixing different ratios of 0.1 M phosphate buffer, 0.1 M phosphorous acid, or 0.1 M di-sodium hydrogen phosphate buffer to obtain the target pH. Total steviol glycosides present in the stability samples were measured by HPLC at baseline as well as various time points over the study period, determined by the sum of the measured concentrations of reb A, D, and M (see Table B.1.3.2-1).

The extent and rate of degradation of steviol glycosides with a high reb M and/or reb D content, based on measured total steviol glycosides, was shown to be dependent on pH, temperature, and time. In general, steviol glycosides with a high reb M and/or reb D content at all pH levels tested (2.0 to 8.0) was most stable when stored at 4°C and the least stable at 56°C. Over the 12-week study period, samples

tested at pH 4.0 to 8.0 at 5, 25, and 37°C remained stable within at least 7% of the starting material percentage value. A significant loss in stability was noted when samples were stored at 56°C at the majority of pH levels, with the pH 4.0 and 5.0 samples remaining the most stable over the 12 weeks. Overall, at pH values ranging from 4.0 to 8.0, no significant degradation was observed over 12 weeks at 5, 25, and 37°C.

A similar test for the general stability at different pH and temperature has been conducted over 8 weeks with steviol glycosides with a high reb AM content from stevia leaf extract. The results presented in Table B.1.3.2-2 demonstrate similar stability trends as reported for steviol glycosides with a high reb M and/or reb D content in Table B.1.3.2-1.

**Table B.1.3.2-1 Stability of Steviol glycosides with a High Reb M and/or Reb D Content Produced by Enzymatic Conversion of Reb A from Stevia Leaf Extract (Lot No. LB110117) in Solution at Varying Temperature and pH**

| Week | pH 2.0 | pH 3.0 | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 | pH 8.0 |
|---|---|---|---|---|---|---|---|
| **4°C** | **Total steviol glycosides (%)[a]** | | | | | | |
| 0 (baseline) | 97.44 | 97.24 | 97.18 | 97.29 | 97.31 | 97.21 | 97.59 |
| 2 | 97.04 | 97.50 | 96.87 | 96.88 | 97.06 | 96.99 | 96.85 |
| 4 | 95.11 | 97.72 | 97.11 | 97.32 | 97.48 | 97.01 | 97.36 |
| 6 | 94.81 | 97.08 | 96.97 | 97.08 | 97.50 | 97.08 | 96.85 |
| 8 | 94.27 | 96.28 | 96.60 | 96.72 | 97.06 | 96.32 | 96.62 |
| 10 | 93.60 | 96.25 | 95.91 | 96.72 | 96.74 | 95.75 | 96.08 |
| 12 | 93.78 | 96.87 | 96.37 | 96.16 | 96.45 | 96.28 | 94.70 |
| **25°C** | **Total steviol glycosides (%)[a]** | | | | | | |
| 0 (baseline) | 97.44 | 97.24 | 97.18 | 97.29 | 97.31 | 97.21 | 97.59 |
| 2 | 92.86 | 96.78 | 96.82 | 96.78 | 96.75 | 96.92 | 96.67 |
| 4 | 83.37 | 96.16 | 97.08 | 97.10 | 97.65 | 96.78 | 96.62 |
| 6 | 79.54 | 95.82 | 97.02 | 97.18 | 97.25 | 97.66 | 96.03 |
| 8 | 70.25 | 94.01 | 96.22 | 96.86 | 96.34 | 96.08 | 95.13 |
| 10 | 66.85 | 93.16 | 95.75 | 96.38 | 95.52 | 94.76 | 94.07 |
| 12 | 64.64 | 93.42 | 96.43 | 96.25 | 95.66 | 95.59 | 94.60 |
| **37°C** | **Total steviol glycosides (%)[a]** | | | | | | |
| 0 (baseline) | 97.44 | 97.24 | 97.18 | 97.29 | 97.31 | 97.21 | 97.59 |
| 2 | 64.01 | 93.46 | 96.34 | 97.29 | 96.73 | 96.26 | 95.71 |
| 4 | 32.01 | 87.34 | 95.97 | 95.83 | 95.27 | 95.22 | 96.01 |
| 6 | 24.28 | 85.21 | 95.90 | 95.50 | 94.33 | 95.34 | 95.85 |
| 8 | 16.89 | 75.69 | 95.37 | 95.30 | 94.13 | 92.79 | 94.50 |
| 10 | 11.64 | 75.10 | 93.46 | 94.61 | 91.52 | 91.15 | 93.07 |
| 12 | 9.16 | 75.18 | 93.99 | 94.92 | 91.76 | 90.49 | 93.19 |
| **56°C** | **Total steviol glycosides (%)[a]** | | | | | | |
| 0 (baseline) | 97.44 | 97.24 | 97.18 | 97.29 | 97.31 | 97.21 | 97.59 |
| 2 | 4.88 | 69.94 | 92.42 | 94.66 | 95.62 | 94.48 | 94.22 |
| 4 | 0.79 | 36.96 | 84.74 | 89.33 | 77.82 | 70.94 | 85.77 |
| 6 | 1.01 | 30.40 | 80.78 | 86.56 | 70.14 | 65.35 | 83.73 |
| 8 | 0.11 | 23.91 | 77.01 | 83.92 | 65.39 | 62.51 | 80.05 |
| 10 | 0.03 | 14.75 | 72.92 | 76.89 | 56.32 | 54.64 | 71.90 |
| 12 | 0.03 | 11.62 | 70.67 | 73.89 | 52.36 | 51.57 | 68.94 |

Reb = rebaudioside.

[a] Sum of the following individual steviol glycosides: rebaudiosides A, D, and M.

**Table B.1.3.2-2 Stability of Steviol glycosides with a High Reb AM Content Produced by Enzymatic Conversion of Stevioside from Stevia Leaf Extract in Solution at Varying Temperature and pH**

| Week | pH 2.0 | pH 3.0 | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 | pH 8.0 |
|---|---|---|---|---|---|---|---|
| **4°C** | **Total steviol glycosides (% of baseline)[a]** | | | | | | |
| 0 (baseline) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| 2 | 100.09 | 99.63 | 99.99 | 100.13 | 99.73 | 99.87 | 100.01 |
| 4 | 98.75 | 99.57 | 99.59 | 100.08 | 100.16 | 99.99 | 100.02 |
| 6 | 97.22 | 100.14 | 100.25 | 99.82 | 100.20 | 100.52 | 99.75 |
| 8 | 97.43 | 99.88 | 99.74 | 100.29 | 100.23 | 100.48 | 100.04 |
| **25°C** | **Total steviol glycosides (% of baseline)[a]** | | | | | | |
| 0 (baseline) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| 2 | 89.35 | 98.14 | 99.79 | 99.88 | 99.70 | 100.04 | 99.81 |
| 4 | 83.54 | 97.93 | 99.82 | 100.17 | 99.99 | 99.88 | 99.75 |
| 6 | 75.66 | 96.45 | 99.53 | 99.81 | 99.96 | 100.48 | 99.10 |
| 8 | 69.49 | 96.36 | 99.32 | 100.18 | 99.90 | 99.85 | 99.03 |
| **37°C** | **Total steviol glycosides (% of baseline)[a]** | | | | | | |
| 0 (baseline) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| 2 | 58.11 | 93.86 | 99.23 | 99.90 | 99.69 | 99.07 | 99.51 |
| 4 | 39.15 | 90.77 | 98.36 | 99.84 | 99.18 | 99.08 | 99.23 |
| 6 | 23.87 | 85.95 | 97.25 | 99.13 | 98.56 | 98.36 | 98.84 |
| 8 | 15.12 | 83.17 | 96.81 | 98.71 | 98.41 | 97.97 | 98.52 |

Reb = rebaudioside.

[a] Sum of the following individual steviol glycosides: rebaudiosides AM, E, and stevioside; although in this case no rebaudioside E or stevioside were detected in any test sample.

Similar to individual steviol glycosides, the stability of steviol glycoside products produced by enzymatic conversion followed the same degradation pathway and was pH-, temperature-, and time-dependent. Therefore, the conclusions regarding the stability of steviol glycosides made by JECFA and other scientific bodies (that steviol glycosides are thermally and hydrolytically stable for use in foods and acidic beverages under normal processing and storage conditions) can be extended to steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract that are the subject of this safety assessment.

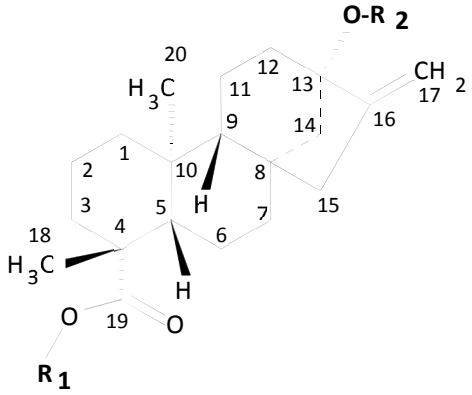
# B.2 Information to Enable Identification of Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside from Stevia Leaf Extract

## B.2.1 Identity of Substance

PureCircle's steviol glycosides produced by enzymatic conversion of highly purified reb A from stevia leaf extract may contain remnants of the starting material reb A (*i.e.,* 4 glucose units conjugated to the steviol backbone), the intermediate glycoside reb D (*i.e.,* 5 glucose units), and the final glycoside reb M (*i.e.,* 6 glucose units). Likewise, steviol glycosides produced by enzymatic conversion of highly purified stevioside from stevia leaf extract may contain remnants of the starting material stevioside (*i.e.,* 3 glucose units conjugated to the steviol backbone), the intermediate glycoside rebaudioside E (reb E) (*i.e.,* 4 glucose units), and will primarily contain the final glycoside reb AM (*i.e.,* 5 glucose units). Reb AM is an isomer of reb D that is found in stevia leaf extract, though at a very small amount, and shows the same metabolic fate as other steviol glycosides, as discussed in Section C.2.

The final purified steviol glycoside products produced by enzymatic conversion contain ≥95% total steviol glycosides, which is consistent with the purity criteria for steviol glycosides established by JECFA and the current purity requirements for "Steviol glycosides from *Stevia rebaudiana* Bertoni" (S3-35) listed in *Schedule 3* of *The Code* (FSANZ, 2019a). All steviol glycosides are glycosylated derivatives of the aglycone steviol and as such, all share the same backbone structure (Figure B.2.1-1) and differ only with respect to the type and number of glycoside units at positions $R_1$ and $R_2$. The chemical structures of the different steviol glycosides that may be present in preparations of steviol glycosides produced by enzymatic conversion are presented in Table B.2.1-1. The enzymatic conversion pathways of highly purified reb A and stevioside to produce reb M and reb AM, respectively, with intermediate production of reb D and reb E are shown in Figure B.2.1-2 (Markosyan *et al.*, 2013, 2018).

**Figure B.2.1-1 Backbone Structure for Steviol Glycosides**



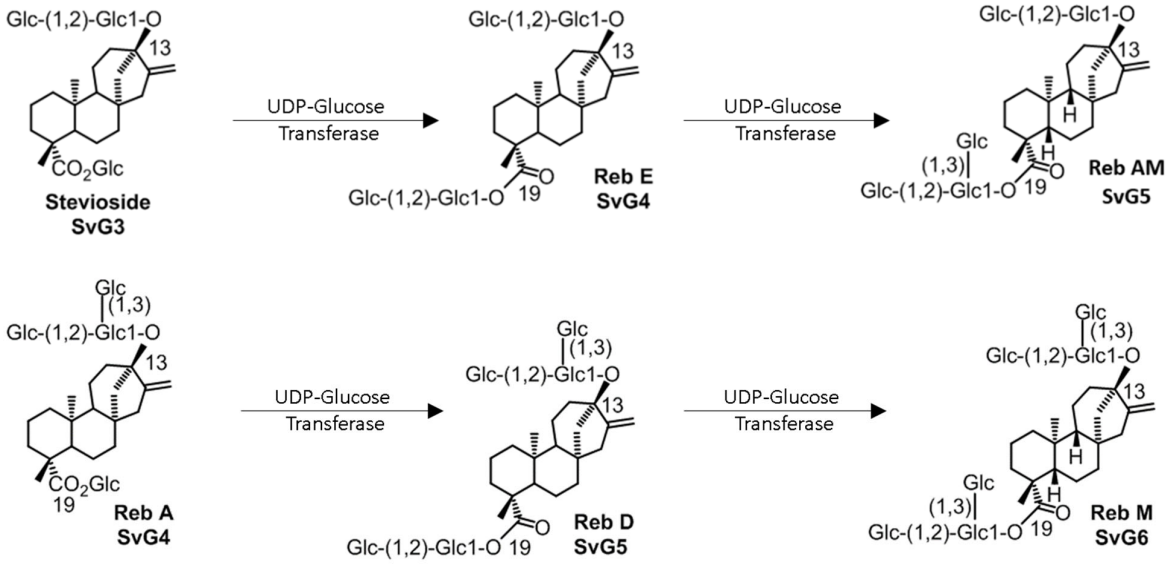

Where $R_1$ and $R_2$ can be 1 or more sugar moieties, including, but not limited to glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, and galactose.

**Table B.2.1-1 Individual Steviol Glycosides that May be Present in Steviol Glycosides Produced by Enzymatic Conversion (see Figure B.2.1-1 for Backbone Structure)**

| Common Name | Trivial Formula | Mol. Wt. | $R_1$ | $R_2$ | Chemical Name | CAS Number | Chemical Formula |
|---|---|---|---|---|---|---|---|
| **Starting material: Rebaudioside A (>95%) from stevia leaf extract** | | | | | | | |
| Rebaudioside A | SvG4 | 967 | Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- | 13-[(2-O-β-D-glucopyranosyl-3-O-β-D- glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16- en-18-oic acid, β-D-glucopyranosyl ester | 58543-16-1 | $C_{44}H_{70}O_{23}$ |
| Rebaudioside D | SvG5 | 1,129 | Glcβ(1-2)Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- | 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester | 63279-13-0 | $C_{50}H_{80}O_{28}$ |
| Rebaudioside M | SvG6 | 1,291 | Glcβ(1-2)[Glcβ (1-3)]Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- | 13-[(O-β- D-glucopyranosyl-(1,2)-O-[ β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-O-β- D-glucopyranosyl-(1,2)-O-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester | 1220616-44-3 | $C_{56}H_{90}O_{33}$ |
| **Starting material: Stevioside (>95%) from stevia leaf extract** | | | | | | | |
| Stevioside | SvG3 | 805 | Glcβ1- | Glcβ(1-2)Glcβ1- | 13-[(2-O-β-D-glucopyranosyl-β-D- glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D- glucopyranosyl ester | 57817-89-7 | $C_{38}H_{60}O_{18}$ |
| Rebaudioside E | SvG4 | 967 | Glcβ(1-2)Glcβ1- | Glcβ(1-2)Glcβ1- | 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester | 63279-14-1 | $C_{44}H_{70}O_{23}$ |
| Rebaudioside AM | SvG5 | 1,129 | Glcβ(1-2)[Glcβ (1-3)]Glcβ1- | Glcβ(1-2)Glcβ1- | 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester | 63279-13-0 | $C_{50}H_{80}O_{28}$ |

Glc = glucose; Mol. Wt. = molecular weight.

**Figure B.2.1-2 Schematic Pathways of Enzymatic Conversion of Highly Purified Stevioside and Reb A from Stevia Leaf Extract to Selected Steviol Glycosides**




Reb = rebaudioside; UDP = uridine diphosphate.

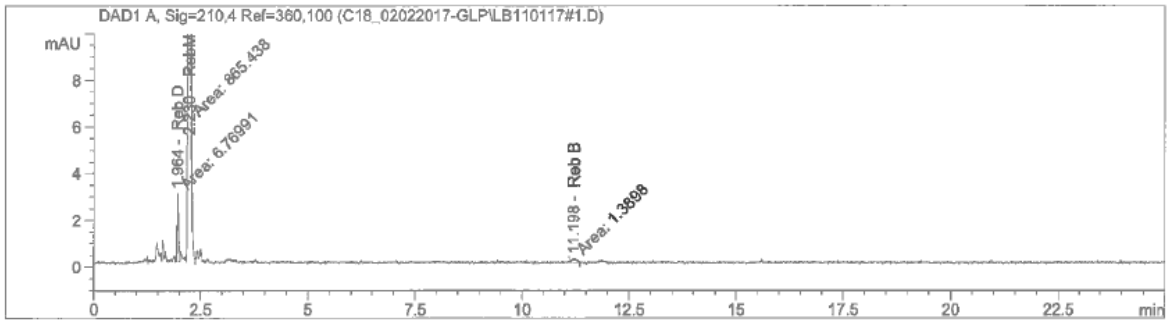
## B.2.2 Equivalence of Steviol Glycosides Produced by Enzymatic Conversion with Steviol Glycosides Extracted from *S. rebaudiana* Bertoni

The presence and specific chemical identity of the individual steviol glycosides present in steviol glycosides with a high reb M and/or reb D content produced by enzymatic conversion of reb A from stevia leaf extract was determined by PureCircle using HPLC. A sample HPLC chromatogram of 1 lot of steviol glycosides with a high reb M and/or reb D content (Lot No. LB110117) is presented in Figure B.2.2-1a. Details of the HPLC method and chromatograms for 2 additional samples (Lot No. LB130117 and LB180117) are available in Appendix E.

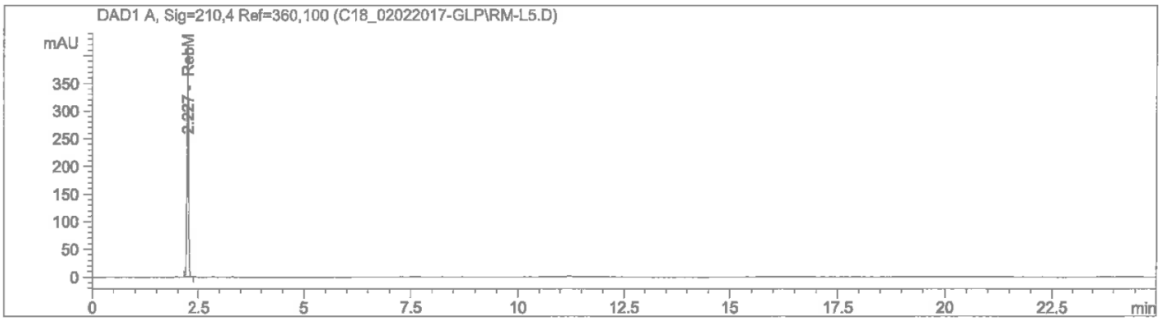
As shown in the figure below, 2 primary peaks were identified in the chromatogram (Lot No. LB110117), corresponding to reb D and reb M, with retention times of 1.964 and 2.230 minutes, respectively. These retention times are comparable to the obtained range of retention times for the standards (extracted from *S. rebaudiana* Bertoni) as follows: reb D, 1.965 to 1.971 minutes; and reb M, 2.225 to 2.231 minutes (see example chromatogram in Figure B.2.2-1b). A minor peak corresponding to reb B was also identified in this sample at 11.198 minutes. These data demonstrate that reb M and reb D produced by enzymatic bioconversion of reb A have the same HPLC retention times as reb M and reb D extracted from the leaves of *S. rebaudiana* Bertoni and establish that steviol glycosides from these 2 sources are chemically identical.

**Figure B.2.2-1 Sample Chromatograms for Steviol Glycosides with a High Reb M and/or Reb D Content Produced by Enzymatic Conversion of Reb A from Stevia Leaf Extract**

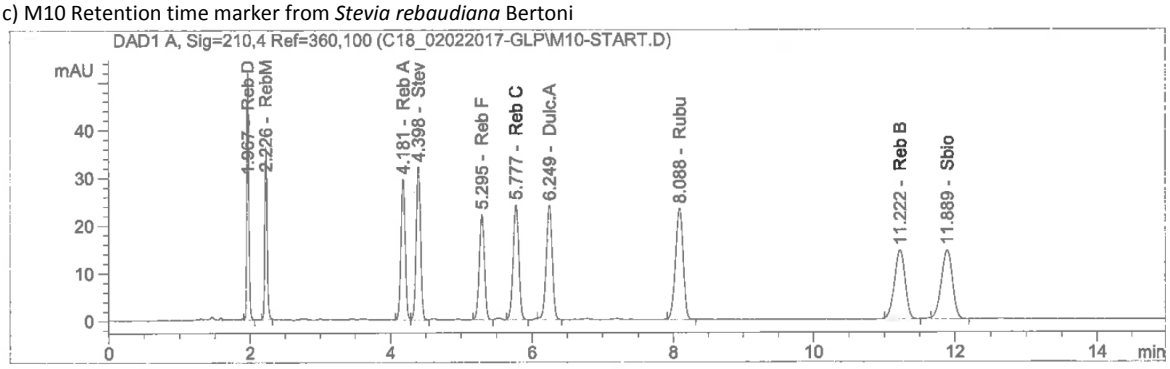
a) HPLC chromatogram for steviol glycosides with a high reb M and/or D content produced by enzymatic bioconversion of reb A (Lot No. LB110117)




b) Reb M standard from *Stevia rebaudiana* Bertoni




c) M10 Retention time marker from *Stevia rebaudiana* Bertoni

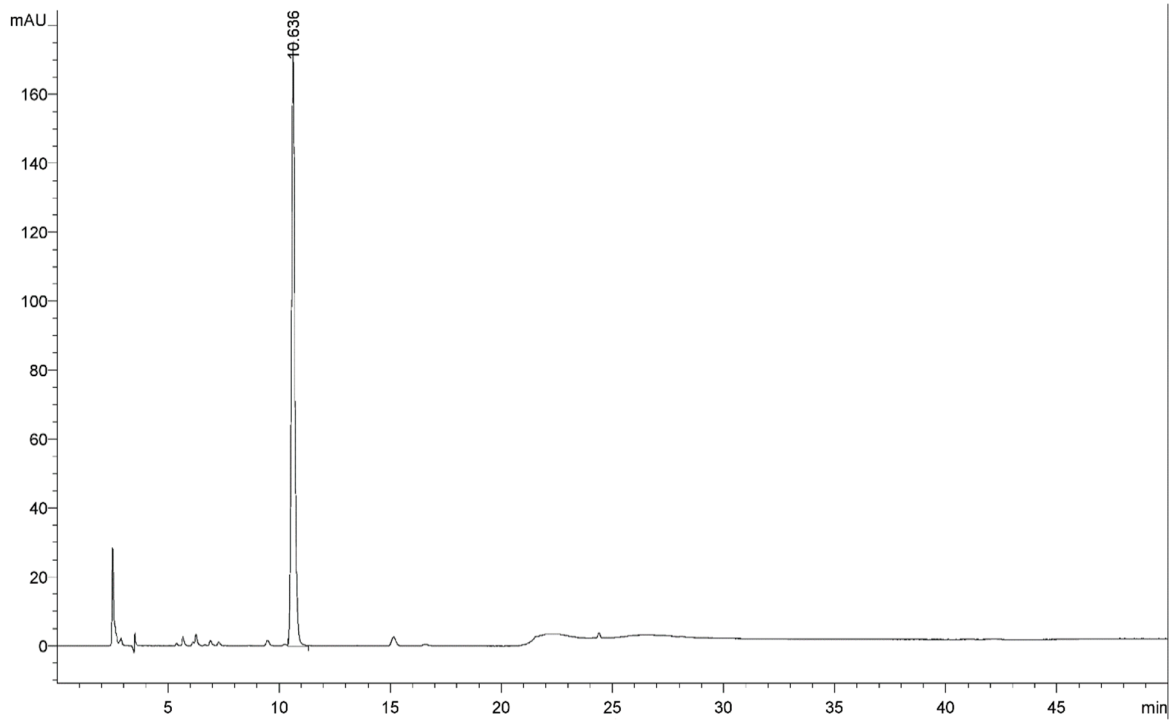



HPLC = high-performance liquid chromatography; Reb = rebaudioside.

## B.2.3 Chromatographic Identity of Reb AM

Reb AM produced by enzymatic conversion of highly purified stevioside (>95%) from stevia leaf extract was analysed by PureCircle using HPLC. Reb AM is a minor steviol glycoside that is also found at very small amounts in stevia leaf extract. Reb AM was analysed by the JECFA HPLC method outlined in the current specifications for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a), and as shown in the figure below, reb AM produced by enzymatic conversion of highly purified stevioside from stevia leaf extract is a high purity product containing 1 primary HPLC peak at 10.636 minutes, which corresponds to reb AM.

**Figure B.2.3-1 HPLC Chromatogram for Reb AM Produced by Enzymatic Conversion of Stevioside (>95%) from Stevia Leaf Extract**




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

## B.3 Information on the Chemical and Physical Properties of Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside from Stevia Leaf Extract

PureCircle's steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are white to off-white powders that have a clean taste with no abnormal of off odour. The preparations are freely soluble in water with a pH of between 4.5 to 7.0 (1% solution), which conforms with the existing specifications for "Steviol glycosides from *Stevia rebaudiana* Bertoni" in Australia and New Zealand. Data supporting the physical properties, particularly for reb AM produced by enzymatic conversion of highly purified stevioside from stevia leaf extract, is considered CCI and is provided in Appendix A.

## B.4 Information on the Impurity Profile

As described in Section B.6.1, PureCircle has established product specifications for steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract 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). Batch analyses showing conformation to the established purity criteria are provided in Section B.6.2. Additional tests have been conducted based on impurities that may arise from the manufacturing process, including pesticide analyses and tests for residual protein and DNA, which are described below.

The starting steviol glycoside materials extracted from the leaves of *S. rebaudiana* Bertoni that are utilised to make reb A and stevioside were subjected to multi-residue pesticide screens that covered a

range of commonly applied pesticides. No pesticide residues were detected in the steviol glycoside starting materials. Example pesticide analysis reports from 5 lots of steviol glycoside starting material extracted from *S. rebaudiana* Bertoni used to prepare reb A and 6 lots of steviol glycoside starting material extracted from *S. rebaudiana* Bertoni used to prepare stevioside are provided in Appendix F.

To confirm the absence of residual proteins in the final steviol glycoside product produced by enzymatic conversion of highly purified stevia leaf extract, steviol glycosides with a high reb M and/or reb D content were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Test samples were dissolved to a concentration of 1,000 ppm, and about 10 µL from each dissolved sample was stained with 3X protein loading dye and loaded onto a precast polyacrylamide gel (10% Mini-PROTEAN® TGX™ Precast Protein Gels, BIORAD). Electrophoresis was conducted at 60 minutes at 130 V and the gel was stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% water for 1 hour. Gels were destained by soaking for 4 hours in a mixture of 10% acetic acid, 50% methanol, and 40% water. If protein is present in the sample, it will be visually detected on the gel (limit of detection = 0.1 µg protein). No visible protein bands have been detected above the limit of detection in any batches of final product, including 4 batches of steviol glycosides with a high reb M and/or reb D content (Reb M Lot Nos. BM050517, SK-B-U2D1, SK-B-U3D1; Reb D Lot No. PTBRD 150218). Certificates of analysis are provided in Appendix F.

The bicinchoninic acid (BCA) assay may also be employed to test the final products for residual protein. The protocol included in the Thermo Scientific Pierce BCA Protein Assay Kit was employed with some minor deviations. Samples were prepared at 1,000 ppm steviol glycosides (1 mg/mL) and the protein content in the solution was measured against the known protein concentration standard (Bovine Serum Albumin 250 µg to 1 mg) provided by the Kit. The assay protocol requires having 1:8 ratio of the sample and working reagents in the final volume (*e.g.,* 125 µL sample: 875 µL working reagent); steviol glycoside concentration in the assay solution is 125 ppm. An incubation for 30 minutes at 37°C was applied to develop the colour. The limit of detection for this assay is 5 ppm. No protein was detected above the limit of detection in 3 non-consecutive lots of steviol glycosides with a high reb M content (Lot Nos. BRM 005-001, BRM 005-002, BRM 005-003), 2 batches of steviol glycosides with a high reb D content (Lot Nos. PTBRD 070818, PTBRD 080818), and 3 batches of steviol glycosides with a high reb AM content (Lot Nos. AM10122018, AM11122018, AM12122018). These data confirm the absence of any residual protein in the final product. The analyses were conducted by an external laboratory and Certificates of Analysis are provided in Appendix F.

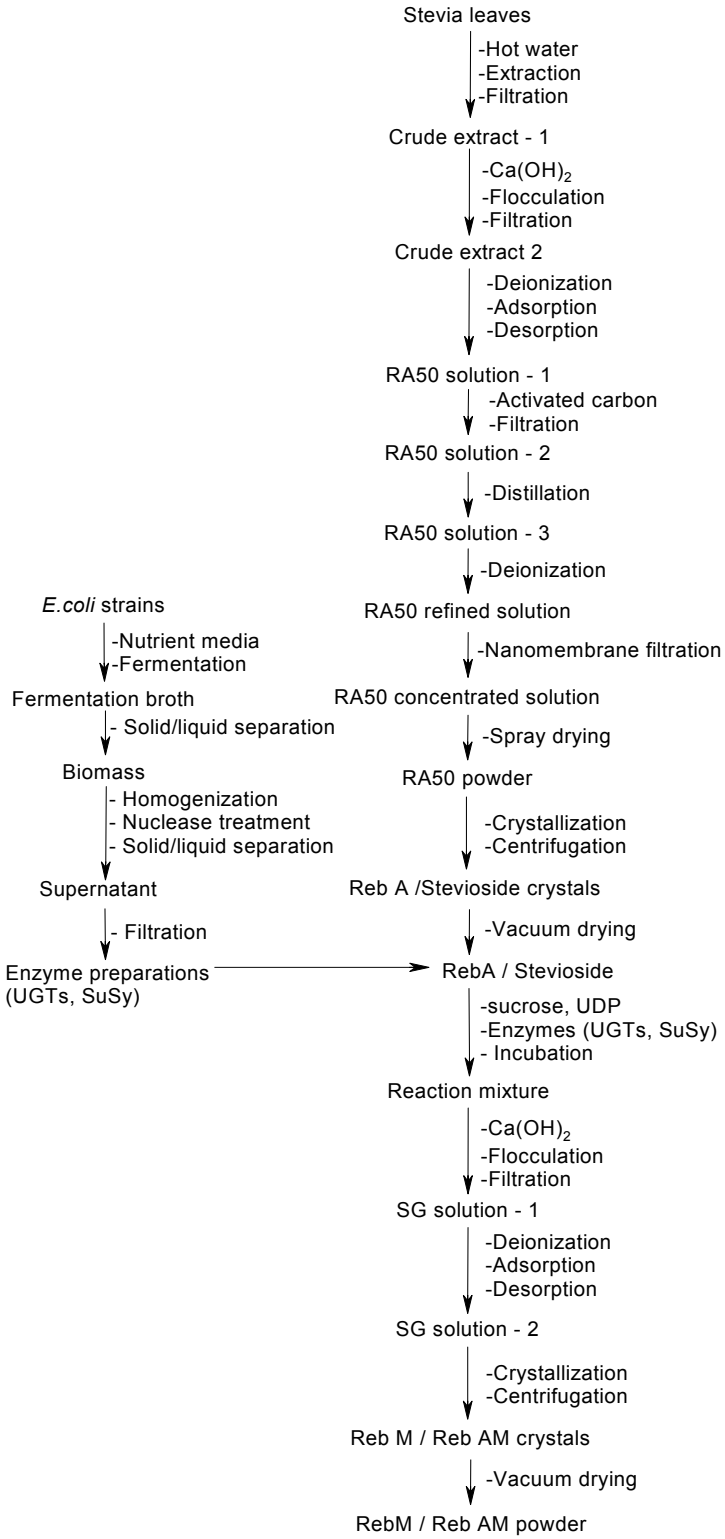
To confirm the absence of residual DNA in the final product, spectrophotometer and polymerase chain reaction (PCR) analytical methods were used. The steviol glycoside sample is extracted using a DNA extraction kit according to manufacturer's protocol. The extracted sample is tested for DNA using a spectrophotometer, which did not detect the presence of DNA in the samples. Additionally, primers were designed for the genes of interest that were inserted into the *E. coli* production organisms. The absence of the gene of interest in extracted samples was confirmed using PCR and a gel imaging system. The thermal profile used for PCR is 2 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C. No PCR products have been detected by the gel imaging system (limit of detection = 0.00002 ng DNA) in any batches of final product, including the same 9 tested lots of steviol glycosides produced by enzymatic conversion of highly purified reb A or stevioside from stevia leaf extract (Reb M Lot No. BM050517, SK-B-U2D1, SK-B-U3D1; Reb D Lot Nos. PTBRD 150218, PTBRD 070818, PTBRD 080818; Reb AM Lot Nos. AM10122018, AM11122018, AM12122018). Certificates of Analysis are provided in Appendix F.

# B.5 Manufacturing Process

## B.5.1 Overview

Selected steviol glycosides are manufactured by enzymatically converting reb A and/or stevioside extracted and purified from the leaves of *S. rebaudiana* Bertoni to reb M, reb D and/or reb AM using UDP-glucosyltransferase and sucrose synthase enzymes derived from strains of genetically modified *E. coli* K-12. A schematic overview of the manufacturing process is illustrated in Figure B.5.1-1. Though Figure B.5.1-1 shows the final product as reb M or reb AM, the end product could also include the intermediate glycoside reb D, depending on the process conditions. The steviol glycoside purification processes utilised prior to and following the enzymatic conversion are consistent with the methodologies for the manufacture of steviol glycosides as described in the Chemical and Technical Assessment (CTA) published by FAO/JECFA (FAO, 2016). Steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are manufactured in a facility certified under Food Safety System Certification 22000:2010.

**Figure B.5.1-1 Schematic Overview of the Manufacturing Process**




RA50 = steviol glycoside mixture containing >50% rebaudioside A; Reb = rebaudioside; SG = steviol glycoside; SuSy = sucrose synthase; UDP = uridine diphosphate; UGT = UDP glucosyltransferase.

## B.5.2 Identity of Raw Materials and Processing Aids

All raw materials, processing aids, and purification equipment used to manufacture steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are food-grade ingredients that are compliant with the specifications set forth in the Food Chemicals Codex (FCC) or equivalent international food or pharmacopeia standards (*e.g.,* JECFA, CODEX, United States Pharmacopeia, European Pharmacopeia). All reagents and materials used to extract and purify reb A and/or stevioside from the leaves of *S. rebaudiana* Bertoni, to produce selected steviol glycosides by enzymatic conversion (*i.e.,* reb M, reb D, reb AM), and to purify the final steviol glycoside products are presented in Table B.5.2-1.

**Table B.5.2-1 Raw Materials, Processing Aids, and Equipment Used in the Manufacturing Process**

| Material | Function |
|---|---|
| *Stevia rebaudiana* Bertoni leaves | Raw material; source of rebaudioside A and/or stevioside |
| Sucrose | Reactant |
| UDP disodium salt (5'-UDP-$Na_2$) | Processing aid |
| UDP-glucosyltransferases and sucrose synthase | Processing aids |
| High-purity calcium hydroxide | Flocculant |
| Ethanol, food-grade | Crystallisation and desorption solvent |
| Activated carbon, food-grade | Decolourising agent |
| Adsorption and ion-exchange resins | Purification |

UDP = uridine diphosphate.

## B.5.3 Details of the Manufacturing Process

In stage 1, *S. rebaudiana* leaves are placed in hot water at 50 to 60°C for 1 to 2 hours in continuous countercurrent extractors. The filtrate is separated using mesh screens, collected in a holding tank, and treated with flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and colouring agents. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionised by ion-exchange resins in ($H^+$) and ($OH^-$) form. The deionised filtrate is fed to a column system packed with macroporous adsorption resin that retains the glycosides. The column is washed with deionised water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. The obtained glycoside solution is treated with activated carbon and the carbon is separated from the solution by plate-and-frame filter press. A standard evaporator is used to remove the ethanol and the resulting aqueous solution is deionised again by ion-exchange resins in ($H^+$) and ($OH^-$) forms. The refined solution is concentrated using a nanofiltration membrane and the concentrated solution is spray dried to yield stevia extract powder containing >50% reb A (RA50). The RA50 powder is further purified by dissolving in aqueous ethanol and incubating at low temperature for several hours to allow for reb A or stevioside to crystallise. The reb A crystals containing >95% reb A or the stevioside crystals contain >95% stevioside are separated by conventional centrifugation and dried in a rotary drum vacuum dryer at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminium foil bags.

In stage 2, the *E. coli* production strains (LE1B109) carrying the expression vectors for the corresponding enzymes are inoculated separately in sterilised culture medium and fermented. The usual fermentation conditions are a pH of between 6 to 8 and a temperature of between 25 to 37°C. The fermentation process is continued until laboratory test data shows the desired enzyme production yield. Usually, after at least 15 hours, the fermentation is stopped. In a subsequent recovery process, the enzyme is isolated from the biomass. In a first solid/liquid separation, the biomass is separated from the culture broth by standard techniques (*e.g.,* is centrifuged and/or filtered). The biomass is homogenised to disrupt the bacterial cells and treated with a nuclease to degrade the DNA/RNA nucleic acids released upon cell disruption. This is followed by solid/liquid separation steps to further remove cell debris and other insoluble matter. The cell-free supernatant is filtered to obtain the purified enzyme preparation. The purified UDP-glucosyltransferase (UGT-Sr and UGT-Sl) and sucrose synthase (SuSy-At) enzymes meet the specifications defined in Tables B.5.4.5-1 to B.5.4.5-3. All raw materials used for fermentation and recovery are of food-grade quality or have been assessed to be fit for their intended use (see Table B.5.3-1). These enzymes are manufactured in accordance with cGMP for food and the principles of Hazard Analysis of Critical Control Points (HACCP).

**Table B.5.3-1 Materials Used for Fermentation of the *Escherichia coli* Enzyme Production Strains**

| Material | Technological Function |
|---|---|
| Glucose | Fermentation nutrient |
| Isopropyl β-D-1-thiogalactopyranoside (IPTG) | Inducer for enzyme expression |
| Defined mineral components | Fermentation nutrients |
| Suitable antifoam agent | Processing aid |
| Nuclease | Processing aid |

In stage 3, the products of stage 1 (purified stevia leaf extract, reb A >95% and/or stevioside >95%) and stage 2 (enzymes, UGT-Sr, UGT-Sl, and SuSy-At) are mixed to initiate the enzymatic conversion process. First, the reb A (>95%) and/or stevioside (>95%) powder and sucrose are dissolved in reverse-osmosis water. Next, 5'-UDP-$Na_2$ and UGT-Sr, UGT-Sl, and SuSy-At enzymes are added to formulate the reaction mixture. The reaction mixture is incubated at 40 to 50°C for 10 to 48 hours. The use of different reaction times yields steviol glycoside mixtures with different ratios of starting glycosides reb A and/or stevioside, intermediate glycosides reb D and/or reb E, and the primary final glycoside reb M and/or reb AM, respectively. The resulting reaction mixture containing a mixture of steviol glycosides is heated to 80 to 100°C for 10 minutes to inactivate the enzymes.

In the last stage of manufacturing, the reaction mixture is treated with a flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and other impurities. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionised by ion-exchange resins in ($H^+$) and ($OH^-$) form. The deionised filtrate is fed to a column system packed with macroporous adsorption resin that retains the reb M, reb AM, and other steviol glycosides. The column is washed with deionised water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. Next, the filtrate is maintained at low temperatures for several hours to allow the steviol glycosides to crystallise. The steviol glycoside crystals are separated by conventional centrifugation and dried in a rotary drum vacuum at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminium foil bags. The bags are placed in high-density polyethylene drums sealed with tamper evident seals.

## B.5.4 Information Regarding the Enzymatic Processing Aids

As described in Section B.5.3 above, UDP-glucosyltransferases (UGT-Sr and UGT-Sl) and sucrose synthase (SuSy-At) are used as enzymatic processing aids to produce reb M, reb D and/or reb AM from highly purified reb A and/or stevioside from stevia leaf exact. The enzymes are derived from 3 *E. coli* K-12 production strains (LE1B109) carrying the expression vectors for the corresponding enzymes. Therefore, information on the enzymes and source organisms is provided in accordance with *Section 3.3.2 – Processing Aids*, subsections A, C, D & E of the *Application Handbook* (FSANZ, 2016a). Information that is considered CCI is presented in Appendix A.

### *B.5.4.1 Information on the Type of Processing Aid*

UDP-glucosyltransferase (EC 2.4.1.X) 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 highly purified reb A and/or stevioside from stevia leaf extract to reb M, reb D, and/or reb AM.

### *B.5.4.2 Information on the Identity of the Enzymes*

**1. UDP-glucosyltransferases**

| | |
|---|---|
| Source (strain): | *E. coli* K-12 production strains (LE1B109) containing DNA sequences encoding UDP-glucosyltransferases (UGT-Sr or UGT-Sl) |
| Common/Accepted Name: | Glucosyltransferase |
| Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB]: | 2.4.1.X |
| Chemical/Systematic Name: | UDP-glucose β-D-glucosyltransferase |
| Chemical Abstracts Service (CAS) Number: | 9030-08-4 |

**2. Sucrose Synthase**

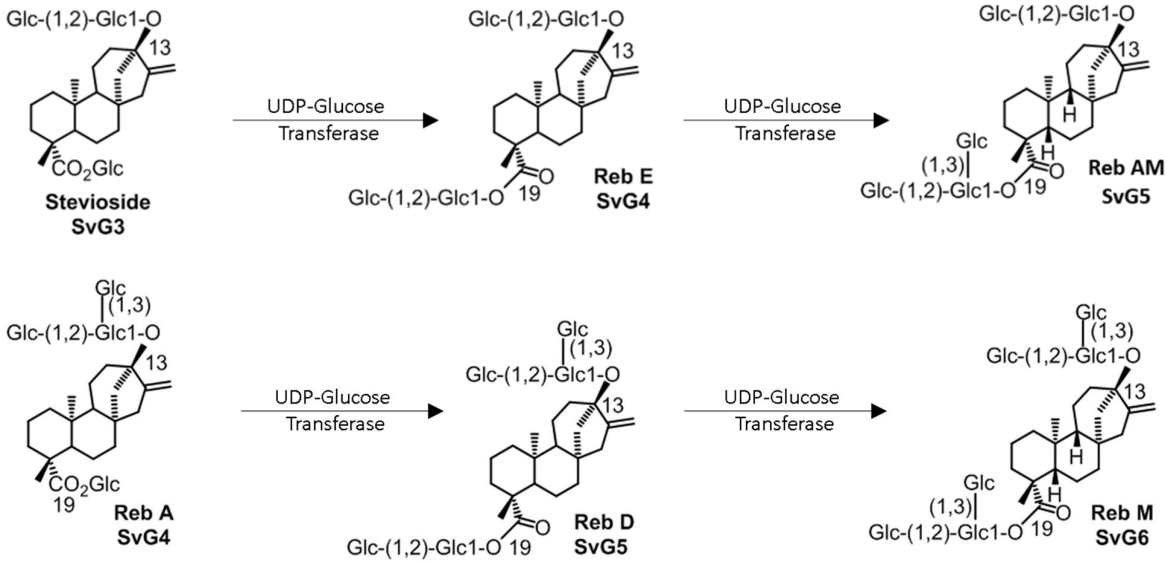
| | |
|---|---|
| Source (strain): | *E. coli* K-12 production strain (LE1B109) containing DNA sequences encoding sucrose synthase (SuSy-At) |
| Common/Accepted Name: | Sucrose synthase |
| Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular 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.5.4.3 Information on the Chemical and Physical Properties of the Enzymes*

Steviol glycosides are synthesised in the *S. rebaudiana* Bertoni plant starting with the backbone molecule steviol. Glucose moieties are added to steviol through the action of UDP-glucosyltransferases that transfer glucose from an activated donor molecule (*e.g.,* UDP-glucose) to the acceptor molecule steviol (Richman *et al.,* 2005). Following the synthesis of steviolmonoside, successive glucose moieties are added, leading to the formation for steviolbioside, and then stevioside *etc. In vitro,* the availability of UDP-glucose for this reaction can be re-generated with the enzyme sucrose synthase that catalyses the conversion of UDP and sucrose to fructose and UDP-glucose (Wang *et al.,* 2015).

The enzymatic conversion pathways of highly purified reb A and stevioside to produce reb M and reb AM, respectively, with intermediate production of reb D and reb E, *via* the catalytic action of UDP-glucosyltransferases (UGT-Sr and UGT-Sl) are shown in the figure below (Markosyan *et al*., 2013, 2018). Sucrose synthase (SuSy-At) is utilised in the reaction to regenerate the glucose source, UDP-glucose.

**Figure B.5.4.3-1 Schematic Pathways of Enzymatic Conversion of Highly Purified Stevioside and Reb A from Stevia Leaf Extract to Selected Steviol Glycosides**




Reb = rebaudioside; UDP = uridine diphosphate.

### *B.5.4.4 Manufacturing Process*

The UGT-Sr, UGT-Sl, and SuSy-At enzymes are produced by microbial fermentation of the *E. coli* K-12 production strains (LE1B109) carrying the expression vectors for the corresponding enzymes. Details of the fermentation conditions and subsequent purification of the enzymes is provided in Section B.5.3 above, stage 2 of the manufacturing process. Information regarding the genetic modifications of the *E. coli* K-12 production strains is considered CCI and is provided in Appendix A.

### *B.5.4.5 Specification for Identity and Purity*

Separate specifications for the 3 enzymes have been established and the analytical results from 3 non-consecutive lots of each enzyme are presented in the tables below (certificates of analysis are provided in Appendix F). These data demonstrate product consistency and the absence of the production microorganism in the final enzyme preparations. Furthermore, the enzymes are free of antibiotics as no antibiotics are used in the manufacturing process. The enzymes are food-grade quality and conform to the recommended purity criteria established by the FCC (FCC, 2018) and JECFA (2006a).

**Table B.5.4.5-1 Product Specifications for Sucrose Synthase SuSy-At**

| Specification Parameter | Specification | Manufacturing Lot | | |
|---|---|---|---|---|
| | | PM2-34-001 | PM-39-001 | PM-40-001 |
| Activity | ≥400 U/mL | 413 | 547 | 512 |
| Total viable count | <50,000 CFU/g | <100 | <100 | <100 |
| *Salmonella* spp. | Absent in 25 g | Conforms | Conforms | Conforms |
| *Escherichia coli* | Absent in 25 g | Conforms | Conforms | Conforms |
| Total coliforms | ≤30 CFU/g | <10 | <10 | <10 |
| Antimicrobial activity | Negative | Negative | Negative | Negative |
| Lead | ≤5 mg/kg | 0.11 | 0.14 | 0.11 |
| TOS (%) | NS | 9.48 | 10.49 | 9.62 |

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

**Table B.5.4.5-2 Product Specifications for UDP-Glucosyltransferase UGT-Sr**

| Specification Parameter | Specification | Manufacturing Lot | | |
|---|---|---|---|---|
| | | FAH-a-U3D1 | FAH-a-U4D1 | FAH3-002 |
| Activity | ≥1 U/mL | 1.22 | 1.66 | 2.00 |
| Total viable count | <50,000 CFU/g | <100 | <100 | <100 |
| *Salmonella* spp. | Absent in 25 g | Conforms | Conforms | Conforms |
| *Escherichia coli* | Absent in 25 g | Conforms | Conforms | Conforms |
| Total coliforms | ≤30 CFU/g | <10 | <10 | <10 |
| Antimicrobial activity | Negative | Negative | Negative | Negative |
| Lead | ≤5 mg/kg | 0.08 | 0.07 | 0.08 |
| TOS (%) | NS | 10.53 | 13.61 | 14.17 |

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

**Table B.5.4.5-3 Product Specifications for UDP-Glucosyltransferase UGT-Sl**

| Specification Parameter | Specification | Manufacturing Lot | | |
|---|---|---|---|---|
| | | SK4-14-001 | SK4-18-001 | SK4-19-001 |
| Activity | ≥7 U/mL | 9.6 | 12.0 | 9.2 |
| Total viable count | <50,000 CFU/g | <100 | <100 | <100 |
| *Salmonella* spp. | Absent in 25 g | Conforms | Conforms | Conforms |
| *Escherichia coli* | Absent in 25 g | Conforms | Conforms | Conforms |
| Total coliforms | ≤30 CFU/g | <10 | <10 | <10 |
| Antimicrobial activity | Negative | Negative | Negative | Negative |
| Lead | ≤5 mg/kg | 0.12 | 0.06 | 0.09 |
| TOS (%) | NS | 10.47 | 13.47 | 11.41 |

CFU = colony forming units; NS = not specified; TOS = total organic solids; U = units (1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0).

### *B.5.4.6 Analytical Method for Detection*

Not required for enzymatic processing aids.

### *B.5.4.7 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 PureCircle to produce selected steviol glycosides by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract. PureCircle's selected steviol glycosides by enzymatic conversion from purified stevia leaf extract are currently manufactured outside of Australia/New Zealand. Furthermore, steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract, using these same enzymes, was determined by an Expert Panel to be GRAS for use in the U.S. as a general purpose sweetener, which was subsequently notified to the U.S. FDA as GRN 744 (PureCircle Limited, 2017). The U.S. FDA raised no objections regarding the GRAS status of PureCircle's steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract for use as general purpose sweeteners in foods (U.S. FDA, 2018).

### *B.5.4.8 Information on the Potential Toxicity of the Enzymes*

The UDP-glucosyltransferase and sucrose synthase enzymes that are introduced into the *E. coli* K-12 recipient strain are derived from plants, including *S. rebaudiana* Bertoni, that have not been associated with any known toxicity. The exact identity of the enzymes is considered CCI and is provided in Appendix A. Despite the known safety of the enzymes, several steps are included in the manufacturing process to inactivate and remove the enzymes, including heating, treatment with flocculant, filtration, ion exchange and adsorption resin purification. Furthermore, using both SDS-PAGE and BCA assay analyses, no protein was detected in over 9 non-consecutive lots of selected steviol glycosides produced by enzymatic conversion of highly purified reb A or stevioside from stevia leaf extract.

To confirm that the UGT-Sr, UGT-Sl, and SuSy-At enzymes are not associated with any toxic potential, the Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information (NCBI) was used to conduct sequence alignment queries of the full-length FASTA protein sequences with curated databases maintained by UniProt containing (i) venom proteins and toxins (UniProtKB/Swiss-Prot Tox-Prot ); and (ii) virulence factors (UniProtKB/Swiss-Prot/TrEMBL). The full search report is considered CCI and is provided in Appendix A. The BLAST searches identified sequence matches with 22 to 64% identity with various animal venom proteins and toxins and virulence factors and associated E-values ranging from 0.00005 to 7.9 and therefore are not considered to share structural homology or similarity (Pearson, 2000). Furthermore, matches were generally less than 50% identical over an approximately 20 amino acid region and are considered to occur by chance and do not indicate structural homology (Pearson, 1996). Sequences with alignments less than 20% identity over a 100 amino acid region are not considered homologs (Hammond *et al.*, 2013). Thus, based on the available data it is anticipated that the UGT-Sr, UGT-Sl, and SuSy-At enzymes are not homologs of any animal venom protein or toxins or virulence factors. It is understood that the amino acid sequence of the enzyme is an important determinant of the 3-dimensional structure and motif which dictate the toxic function of the protein (Dunker *et al.*, 2008; Hammond *et al.*, 2013; Negi *et al.*, 2017). Given the low structural homology between UGT-Sr, UGT-Sl, and SuSy-At with known animal venom proteins and toxins and virulence factors, it is expected that these enzymes do not share the protein domains necessary for toxic function. Evolutionary changes resulting in amino acid substitutions are conservative in which the stability of the protein is maintained; as such, enzymes retain the 3-dimensional structure and functional characteristics of the enzyme family from which they were derived and exhibit similar variation in amino acids than what occurs through natural sequence variation (Pariza and Cook, 2010; Hammond *et al.*, 2013). As confirmed by bioinformatics analysis using the amino acid sequences of UGT-Sr, UGT-Sl, and SuSy-At, the amino acid sequences do not share structural homology to known

protein toxins. Therefore, no toxic potential is anticipated with the use of these enzymes in the production of selected steviol glycosides by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract.

### *B.5.4.9 Information on the Potential Allergenicity of the Enzymes*

In order to confirm the lack of potential for cross-reactivity among the inserted heterologous gene sequences in the production strain, 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 23 March 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2018). This was done to determine whether the UGT-Sr, UGT-Sl, and SuSy-At enzymes contain amino acid sequences similar to other known allergens that might produce an allergenic response. The database contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. The full report is considered CCI and is provided in Appendix A.

A FASTA 35.04 overall search of AllergenOnline was conducted using default settings (E-value/score cut-off = 1 and maximum alignments of 20). Several matches to known allergen sequences were identified for all 3 enzyme sequences. E values/scores for these matches were between 0.13 and 0.76, indicating that these alignments are not significant as E-values/scores larger than $1 \times 10^{-7}$ are unlikely to identify proteins that may share immunologic or allergic cross-reactivity to known allergens (Hileman *et al.*, 2002). Additionally, none of these sequences shared greater than 50% identity with the identified allergens, indicating the unlikely potential for cross-reactivity to these allergens (Aalberse, 2000).

Additionally, in accordance with the Codex Alimentarius criterion for use in flagging proteins that might be of some concern of cross-reactivity for genetically engineered plants, an 80-amino acid sliding window (segments 1–80, 2–81, 3–82, *etc*.) was used to scan the amino acid sequence of each heterologous gene inserted in the production strain against the allergen database using FASTA to search for matches of >35% identity (Codex Alimentarius, 2003, 2009). No matches greater than 35% were identified among the 80-mer sliding windows in comparison to known allergens.

### *B.5.4.10 Information on the Source Microorganism*

*E. coli* K-12 substrain W3110 was used as the parental microorganism to construct the source microorganisms for the UDP-glucosyltransferases and sucrose synthase. Further details regarding the origins of the source microorganism are considered CCI and are provided in Appendix A. *E. coli* K-12 belongs to the Enterobacteriaceae family and its taxonomic identity is presented in Table B.5.4.10-1.

**Table B.5.4.10-1 Taxonomic Identity of *Escherichia coli* K-12**

| | |
|---|---|
| **Kingdom** | Bacteria |
| **Phylum** | Proteobacteria |
| **Class** | Gammaproteobacteria |
| **Order** | Enterobacteriales |
| **Family** | Enterobacteriaceae |
| **Genus** | *Escherichia* |
| **Species** | *Escherichia coli* |
| **Strain** | K-12 |
| **Substrain** | W3110 |

*E. coli* K-12 substrain W3110 has a long history of safe use in the production of enzymes used in the food industry, has been safely used as a laboratory organism for more than 50 years, and is one of the most extensively characterised bacteria (Bachmann, 1972; Jensen, 1993). For example, in Australia and New Zealand, the food enzyme chymosin (EC 3.4.23.4) derived from *E. coli* K-12 strain GE81 is listed in *Schedule 18 – Processing Aids* as a permitted processing aid (FSANZ, 2019b). Likewise, chymosin obtained from a genetically modified *E. coli* K-12 strain was affirmed as GRAS by the U.S. FDA in 1990 (Flamm, 1991; Olempska-Beer *et al.*, 2006) and has been used safely for cheese production worldwide. Also, in the U.S., D-allulose 3-epimerase has recently been the subject of a GRAS notification, receiving no questions from the U.S. FDA (U.S. FDA, 2016). In the EU, 2 different cyclomaltodextrin glucotransferases derived from *E. coli* K-12 have been safely used for years in the production of the novel food ingredients alpha- and gamma-cyclodextrin, authorised for use by the European Commission in 2008 and 2012, respectively. Furthermore, *E. coli* K-12 also has a long history of safe use in the industrial production of specialty chemicals and human drugs (U.S. EPA, 1997).

### *B.5.4.11 Pathogenicity and Toxicity of the Source Microorganism*

The parental organism used to generate the enzymes, *E. coli* K-12 substrain W3110, is non-pathogenic and non-toxicogenic and has a long history of safe use in the production of enzymes used in the food industry. As indicated previously, the UDP-glucosyltransferase and sucrose synthase enzymes that are introduced into the *E. coli* K-12 recipient strain are derived from plants, including *S. rebaudiana* Bertoni, that have not been associated with any known toxicity. The *E. coli* LE1B109 production organism is a biosafety level 1. Specifications that have been established for the 3 enzymatic processing aids ensure that the production microorganism is absent from the final enzyme preparations (*i.e., E. coli* must be absent in 25 g), as confirmed by the analytical results of3 non-consecutive lots for each enzyme (see Section B.5.4.5). Furthermore, using PCR analysis, no DNA was detected in 9 non-consecutive lots of selected steviol glycosides produced by enzymatic conversion of highly purified reb A or stevioside from stevia leaf extract. Likewise, standard microbiological impurity testing for *E. coli* is conducted on all lots of final product and was demonstrated to be absent (MPN/g) in the 9 non-consecutive lots (see Table B.6.2.1-1), further confirming that the production strain is not present in the final product.

### *B.5.4.12 Genetic Stability of the Source Microorganism*

Information on the genetic stability of the source microorganism is considered CCI and is provided in Appendix A.

### *B.5.4.13 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 parental organism to generate the source organisms expressing the UDP-glucosyltransferases and sucrose synthase is considered CCI and is provided in Appendix A.

# B.6 Specification for Identity and Purity of Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside From Stevia Leaf Extract

## B.6.1 Product Specifications for Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside From Stevia Leaf Extract

Product specifications for steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract, as defined in S3—35 of Schedule 3, are outlined in Table B.6.1-1. All methods of analysis are internationally-recognised methods.

**Table B.6.1-1 Product Specifications for Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside From Stevia Leaf Extract**

| Specification Parameters | FSANZ (Steviol glycosides from *S. rebaudiana* Bertoni, S3-35) | JECFA (Steviol glycosides from *S. rebaudiana* Bertoni) | PureCircle's Steviol Glycosides from Enzymatic Bioconversion of Stevia Extract | PureCircle's Method of Analysis |
|---|---|---|---|---|
| Appearance | Powder | Powder | Powder | Sensory evaluation |
| Colour | White to light yellow | White to light yellow | White to light yellow | Sensory evaluation |
| Purity | ≥95%total steviol glycosides | ≥95%total steviol glycosides | ≥95% total steviol glycosides | HPLC |
| Solubility | Freely soluble in water | Freely soluble in a mixture of ethanol and water (50:50) | Freely soluble in water | JECFA Vol. 4 |
| pH (1% solution) | 4.5 to 7.0 | 4.5 to 7.0 | 4.5 to 7.0 | JECFA Vol. 4 |
| Total ash | ≤1% | ≤1% | ≤1% | AOAC 945.46 |
| Loss on drying | ≤6% | ≤6% | ≤6% | JECFA Vol. 4 |
| Residual methanol | ≤200 mg/kg | ≤200 mg/kg | ≤200 mg/kg | USP 467 |
| Residual ethanol | ≤5,000 mg/kg | ≤5,000 mg/kg | ≤5,000 mg/kg | USP 467 |
| Arsenic | ≤1 mg/kg | ≤1 mg/kg | ≤1 mg/kg | AOAC 993.14 |
| Lead | ≤1 mg/kg | ≤1 mg/kg | ≤1 mg/kg | AOAC 993.14 |
| Cadmium | ≤1 mg/kg | Not specified | ≤1 mg/kg | AOAC 993.14 |
| Mercury | ≤1 mg/kg | Not specified | ≤1 mg/kg | AOAC 993.14 |
| Total plate count | Not specified | ≤1,000 CFU/g | ≤1,000 CFU/g | AOAC 966.23 |
| Yeast and moulds | Not specified | ≤200 CFU/g | ≤200 CFU/g | AS 1766.2.2 |
| *Escherichia coli* | Not specified | Negative in 1 g | Negative in 1 g | ISO 7251 |
| *Salmonella* spp. | Not specified | Negative in 25 g | Negative in 25 g | ISO 6579 |

AOAC = Association of Official Analytical Chemists; CFU = colony forming units; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; 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*

A total of 9 non-consecutive lots of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract were analysed for conformance with the product specifications. The results are summarised in Table B.6.2.1-1 and certificates are available in Appendix F.

**Table B.6.2.1-1 Results of Batch Analyses for 9 Non-Consecutive Lots of Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside From Stevia Leaf Extract**

| **Current Specifications for Steviol Glycosides** | | **Reb M Manufacturing Lot** | | | **Reb D Manufacturing Lot** | | | **Reb AM Manufacturing Lot** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | **Limit** | **BM050517** | **SK-B-U2D1** | **SK-B-U3D1** | **PTBRD 150218** | **PTBRD 070818** | **PTBRD 080818** | **AM 10122018** | **AM 11122018** | **AM 12122018** |
| Appearance | Powder | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms |
| Colour | White to light yellow | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms | Conforms |
| Purity | ≥95%total steviol glycosides | 97.97 | 96.37 | 96.27 | 97.78 | 98.56 | 97.58 | 99.19 | 99.07 | 98.78 |
| Solubility | Freely soluble in water | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass | Pass |
| pH (1% solution) | 4.5 to 7.0 | 6.32 | 5.99 | 5.89 | 6.32 | 6.63 | 6.61 | 5.87 | 5.95 | 5.88 |
| Total ash | ≤1% | 0.05 | <0.005 | 0.02 | <0.005 | 0..001 | <0.005 | <0.005 | <0.005 | <0.005 |
| Loss on drying | ≤6% (105°C, 2h) | 1.64 | 1.64 | 3.85 | 2.80 | 1.50 | 1.94 | 3.80 | 4.46 | 3.23 |
| Residual methanol | ≤200 mg/kg | ND | 10 | 10 | 10 | 10 | 10 | 20 | 20 | 20 |
| Residual ethanol | ≤5,000 mg/kg | 410 | 1,340 | 1,330 | 3,420 | 770 | 710 | 30 | 20 | 20 |
| Arsenic | <1 mg/kg | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | 0.005 | <0.005 |
| Lead | <1 mg/kg | 0.021 | 0.035 | 0.038 | 0.012 | 0.012 | 0.012 | 0.015 | 0.011 | 0.010 |
| Cadmium | <1 mg/kg | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| Mercury | <1 mg/kg | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 | <0.005 |
| Total plate count | <1,000 CFU/g | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Yeast and moulds | <200 CFU/g | ND | ND | ND | ND | 40 | ND | ND | ND | ND |
| *Escherichia coli* | Negative in 1 g | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |
| *Salmonella* spp. | Negative in 25 g | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent |

CFU = colony forming units; h = hour(s); ND = not detected; Reb = rebaudioside.

## B.7 Information for Food Labelling

Under *Schedule 3*, steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract would be classified as steviol glycosides, with INS number 960. When added to food products, steviol glycosides are classified as intense sweeteners and flavour enhancers. The food labelling of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract will reflect the labelling of current steviol glycoside preparations [*i.e.,* sweetener (960) or sweetener (steviol glycosides)].

## B.8 Analytical Method for Detection

The analytical methods used to ensure that PureCircle's steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract meet the purity specifications established in Australia and New Zealand for steviol glycosides (Section B.6.1) are internationally recognised [*e.g.*, Association of Analytical Communities (AOAC), U.S. Pharmacopeia (USP), JECFA]. The steviol glycoside purity of the final product is measured using the JECFA HPLC method for steviol glycosides described in FAO JECFA Monograph 20 for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2017a).

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

Pure Circle's steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract may be used to replace the sweetness provided by sugar in food products. Since it is a low-energy sweetener product, steviol glycosides may be used by consumers as a low-calorie alternative to sugar, which may in turn assist with weight loss and/or managing diabetes.

# C. INFORMATION RELATED TO THE SAFETY OF THE FOOD ADDITIVE

In accordance with *Section 3.3.1 – Food Additives* of the FSANZ *Application Handbook* (FSANZ, 2016a) the following safety information is 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, its 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 FSANZ *Application Handbook* (FSANZ, 2016a) 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 were pivotal to establishing the safety of steviol glycosides are outlined below.

## C.1 Introduction

The safety of steviol glycosides has already been thoroughly investigated by several advisory scientific bodies including FSANZ, JECFA, EFSA, and Health Canada and it has been concluded that steviol glycosides, as a group of substances, share a similar structure (*i.e.*, glycosylated derivatives of the aglycone steviol with various sugar units attached at the $R_1$ and $R_2$ positions), and that these substances undergo a common metabolic pathway following ingestion (*i.e.*, hydrolysation in the large intestine to steviol followed by absorption, glucuronidation, and elimination). The toxicological conclusions drawn by the agencies during the initial evaluations of steviol glycosides were based on the fact that steviol was the only compound available systemically. As such, safety studies conducted with an individual steviol glycoside can extend to other steviol glycosides due to the shared metabolic fate.

Information related to the safety of steviol glycosides as previously evaluated by FSANZ and other agencies will not be re-evaluated in the sections below; however, any new information that has become available since the 2018 FSANZ evaluation of a similar application (A1157 – FSANZ, 2018) that included a current assessment of the safety of steviol glycosides is summarised and discussed in the sections that follow. Considering, however, the importance of the metabolism of individual steviol glycosides in the assessment of their safety, a detailed overview of the general metabolic fate of steviol glycosides is included in Section C.2.

In order to identify any new data related to the safety of steviol glycosides published since the most recent evaluation of the safety of steviol glycosides by FSANZ, an updated search of the scientific literature was conducted on 29 January 2019 (*i.e.*, studies published between 01 January 2018 and the date of the search). 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®. Summaries of the studies deemed relevant to the safety of steviol glycosides are provided in the sections that follow.

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

*In vitro* and *ex vivo* studies have demonstrated that steviol glycosides are not hydrolysed by digestive enzymes of the upper gastrointestinal tract due to the presence of β-glycosidic bonds and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea *et al*., 1997; Koyama *et al*., 2003a; Geuns *et al*., 2003, 2007). Therefore, steviol glycosides enter the colon intact, where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol (Gardana *et al*., 2003; Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon, reviewed extensively by Renwick and Tarka (2008), have confirmed the ability of gut microflora from mice, rats, hamsters, and humans to hydrolyse steviol glycosides completely to steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Nikiforov *et al.*, 2013; Purkayastha *et al.,* 2016).

Steviol glycosides are hydrolysed sequentially, removing 1 sugar moiety at a time, with differences in the degradation rates depending on the structural complexities of each steviol glycoside (Wingard *et al*., 1980; Koyama *et al*., 2003b). Stevioside, for example, is degraded to steviolbioside, steviolmonoside, and finally to steviol, with glucose released with each sequential hydrolysis, whereas reb A is first converted to either stevioside (major pathway) or reb B (minor pathway) prior to being ultimately degraded to steviol (Nakayama *et al*., 1986; Gardana *et al*., 2003; Koyama *et al*., 2003b). Despite these structural differences, several parallel *in vitro* comparisons between reb A and various individual steviol glycosides have demonstrated a remarkable similarity with respect to the rate of hydrolysis of different steviol glycosides to steviol in the presence of human faecal homogenates, particularly during the first

24 hours of incubation (Purkayastha *et al.,* 2014, 2015, 2016). For example, reb M and reb A (0.2 mg/mL) were incubated with human faecal homogenates samples at 37°C for up to 24 hours under anaerobic conditions, and by 16 hours both compounds were reported to be completely metabolised to steviol (Purkayastha *et al.,* 2016). These experiments demonstrate that steviol glycosides are metabolised by human faecal homogenates to steviol at generally similar hydrolysis rates, indicating that the number and location of sugar units attached to the steviol backbone does not significantly affect the rate of hydrolysis.

Steviol is absorbed systemically into the portal vein and distributed to a number of organs and tissues, including the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Koyama *et al*., 2003b; Wang *et al*., 2004; Roberts and Renwick, 2008). In the liver, steviol is conjugated to glucuronic acid to form steviol glucuronide. In rats, free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) were identified in the plasma 8 hours after oral administration with either reb A or stevioside (Roberts and Renwick, 2008). Similarly, in humans steviol glucuronide was detected in the plasma following ingestion of stevioside or reb A, with maximal concentrations detected 8 and 12 hours after administration, respectively (Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The toxicokinetic/pharmacokinetic differences of steviol and steviol glucuronide were recently examined in rats and humans by Roberts *et al.* (2016) following administration of stevioside (40 mg/kg body weight). Peak plasma concentrations ($C_{max}$) of steviol were similar in both rats and humans but were slightly delayed in humans compared to rats. Similarly, $C_{max}$ values for steviol glucuronide were also delayed in humans but were approximately 25-fold higher in humans than rats. Systemic exposure to steviol and steviol glucuronide based on the area under the curve ($AUC_{0\text{-}72h}$) was reported to be 2.8-fold and 57-fold greater in humans, when compared to rats, respectively. These data show that the extent of conjugation of steviol to glucuronic acid is higher in humans than in rats.

In rats, free and conjugated steviol, as well as any un-hydrolysed fraction of the administered glycosides, are excreted primarily in the faeces *via* the bile (generally within 48 hours), with smaller amounts appearing in the urine (less than 3%) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002 [unpublished]; Roberts and Renwick, 2008). In contrast, steviol glycosides are excreted in humans primarily as steviol glucuronide *via* the urine, along with very small amounts of the unchanged glycoside or steviol. Relative to amounts recovered in urine, larger amounts of steviol (unabsorbed steviol released from steviol glycosides in the colon or from small amounts of steviol glucuronide secreted back into the gut *via* the bile) were also eliminated in the faeces in humans (Kraemer and Maurer, 1994; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). The inter-species difference in the route of elimination of systemically absorbed steviol as steviol glucuronide occurs as a result of the lower molecular weight threshold for biliary excretion in rats (325 Da) as compared to humans (500 to 600 Da; molecular weight of steviol glucuronide is 495 Da) (Renwick, 2007). The difference in the route of elimination is considered to be of no toxicological significance due to the fact that the water-soluble phase II metabolites are rapidly cleared in both species. Therefore, toxicology data generated in rats are considered applicable to the assessment of the safety of steviol glycosides in humans given the similarities in metabolic fate.

In summary, with the exception of having different numbers and types of sugar moieties, steviol glycosides share the same structural backbone, steviol. Steviol glycosides pass undigested through the upper portion of the gastrointestinal tract and enter the colon intact where they are subject to microbial degradation by members of the *Bacteroidaceae* family, resulting in the release of the aglycone steviol. This common metabolite steviol is absorbed systemically, conjugated to glucuronic acid, and eliminated primarily *via* the urine in humans. Numerous *in vitro* studies have demonstrated that steviol glycosides have very similar rates of microbial hydrolysis in the gastrointestinal tract, despite differences in the number of sugar units attached to the steviol backbone. Therefore, the safety database that has been

established for individual steviol glycosides (*e.g.,* stevioside, reb A, reb D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless of the steviol glycoside distribution of the preparation or the manufacturing process employed, including steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract.

### C.2.1 Data Supporting the Common Metabolic Fate of Steviol Glycosides Produced by Enzymatic Conversion

To demonstrate that steviol glycosides produced by enzymatic conversion of reb A or stevioside from stevia leaf undergo a similar metabolic fate as steviol glycosides extracted from *S. rebaudiana* Bertoni, detailed microbial metabolism studies were conducted *in vitro* (see Kwok, 2018 [unpublished] and Kwok, 2019 [unpublished] for full study reports in Appendix A). Human faecal homogenate samples were prepared based on the pooling of faecal samples from 6 healthy male and 6 healthy female volunteers. In the first test, a steviol glycoside preparation with a high reb D content (Lot No. PTBRD 150218) and a steviol glycoside preparation with high reb M content (Lot No. SK-B-U3D1), both produced by enzymatic conversion of reb A from stevia leaf extract, were mixed in equal parts and incubated in 3 (n=2 pooled) adult male and adult female pooled faecal homogenate samples at concentrations of 0.20 mg/mL under anaerobic conditions at 37°C for between 4 to 72 hours in triplicate. In the second test, reb AM (Lot No. PT170418) produced by enzymatic conversion of stevioside (>95%) from stevia leaf extract was mixed with other minor glycosides from the stevia leaf (*i.e.,* rebaudiosides W2, Y, U2, V, N, and O) at comparable concentrations and incubated in 3 (n=2 pooled) adult male and adult female pooled faecal homogenate samples at concentrations of 0.20 mg/mL and 0.40 mg/mL under anaerobic conditions at 37°C for between 4 to 48 hours in triplicate. To demonstrate the complete metabolic hydrolysis of reb D (Lot No. PTBRD 150218), reb M (Lot No. SK-B-U3D1), and reb AM (Lot No. PT170418) produced by enzymatic conversion, the disappearance of reb D, reb M, and reb AM over time was assayed using an established liquid chromatography-mass spectrometry (LC/MS) method. In addition, a LC/MS assay of steviol was performed to provide metabolic mass balance on the molar equivalent formation of the steviol metabolite over the 48- or 72-hour time-course. The expected molar equivalent steviol metabolite in the enzymatic conversion reb D, reb M, and reb AM samples/mixtures was determined based on the molecular weight and relative abundance of each steviol glycoside component to provide a total molar equivalent steviol metabolite in the incubation sample. Reb A from *S. rebaudiana* Bertoni, a steviol glycoside known to be completely metabolised to steviol in the presence of human faecal homogenates, was studied as a metabolic activity positive control in parallel compare the rate and degree of hydrolysis of the steviol glycoside material produced *via* enzymatic conversion.

A summary of the mean reb D and reb M remaining and the mean steviol metabolite concentrations formed in adult male and adult female faecal homogenates is presented in Table C.2.1-1 and the full study report is provided in Kwok (2018 [unpublished]). Likewise, a summary of the mean reb AM and other minor glycosides remaining (0.4 mg/mL test concentration) and the mean steviol metabolite concentrations formed in adult male and adult female faecal homogenates is presented in Table C.2.1-2 and the full study report is provided in Kwok (2019 [unpublished]). For comparison, the data for reb A is presented in Table C.2.1-3. These data indicate rapid and near complete deglycosylation of reb D, reb M, and reb A produced by enzymatic conversion within an incubation period of 12 hours in pooled faecal homogenates, with 4.6% or 4.5%, 2.9% or 2.7%, and 2.1% or 1.1% of reb D, M, and AM remaining after 12 hours in male or female samples, respectively. This is in agreement with near complete deglycosylation at the 12-hour time point when the total molar equivalent steviol metabolite was observed at a mean of 97.4 ± 4.3% in male samples and 98.5 ± 6.2% in female samples for the reb D and reb M test mixture. Similarly, for the reb AM test sample, at 12 hours 60.5 ± 15.6% and 67.7 ± 8.1% total steviol molar equivalent was formed in male and female samples, respectively, and at 24 hours 103 ± 13.5% and 95.4 ± 3.8% total steviol molar equivalent was formed. The results for the positive control reb A material provided a similar rate and overall degree of hydrolysis to the steviol glycoside

materials produced *via* enzymatic conversion. Overall, these data demonstrate clearly that reb D, reb M, and reb AM produced by enzymatic conversion in the presence of human faecal homogenates are metabolised completely to steviol within 12 hours and confirm that steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract undergo an almost identical rate and degree of hydrolysis as steviol glycosides from *S. rebaudiana* Bertoni. The similarity in the degree and rate of hydrolysis of the steviol glycoside materials produced *via* enzymatic conversion further demonstrates there will be a similar rate and level of absorption of steviol, leading to the conclusion that the overall pharmacokinetics of the products will be identical leading to a lack of concern regarding differences in exposure and elimination. Furthermore, the overall similarity in the metabolic profile of steviol glycosides produced by enzymatic conversion and steviol glycosides extracted from *S. rebaudiana* Bertoni indicates the acceptability to be able to bridge to the steviol glycoside safety data base thereby underpinning the generalisability of the safety of steviol glycosides produced by enzymatic conversion.

**Table C.2.1-1 Incubation of Steviol Glycosides with a High Reb M and Reb D Content (Equal Parts of Lot No. PTBRD 150218 and SK-B-U3D1) Produced by Enzymatic Conversion from Reb A in Pooled Faecal Homogenates**

| Subjects | Human Faecal Homogenate Incubation Time (h) | Mean Reb M Conc (µg/mL) | SD | Mean % Reb M Remaining Relative to T=0 | SD | Mean Reb D Conc (µg/mL) | SD | Mean % Reb D Remaining Relative to T=0 | SD | Mean Steviol Conc (µg/mL) | SD | Mean % Steviol Metabolite Formed | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0.0 | 116 | 10.7 | 100 | NA | 122 | 9.12 | 100 | NA | 0.00 | 0.00 | 0.0 | 0 |
| | 4.0 | 76.1 | 18.2 | 65.5 | 17.0 | 22.6 | 10.3 | 18.6 | 9.7 | 6.41 | 1.93 | 10.1 | 3.5 |
| | 8.0 | 10.8 | 2.58 | 9.3 | 2.5 | 4.65 | 0.771 | 3.8 | 0.7 | 52.8 | 6.84 | 83.0 | 10.4 |
| | 12.0 | 5.39 | 0.369 | 4.6 | 0.4 | 3.24 | 0.049 | 2.7 | 0.0 | 62.1 | 4.56 | 97.4 | 4.3 |
| | 16.0 | 5.93 | 1.56 | 5.1 | 1.5 | 3.07 | 0.413 | 2.5 | 0.4 | 57.8 | 5.39 | 90.7 | 8.2 |
| | 24.0 | 5.21 | 1.27 | 4.5 | 1.3 | 2.78 | 0.230 | 2.3 | 0.2 | 61.4 | 2.89 | 96.5 | 1.0 |
| | 48.0 | 3.71 | 0.647 | 3.4 | 0.6 | 2.83 | 0.089 | 2.3 | 0.0 | 66.1 | 4.80 | 103.8 | 1.4 |
| | 72.0 | 3.83 | 0.710 | 3.3 | 0.7 | 2.92 | 0.075 | 2.4 | 0.0 | 63.2 | 4.96 | 99.3 | 5.0 |
| Female | 0.0 | 113 | 7.49 | 100 | NA | 123 | 8.47 | 100 | NA | 0.00 | 0.00 | 0.0 | 0.0 |
| | 4.0 | 46.5 | 20.8 | 41.1 | 21.0 | 5.28 | 2.65 | 4.3 | 2.5 | 13.8 | 3.85 | 21.7 | 6.8 |
| | 8.0 | 7.27 | 2.32 | 6.4 | 2.4 | 3.34 | 0.086 | 2.7 | 0.1 | 65.3 | 6.78 | 102.6 | 9.9 |
| | 12.0 | 5.13 | 0.768 | 4.5 | 0.8 | 3.62 | 0.668 | 2.9 | 0.6 | 62.7 | 6.10 | 98.5 | 6.2 |
| | 16.0 | 4.81 | 0.665 | 4.3 | 0.7 | 2.70 | 0.090 | 2.2 | 0.1 | 59.8 | 4.76 | 94.0 | 6.7 |
| | 24.0 | 4.32 | 0.036 | 3.8 | 0.03 | 2.65 | 0.092 | 2.2 | 0.04 | 60.0 | 5.65 | 94.3 | 2.7 |
| | 48.0 | 3.28 | 0.111 | 2.9 | 0.09 | 2.89 | 0.163 | 2.4 | 0.15 | 64.2 | 4.37 | 101.4 | 2.2 |
| | 72.0 | 3.16 | 0.048 | 2.8 | 0.02 | 2.79 | 0.073 | 2.3 | 0.02 | 59.2 | 5.48 | 93.0 | 3.4 |

Conc = concentration; h = hour(s); NA = not applicable; Reb = rebaudioside; SD = standard deviation.

**Table C.2.1-2 Incubation of a Steviol Glycoside Mixture Prepared with Minor Steviol Glycosides (Reb AM, Reb W2, Reb Y, Reb U2, Reb V, Reb N, and Reb O) in Pooled Faecal Homogenates at 0.4 mg/mL**

| Subjects | Human Faecal Homogenate Incubation Time (h) | Mean Reb AM Response | SD | Mean % Reb AM Remaining Relative to T=0 | SD | Mean Reb U2 + Reb W2 Response | SD | Mean % Reb U2 + W2 Remaining Relative to T=0 | SD | Reb V + Reb Y Response | SD | Mean % Reb V + Reb Y Remaining Relative to Time 0 | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 18921 | 2478 | 100 | NA | 18026 | 1773 | 100 | NA | 14738 | 1855 | 100 | NA |
| | 4 | 12741 | 1896 | 67.3 | 5.3 | 15114 | 6375 | 83.8 | 13.5 | 11333 | 975 | 76.9 | 3.2 |
| | 12 | 403 | 219 | 2.1 | 1.3 | 1345 | 784 | 7.5 | 5.2 | 662 | 380 | 4.5 | 2.7 |
| | 24 | 128 | 104 | 0.7 | 0.5 | 232 | 165 | 1.3 | 1.0 | 168 | 95 | 1.1 | 0.7 |
| | 48 | 48 | 15 | 0.3 | 0.05 | ND | ND | ND | 0.00 | 127 | 42 | 0.9 | 0.54 |
| Female | 0 | 17898 | 2128 | 100 | NA | 18479 | 1051 | 100 | NA | 13955 | 1727 | 100 | NA |
| | 4 | 6669 | 4239 | 37.3 | 26.0 | 15520 | 1709 | 84.0 | 7.6 | 10191 | 2284 | 73.0 | 23.2 |
| | 12 | 204 | 164 | 1.1 | 1.0 | 684 | 480 | 3.7 | 2.9 | 662 | 380 | 4.7 | 2.3 |
| | 24 | 61 | 36 | 0.3 | 0.2 | 174 | 33 | 0.9 | 0.2 | 145 | 52 | 1.0 | 0.4 |
| | 48 | 50 | 16 | 0.3 | 0.1 | ND | ND | ND | NA | ND | ND | ND | NA |

**Table C.2.1-2 Incubation of a Steviol Glycoside Mixture Prepared with Minor Steviol Glycosides (Reb AM, Reb W2, Reb Y, Reb U2, Reb V, Reb N, and Reb O) in Pooled Faecal Homogenates at 0.4 mg/mL**

| Subjects | Human Faecal Homogenate Incubation Time (h) | Reb N Response | SD | Mean % Reb N Remaining Relative to Time 0 | SD | Reb O Response | SD | Mean % Reb O Remaining Relative to Time 0 | SD | Mean Steviol Conc (µg/mL) | SD | Mean % Steviol Molar Equivalent Formed | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 1208 | 103 | 100 | NA | 1707 | 268 | 100 | NA | ND | ND | ND | ND |
| | 4 | 987 | 381 | 81.7 | 18.9 | 910 | 322 | 53.3 | 17.4 | 3.82 | 1.21 | 4.2 | 1.5 |
| | 12 | 106 | 13 | 8.7 | 0.3 | 39 | 30 | 2.3 | 1.7 | 54.5 | 12.2 | 60.5 | 15.6 |
| | 24 | 65 | 21 | 5.4 | 1.6 | 18 | 8 | 1.1 | 0.4 | 92.8 | 11.4 | 103 | 13.5 |
| | 48 | ND | ND | ND | 3.07 | ND | ND | ND | 0.00 | 91.6 | 3.10 | 102 | 2.1 |
| Female | 0 | 1204 | 147 | 100 | NA | 1596 | 235 | 100 | NA | ND | ND | ND | ND |
| | 4 | 356 | 86 | 29.6 | 4.6 | 237 | 134 | 14.8 | 7.4 | 4.67 | 0.709 | 5.3 | 1.0 |
| | 12 | 51 | 12 | 4.2 | 0.4 | 29 | 11 | 1.8 | 0.4 | 60.9 | 6.69 | 67.7 | 8.1 |
| | 24 | 48 | 19 | 4.0 | 1.4 | 15 | 3 | 1.0 | 0.1 | 85.9 | 5.76 | 95.4 | 3.8 |
| | 48 | ND | ND | ND | NA | ND | ND | ND | NA | 90.7 | 4.48 | 101 | 4.0 |

Conc = concentration; h = hour(s); NA = not applicable; ND = not detected; Reb = rebaudioside; SD = standard deviation.
The concentration of these minor glycosides in stevia leaf extract ranges between 0.1 to 0.3%. Reb AM is prepared by enzyme conversion of stevioside.

**Table C.2.1-3 Incubation of Rebaudioside A in Pooled Faecal Homogenates**

| Subjects | Human Faecal Homogenate Incubation Time (h) | Mean Reb A Response | SD | Mean % Reb A remaining relative to time 0 | SD | Mean Steviol Response | SD | Mean % Steviol Metabolite Formed | SD |
|---|---|---|---|---|---|---|---|---|---|
| Male | 0 | 39822 | 1,032 | 100.0 | NA | ND | NA | ND | NA |
| | 4 | 25892 | 2,726 | 65.0 | 3.0 | 307660 | 95,999 | 4.4 | 1.4 |
| | 12 | 1213 | 505 | 3.0 | 1.4 | 6000338 | 738,155 | 86.2 | 12.0 |
| | 24 | 235 | 139 | 0.6 | 0.4 | 5921920 | 365,690 | 85.0 | 6.2 |
| | 48 | 346 | 277 | 0.9 | 0.8 | 6969233 | 118,318 | 100.0 | NA |
| Female | 0 | 37142 | 2,731 | 100.0 | NA | ND | NA | ND | NA |
| | 4 | 22164 | 3,709 | 59.7 | 9.6 | 711251 | 163,807 | 8.1 | 2.3 |
| | 12 | 266 | 35 | 0.7 | 0.1 | 6653352 | 95,820 | 92.8 | 1.2 |
| | 24 | 88 | 54 | 0.2 | 0.1 | 6094822 | 152,765 | 85.1 | 2.0 |
| | 48 | 95 | 31 | 0.3 | 0.1 | 7166587 | 126,717 | 100.0 | NA |

h = hour(s); NA = not applicable; ND = not detected.

## C.2.2 Recent Pharmacokinetic Studies

The toxicokinetic profiles of steviol and steviol glucuronide following administration of high dose stevioside were determined in 2 groups of male and female Sprague-Dawley [Crl:CD(SD)] rats (72 animals/sex per dose group) (Roberts *et al.,* 2016). Animals were administered single oral doses of stevioside (40 or 1,000 mg/kg body weight, equivalent to 16 or 396 mg/kg body weight, as steviol), ≥95% purity, by gavage. Distilled water was used as vehicle for the 40 mg/kg body weight dose group, whereas polyethylene glycol (PEG) 400 was used as the vehicle in the 1,000 mg/kg body weight dose group for solubility purposes. Blood samples were collected from 6 animals/sex per dose group per time point from the inferior vena cava prior to dosing and at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, and 72 hours post-dosing. Generally, when administered 40 mg/kg body weight, plasma steviol concentrations increased from 2 hours post-administration through 4 and 6 hours post-administration (76.0 and 87.1 ng/mL, respectively), after which decreased to below the limits of quantification after 36 and 24 hours post-administration in males and females, respectively. Among rats administered 1,000 mg/kg body weight, plasma steviol concentrations increased from 1 hour post-administration through 6 and 12 hours post-administration (539 and 1,960 ng/mL) in males and females, respectively, and decreased through the last time point measured. However, plasma steviol concentrations from males administered 1,000 mg stevioside/kg body weight remained constant between 12 and 24 hours post-administration. Plasma steviol glucuronide concentrations also generally increased from 1 hour post-administration until 6 and 4 hours post-administration in males and females, respectively (160 and 200 ng/mL, respectively) and then decreased below limits of quantification at the last time point measured. When administered 1,000 mg stevioside/kg body weight, plasma concentrations of steviol glucuronide increased from 30 minutes post-administration through 8 and 12 hours post-administration (1,410 and 6,650 ng/mL, respectively) in males and females, respectively. However, similar to plasma steviol concentrations, steviol glucuronide concentrations remained constant between 12 and 24 hours post-administration among males administered both doses of stevioside.

When administered 1,000 mg stevioside/kg body weight, $C_{max}$ for free steviol and steviol glucuronide was approximately 4- and 5-fold higher for females in comparison to males, respectively, and the area under the concentration-time curve to time of the last measurable concentration ($AUC_{last}$) for both free steviol and steviol glucuronide was approximately 3-fold higher in females as compared to males. In comparison to dose administered, a 25-fold increase in the stevioside dose resulted in 7- and 9-fold increases in $C_{max}$ and 16- and 13-fold increases in $AUC_{last}$ for steviol and steviol glucuronide, respectively, in males. In females, a 25-fold increase in stevioside dose resulted in 23- and 33-fold increases in $C_{max}$ and 42- and 32-fold increases in $AUC_{last}$ for steviol and steviol glucuronide, respectively. The remainder of the toxicokinetic parameters for free steviol and steviol glucuronide were comparable between males and females regardless of stevioside dose. The differences in the toxicokinetic parameters for males and females suggest that there may have been incomplete absorption of the bolus stevioside dose of 1,000 mg/kg body weight in males, but not in females.

The pharmacokinetics of steviol and steviol glucuronide following consumption of stevioside (≥95% purity) were examined in an open-label, single dose trial in 10 healthy adult males between the ages of 20 and 45 years (Roberts *et al.,* 2016). The subjects were provided 40 mg/kg body weight of stevioside (equivalent to 16 mg steviol equivalents/kg body weight) in an aqueous solution. Blood samples were collected pre-dose and through 72 hours post-dose and were assayed for steviol and steviol glucuronide. Steviol was detected in the plasma in 9 out of 10 subjects, whereas steviol glucuronide was detected in all study participants following administration of stevioside. The maximum plasma concentrations of steviol glucuronide were approximately 58-fold higher than that of steviol. The time to reach maximum plasma concentrations of steviol and steviol glucuronide were 19 to 20 hours and 21 to 22 hours, respectively. The AUC for the plasma concentration-time data from pre-administration (-0.75 hour) to 24, 48, and 72-hour post-administration for steviol glucuronide were approximately 61-, 76-, and 83-fold higher in comparison to the AUC for steviol. Plasma glucuronide

was eliminated approximately 18 hours after reaching peak concentrations. No adverse events were reported by the study subjects throughout the study.

The toxicokinetic data obtained from rats and humans administered 40 mg/kg body weight of stevioside was compared. The AUC from time 0 to infinity and $C_{max}$ data were used to determine the appropriate chemical specific adjustment factor (CSAF) to account for the toxicokinetic differences between rats and humans following administration of steviol glycosides. The $AUC_{0-72}$ for free steviol in humans (1,631 ng·h/mL) is higher than the $AUC_{last}$ in male and female rats (581 and 605 ng·h/mL, respectively). The ratio of AUC between humans and rats is 2.8. Conversely, the $C_{max}$ values in humans (77.21 ng/mL) are approximately equivalent to those in male and female rats (76.0 and 87.1 ng/mL, respectively). The ratio of $C_{max}$ values between humans and rats is approximately 1. Both of the ratios of the AUC and $C_{max}$ between humans and rats derived from the recent studies are lower than the default uncertainty factor of 4.0 for interspecies toxicokinetic differences. Therefore, based on this data, the uncertainty factor for interspecies differences when calculating the ADI for steviol glycosides can be revised to 2.5 (1 for toxicokinetic x 2.5 for toxicodynamic differences) to 7 (2.8 for toxicokinetic x 2.5 for toxicodynamic differences) from the default value of 10, when based on differences in $C_{max}$ and AUC values, respectively, between humans and rats. The use of a CSAF therefore increases the ADI to between 6 and 16 mg/kg body weight, expressed as steviol, based on the NOAEL of 970 mg/kg body weight/day (or 383 mg/kg body weight/day expressed as steviol) in the 2-year rat study (Roberts *et al.,* 2016).

## C.3 Information on the Toxicity of Steviol Glycosides

### C.3.1 Toxicological Studies

#### *C.3.1.1 Repeat-Dose Toxicity*

The repeat-dose toxicity of steviol glycosides has been addressed in recent applications submitted to FSANZ, and within safety evaluations conducted by scientific bodies and regulatory agencies (see Section C.4). Additional studies were not identified in the updated search of the literature.

#### *C.3.1.2 Genotoxicity*

The genotoxic potential of stevia was also evaluated using *in vitro* micronucleus and chromosomal aberration tests in human lymphocytes (Uçar *et al*., 2018). Peripheral venous blood from healthy non-smoking males and females was used for the collection of human lymphocytes. In both tests, cell cultures were incubated at 37°C for 72 hours and, after 24 and 48 hours of incubation, treated with stevia (steviol glycoside purity of 99%) at concentrations of 0 (negative control), 1, 2, 4, 8, and 16 µg/mL. Both tests were carried out in duplicate and included a positive control group, treated with 0.2 µg/mL mitomycin C. After 70 hours of incubation in the chromosome aberration test, 0.06 µg/mL colchicine was added, then cells were harvested and prepared for analysis at 72 hours. One-hundred metaphases/donor/concentration were analysed, totalling 400 metaphases/concentration. In the micronucleus test, after cells were collected and prepared, a total of 4,000 binucleated cells/concentration were analysed. No significant increases in the number of chromosomal aberrations or micronuclei were reported at any stevia test concentration when compared to the negative control. Conversely, a significant increase in the number of chromosomal aberrations and micronuclei was reported for the positive control in each respective assay. The authors concluded that stevia does not have genotoxic potential based on these results, which remains consistent with the results of other similarly conducted studies.

### *C.3.1.3 Long-term Toxicity and Carcinogenicity*

The long-term toxicity and carcinogenicity of steviol glycosides has been addressed in recent applications submitted to FSANZ, and within safety evaluations conducted by scientific bodies and regulatory agencies (see Section C.4). Additional studies were not identified in the updated search of the literature.

### *C.3.1.4 Reproductive and Developmental Toxicity*

The potential effect of reb A (R107558; obtained from Aladdin Co., Ltd., China) on ovary function in rats was investigated by Jiang *et a*l. (2018). Reb A was provided in drinking water to weanling Sprague-Dawley rats (n=6 females/group) for a total of 48 days at dose levels of 0, 0.5, or 2.5 mM (reported to be equivalent to 0, 210, or 1,430 mg/kg body weight/day). Food and water were provided *ad libitum*. In the high dose group, body weight was significantly decreased from Day 18 to Day 30; however, at the end of the study, no significant difference in body weight between the high dose reb A and control groups was observed. Water consumption was significantly increased in the high-dose group during the entire study period, while the low-dose group was reported to be notably different from the control group after 21 days of treatment. Furthermore, the high-dose group water consumption was reported to be remarkable higher than the low-dose group in the last 3 weeks of treatment. Conversely, reb A was not reported to influence food intake in either group. No significant differences between the control and test-article treated groups were reported for puberty onset or body weights at puberty, nor were differences in oestrous cycles observed. No morphological changes in the ovaries of the reb A treated groups were reported. Serum levels of progesterone in the reb A groups were significantly decreased compared to the control group and decreased expression of 3β- hydroxysteroid dehydrogenase, an enzyme involved in progesterone synthesis, was measured in the ovaries *via* western blotting. Several other steroidogenesis-related factors were also reported to decrease based on the western blot results; however, the significance of these findings with respect to safety are limited as no effects on ovarian morphology or oestrous cyclicity were reported following reb A exposure for 48 days.

Gharheri *et al.* (2018) investigated the effects of *S. rebaudiana* extract (purity not reported) on reproduction function in diabetes-induced healthy adult male albino rats of Wistar strain. Diabetes mellitus was induced in rats *via* intraperitoneal injection of 50 mg streptozotocin/kg. The rats that reached fasting glucose levels greater than 250 mg/dL after 72 hours were selected for the study. Animals (7/group) were administered stevia extract at doses of 5, 50, or 100 mg/kg by gavage for 28 days. A diabetic and non-diabetic control group received 2 mL distilled water only. Sexual behaviours of the rats were recorded for 30 minutes every 2 weeks for 1 month, including mount latency, intromission latency, mount frequency, intromission frequency, ejaculation latency, the mount latency post ejaculation, and ejaculation frequency. Following the study period, animals were killed, and serum concentration of testosterone was measured. Histological examination was carried out on the right testis and epididymis. In diabetic rats, a significant increase in the frequency of intromission was observed in the low-dose group, compared to diabetic control rats. In addition, diabetic rats of the low-dose group showed a significant increase in the frequency of ejaculation, compared to the diabetic control and high-dose animals. However, a significant decrease in the latency of ejaculation was observed in the low-dose group when compared to the high-dose animals, although, the effect was not significant between the treated animals and the controls. Significant differences in other sexual behaviour parameters measured were not observed in the animals. Furthermore, a significant reduction in the number of Leydig cells in high-dose animals was noted, compared to the non-diabetic control group; however, this effect was not significantly different compared to the diabetic control rats. Organ weights and serum testosterone levels showed no significant differences among the study animals. Based on the results of the study, the authors concluded that there is no risk to reproductive parameters and consumption of stevia. The authors reported that intake of stevia may be effective in the promotion of blood glucose reduction and preventing destruction of Leydig cells.

### *C.3.1.5 Immune Function/Immunotoxicity*

Immune function/immunotoxicity of steviol glycosides has been addressed in recent applications submitted to FSANZ, and within safety evaluations conducted by scientific bodies and regulatory agencies (see Section C.4). Additional studies were not identified in the updated search of the literature.

### *C.3.1.6 Antidiabetic Effects*

Ahmad and Ahmad (2018) examined the antidiabetic effects of aqueous extract of *S. rebaudiana* leaves (purity not reported) in rats with streptozotocin-induced diabetes. Sixty adult male albino rats (body weight 152.53 g; n=10/group) were provided a basal diet *ad libitum* for 2 weeks. The diabetic rats were orally administered *S. rebaudiana* Bertoni aqueous extract dissolved in distilled water at doses of 200, 300, 400, and 500 ppm/kg body weight (doses not reported on a mg/kg body weight/day basis) for 8 weeks. The non-diabetic and diabetic control rats were provided with distilled water. Food and water consumption were measured daily, and body weight gain was estimated weekly. Following the study period, rats were fasted overnight and terminated. Blood samples were collected, and the serum of the rats were analysed for the following biochemical parameters: blood glucose, glycosylated haemoglobin (HbA1c), insulin, and liver glycogen levels. Random blood glucose and fasting blood glucose levels were significantly decreased in diabetic rats when compared to the controls after 8 weeks. While fasting blood glucose levels significantly increased in diabetic control rats compared to non-diabetic control rats. HbA1c levels were also significantly decreased after 8 weeks in diabetic rats compared to the controls. Although, HbA1c levels significantly increased in diabetic control rats compared to non-diabetic control rats. Insulin and liver glycogen levels significantly improved in diabetic rats following the 8-week study period compared to the controls. However, when compared to the non-diabetic controls, a significant decrease in liver glycogen levels was observed in diabetic control rats. The authors concluded that stevia extract ameliorate diabetic effects in diabetic rats.

The effects of stevioside on skeletal muscle metabolic dysfunctions of diabetic rats was investigated by El-Mesallamy *et al.* (2018). Male Sprague Dawley rats (10/group) were administered a 50 mg/kg body weight intraperitoneal dose of streptozotocin to induce type 1 diabetes. Diabetes was confirmed by measuring fasting blood glucose 1 week after administration. The diabetic rats were then provided *via* gavage a dose of 0.5 mL saline (control) or 2 mg/kg body weight/day pure stevioside for 4 weeks. Additional groups receiving separate treatments were included in the study, including a non-diabetic control group receiving 0.5 mL saline. At study termination, blood samples were collected and analysed for changes in blood glucose, HBA1c, fasting serum insulin, and homeostatic model assessment (HOMA)-insulin resistance. Diabetic rats were confirmed to have significantly higher fasting blood glucose, HOMA-IR and Hb1Ac and significantly reduced body weight and insulin levels in comparison to the non-diabetic control. Stevioside treatment was reported to reverse these effects with statistical significance. Oxidative stress in the soleus muscle was measured, and it was reported diabetes caused a statistically significant reduction in malondialdehyde concentration, and a statistically significant increase in glutathione peroxidase activity, superoxide dismutase activity and catalase activity. Stevioside treatment reversed these effects with statistical significance, when compared to the diabetic control. AMP-activated protein kinase activity in soleus muscle tissue and expression of GLUT4 gene mRNA were also decreased in diabetic rats, with the stevioside treatment statistically reversing these effects compared to the diabetic control. Overall, it was concluded that stevioside treatment improved skeletal muscle metabolic dysfunctions in diabetic rats.

The effects of 2 different stevia residue extracts on mice with impaired glucose regulation was investigated by Zhao *et al.* (2018). Male ICR mice consuming a high fat/high fructose diet (10/group) received *via* gavage either 50 or 200 mg/kg body weight/day stevia residue extract 1 or 2 for 12 weeks. The stevia residue extracts were obtained from aqueous extracts of stevia leaves and differed based on phenolic compound content (steviol glycoside content and purity not reported). Several control groups were included, in particular a group consuming the same high fat/high fructose diet in the absence of stevia residue extract, and a normal diet control group. An oral glucose tolerance test was conducted after a 12-hour fast on the third, sixth, and ninth week of the study and consumption of the high fat/high fructose was confirmed to induce an impaired glucose tolerance that was reported to be reduced to normal diet levels in the presence of stevia, in particular with the high dose of stevia residue extract 1. Serum lipids, including total cholesterol, triglycerides, LDL-C, and HDL-C were assayed on the fifth and tenth week of study and consumption of the high fat/high fructose diet was confirmed to induce statistically significant increases in these lipid metabolic parameters, which were mostly reduced to normal diet levels in the presence of the high dose of stevia residue extract 1. At the end of the study serum antioxidant status was evaluated based on levels of superoxide dismutase, malondialdehyde, and total antioxidant activity, which was confirmed to be statistically significantly reduced following consumption of the high fat/high fructose diet. The high dose of stevia residue extract 1 was reported to restore the serum antioxidant activity to normal diet levels. Lastly, liver histology showed increased lipid deposits in mice consuming the high fat/high glucose diet compared to the normal diet control and the high dose of stevia residue extract 1 was reported to most significantly decrease the level of lipid droplets induced by the high fat/high fructose diet. Overall, the results of this study suggest that certain stevia residue extracts may have beneficial effects on impaired glucose regulation in mice.

### C.3.2 Human Studies

Ahmad *et al.* (2018) investigated the potential effects of stevia leaf powder (prepared from dried stevia leaves; steviol glycoside content not reported) on postprandial glycemia, appetite, palatability, gastrointestinal discomfort, and anthropometric parameters in a randomised single-blind, crossover placebo-controlled study in healthy humans. Healthy males and females [10/group; mean age 24.1 ± 1.33 years; body mass index (BMI) 22.09 ± 3.88 kg/m$^2$] were fasted overnight and provided with either a placebo (140 g cookie made from 100% wheat flour) or cookies containing stevia leaf powder (3% w/w; approximately equivalent to 4.2 g stevia) once in the morning. A 1- to 2-week washout period was carried out before and after each treatment period. The subjects were to avoid any vigorous physical activity prior to each study visit, while also maintaining the same dietary patterns in the evening prior to each visit. At baseline and following each treatment, fasting blood glucose concentration, appetite, hunger levels, and gastrointestinal discomfort were measured. The following parameters were also measured: blood pressure, weight, height, and BMI. The palatability of test foods was also recorded using a 9-point hedonic scale. Following the consumption of stevia, a decrease in appetite was noted by the authors, compared to the control cookies. However, this observed effect was only significant at 30 minutes following intake. In addition, the stevia cookies had a lower rating for texture based on the palatability testing, when compared to the control cookies. No other significant differences were observed in relation to palatability parameters, and the stevia-containing cookies did exceed the score required to be considered acceptable. The results also demonstrated no significant effects on any of the anthropometric parameters, blood glucose response, or gastrointestinal discomfort. The study authors concluded that consumption of stevia leaf powder in cookies decreased hunger, when compared to the control cookies.

Rizwan *et al.* (2018) investigated the beneficial effect of *S. rebaudiana* (purity not reported) in a prospective, interventional, randomised, single-blind, placebo-controlled preliminary trial in stage I to stage III chronic kidney disease (CKD) patients. Ninety-seven male and female patients [Group 1 (n=44): 55 ± 11.75 years, BMI 26.34 ± 3.46 kg/m$^2$; Group 2 (n=43): 53.60 ± 11.27 years, BMI 25.79 ± 3.31 kg/m$^2$; Group 3 (n=10): 47.20 ± 4.87 years, BMI 25.45 ± 4.11 kg/m$^2$) were enrolled in the study and received either a stevia capsule (250 mg; purity not reported) or matching placebo twice daily, as well as Angiotensin-II Receptor Blocker (ARB) and/or Calcium Channel Blocker (CCB) for 9 months. The patients were separated into 3 groups as follows: study group-1 (STV), stevioside capsule plus conventional antihypertensive treatment and CKD treatment; study group-2 (PLC), matching placebo and a similar treatment regimen; study group-3 (CL), control group with healthy participants. Follow-up visits were scheduled every 3 months, and a washout period was conducted after 9 months. Data from the first 3 months of the study were only considered in this study. Blood and urine samples were collected at the first follow-up, 3 months after the initial stage of the study (baseline). Significant changes were observed in systolic and diastolic blood pressure, serum creatinine, serum uric acid, fasting blood sugar, postprandial blood sugar, and microalbumin levels in Group 1 compared to baseline. In comparison, significant differences in systolic and diastolic blood pressure, serum uric acid, sodium, chloride, urine for albumin, and urine for protein were observed in the Group 2 compared to baseline. Moreover, significant differences at baseline were observed in diastolic blood pressure, blood urea, serum creatinine, serum total protein, calcium, and inorganic phosphate between Groups 1 and 2. In the first follow-up (3 months), significant differences were observed in diastolic blood pressure and urine for protein between Groups 1 and 2. In addition, very highly significant ($p<0.001$) results were observed for estimated glomerular filtration rate in Group 1 compared with controls. Based on the results of the study, the authors concluded that stevia has the potential to significantly improve some biochemical parameters in CKD patients after 3 months of treatment, and the constructive effect of stevia can be confirmed after 9 months of treatment.

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

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

The safety of steviol glycosides was reviewed by JECFA at 5 separate meetings (51$^{st}$, 63$^{rd}$, 68$^{th}$, 69$^{th}$, and 82$^{nd}$) in 1998, 2004, 2007, 2008, and 2016 (JECFA, 1999, 2006b, 2007b, 2009, 2017b). At the first meeting in 1998, JECFA was asked to specifically review the safety of stevioside. Following review of the available information, the Committee concluded that the data on stevioside were limited and highlighted the need for specifications for commercial materials. An ADI could not be established.

Subsequently in 2004, the Committee determined that the material of commerce for which tentative specifications were developed should be known as “steviol glycosides”. New data as per the requests made at the earlier meeting were provided to the Committee for review. The Committee reviewed the newly available data which demonstrated that stevioside and reb A were not genotoxic and that the positive *in vitro* results for steviol and its oxidative derivatives were not confirmed *in vivo*. Although the Committee reviewed the results of a developmental study showing adverse effects on fertility following treatment of male rats with a crude aqueous extract of *S. rebaudiana*, the Committee referred back to the studies reviewed at the preceding meeting noting that in studies conducted with higher purity material, no reproductive or developmental effects were observed, and thus, the reproductive effects noted following administration of the crude extract were unlikely to be related to steviol glycosides. Although the Committee did not raise any further questions regarding the potential toxicity of steviol glycosides at this review, the Committee noted that pharmacological effects in patients with hypertension or type 2 diabetes were observed at doses of 12.5 to 25 mg/kg body weight/day of steviol glycosides (5 to 10 mg/kg body weight/day as steviol equivalents). Consequently, further information regarding the potential effects of steviol glycosides in subjects with diabetes and in normotensive and

hypotensive populations was requested. At this time, a temporary ADI of 2 mg/kg body weight (expressed as steviol) for steviol glycosides was allocated, based on a no-observed-adverse-effect level (NOAEL) of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and application of a safety factor of 200 (JECFA, 2006b).

In 2007, the Committee received additional data pertaining to the potential pharmacological effects of steviol glycosides in humans; yet, none of these studies were conducted with a material that met the specifications for steviol glycosides. However, the Committee was made aware of an ongoing human study that was designed to specifically address the Committee's previous concerns (Maki *et al*., 2008a,b) and thus the temporary ADI was extended until 2008. The specifications were revised, and the tentative designation was removed.

In 2008, the Committee was presented with new data pertaining to the metabolic fate of steviol glycosides in rats and humans (Roberts and Renwick, 2008; Wheeler *et al*., 2008), subchronic and reproductive/developmental toxicity of reb A specifically (Curry and Roberts, 2008; Curry *et al*., 2008; Nikiforov and Eapen, 2008), and the potential pharmacological effects of steviol glycosides in diabetic populations and individuals with normal or low-normal blood pressure (Maki *et al*., 2008a,b). The Committee concluded that the results of the human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose were sufficient to remove the additional safety factor of 2 and establish a full ADI of 0 to 4 mg/kg body weight (expressed as steviol) for steviol glycosides. The specifications for steviol glycosides were revised further, requiring not less than 95% of the 7 named steviol glycosides (stevioside, reb A, B, C, dulcoside A, rubusoside, and steviolbioside).

During the Committee's 73rd meeting in 2010, JECFA revised the specifications for steviol glycosides to include 2 additional steviol glycosides, reb D and reb F, within the purity criteria (JECFA, 2010). Although no specific studies have been conducted with these steviol glycosides individually, their inclusion within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all steviol glycosides are degraded to the aglycone steviol and that the safety demonstrated for one glycoside is relevant to all glycosides in general.

At the 82nd meeting, the Committee reviewed data related to the safety of steviol glycosides that had become available since the 69th meeting and confirmed the ADI of 0 to 4 mg/kg body weight, expressed as steviol (FAO, 2016). A new specifications monograph was prepared for "Rebaudioside A from Multiple Gene Donors Expressed in *Yarrowia lipolytica*" (the Committee also confirmed its inclusion in the ADI) based on details of a new manufacturing process that utilises a strain of genetically modified *Yarrowia lipolytica* overexpressing the steviol glycoside biosynthetic pathway to produce reb A (JECFA, 2016). New 'tentative' specifications were established for "Steviol Glycosides from *Stevia rebaudiana* Bertoni", showing a separation of the specifications based on source material used in the manufacturing process. At the 84th meeting the tentative designation was removed, and the specifications were finalised to recognise commercial products that contain not less than 95% of total steviol glycosides (on a dried basis), where steviol glycosides are defined as "*a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni*" (JECFA, 2017a).

### C.4.2 Food Standards Australia/New Zealand (FSANZ)

Immediately prior to JECFA's 69th meeting, FSANZ conducted their own evaluation of the safety of steviol glycosides (FSANZ, 2008). In its safety assessment, FSANZ considered the data previously reviewed by JECFA (2010), as well as supplementary data consisting of published and unpublished studies. FSANZ considered the toxicological database for stevioside to cover a range of toxicological endpoints and concluded that the supplementary data were sufficient to revise JECFA's temporary ADI to a full ADI of 4 mg/kg body weight by removing the additional uncertainty factor of 2. Consistent with

the expansion of the definition of steviol glycosides in Europe to include reb M, FSANZ also evaluated the safety of reb M and amended the steviol glycoside specification to include reb M along with the 9 other glycosides (FSANZ, 2015). Recently, FSANZ has reviewed the safety of all steviol glycosides from *S. rebaudiana* (FSANZ, 2017), similar to JECFA, which resulted in updated specifications that broaden the definition to include all individual steviol glycosides present in the *S. rebaudiana* Bertoni leaf, so long as the total steviol glycoside content is not less than 95% on the dried basis (FSANZ, 2017). In 2018, FSANZ evaluated a similar application to include an alternative manufacturing process for reb M produced by enzymatic bioconversion of stevia leaf extract using UDP-glucosyltransferase and sucrose synthase derived from strains of *P. pastoris* (A1157 – FSANZ, 2018), which resulted in updated specifications for steviol glycosides from *S. rebaudiana* Bertoni to include this new manufacturing method.

### C.4.3 European Food Safety Authority (EFSA)

In 1985, the European Commission's Scientific Committee on Food (SCF) evaluated stevioside as a sweetener and concluded that its use was "not toxicologically acceptable" due to limited data on metabolism, mutagenicity, long-term, and reproductive and developmental toxicity (SCF, 1985). In a subsequent evaluation, the SCF examined newly available data on metabolism, genotoxicity, and long-term toxicity, but maintained that these data were inadequate to sufficiently assess the safety of stevioside (SCF, 1999). Specifically, the SCF continued to raise concerns related to the potential reproductive effects of steviol glycosides and recommended that a study in a rat strain other than the F344 rat be conducted [rat strain used in the 2 carcinogenicity studies on stevioside (Yamada *et al*., 1985; Toyoda *et al*., 1997)], since it was not possible to evaluate any potential effects on the testicular system as this strain of rats seemed to generally develop testicular changes. The SCF (1999) also questioned the relevance of numerous other studies because the composition of the test material was not clearly defined. The potential mutagenic effects of steviol also continued to be a concern (SCF, 1999). Based on the SCF's review of stevioside, the European Commission rejected *Stevia* and stevioside for use as a sweetener (Geuns, 2003). However, in an independent review of the safety data previously reviewed by JECFA at its 69$^{th}$ meeting, EFSA corroborated JECFA's conclusion regarding the safety and concurred with the ADI previously established by JECFA of 0 to 4 mg/kg body weight for steviol glycosides, expressed as steviol equivalents (EFSA, 2010). Moreover, in a subsequent examination of steviol glycoside safety, in response to a request to amend the specifications for steviol glycosides, EFSA concluded that safety studies conducted with reb A and stevioside (*i.e.,* individual steviol glycosides) can extend to other steviol glycosides due to the shared metabolic fate (EFSA, 2015). The EFSA Panel concluded that "*extending the current specifications to include* [two additional steviol glycosides]*, rebaudiosides D and M, as alternatives to rebaudioside A in the predominant components of steviol glycosides would not be of safety concern*" and further to that, "*considered that the ADI of 4 mg/kg body weight/day can also be applied where total steviol glycosides comprise more than 95% of the material*".

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).

### C.4.4 Health Canada

Health Canada conducted its own independent review of the available safety data for steviol glycosides (Health Canada, 2012) and corroborated the safety conclusions by JECFA, FSANZ, and EFSA. Health Canada also established an ADI of 4 mg/kg body weight for steviol glycosides, expressed as steviol glycosides, based on the NOAEL from the 2-year carcinogenicity study conducted by Toyoda *et al*. (1997). Consistent with the expansion of the definition of steviol glycosides in Europe to include reb M, Health Canada also evaluated the safety of reb M and expanded the definition of steviol glycosides to include reb M (Health Canada, 2016). Based on their most recent safety review, Health Canada expanded the definition of steviol glycosides to include all steviol glycosides in the *S. rebaudiana* Bertoni plant and no safety concerns were raised in their assessment (Health Canada, 2017).

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

In accordance with *Section 3.3.1 – Food Additives* of the FSANZ *Application Handbook* (FSANZ, 2016a) the following dietary exposure information is 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. The percentage of the food group in which the food additive is proposed to be used or the percentage of the market likely to use the new food additive; and

4. Information relating to the use of the food additive in other countries.

## D.1 Proposed Food Uses and Use-Levels of Steviol Glycosides Produced by Enzymatic Conversion of Highly Purified Reb A and/or Stevioside From Stevia Leaf Extract

The uses and use-levels for steviol glycosides in Australia and New Zealand as defined in *Schedule 15 – Substances that may be used as Food Additives* of *The Code* are presented in Table D.1-1 (FSANZ, 2019c). The current application being submitted by PureCircle is intended to amend the current specification of steviol glycosides to include the new manufacturing method. Thus, steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are proposed for use as a high-intensity sweetener in food and beverages under the same conditions as those already approved for steviol glycosides in Australia and New Zealand.

**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

Since steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are intended for use as a high-intensity sweetener in foods and beverages under the same conditions of use as presently authorised for steviol glycosides, and either in place of existing steviol glycoside preparations already in the Australia/New Zealand marketplace or in combination with such preparations up to but not greater than the maximum permitted use-levels (as steviol equivalents), intakes of steviol glycosides (as steviol equivalents) will remain the same. Accordingly, a separate intake assessment for steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract was not performed for the purpose of this food additive amendment. It should be further noted that the use-levels for steviol glycosides as adopted in *Schedule 15* of *The Code* are expressed as steviol equivalents and as such are not specified for any one particular steviol glycoside, but rather 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., PureCircle's steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract has GRAS status for use as general purpose sweetener in foods. Specifically, GRN 744 was submitted by PureCircle to the FDA (PureCircle Limited, 2017) and considered the safety of steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract, equivalent to the steviol glycoside preparation presented herein. The FDA raised no objections regarding the GRAS status of PureCircle's steviol glycosides with a high reb M/reb D content produced by enzymatic conversion of reb A from stevia leaf extract for use as general purpose sweetener in foods, excluding meat and poultry products and infant formula, at levels in accordance with cGMP (U.S. FDA, 2018).

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# Statutory Declaration – Australia

## STATUTORY DECLARATION

*Statutory Declarations Act 1959* [1]

Head of Global Scientific & Regulatory Affairs, PureCircle Limited,

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 200 W Jackson Blvd, 8th Floor, Chicago, Illinois, 60606, USA on 14 of March 2019

Before me,

200 W Jackson Blvd, 8th Floor
Chicago, IL 60606 USA

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

## Statutory Declaration – New Zealand

**STATUTORY DECLARATION**

*Oaths and Declarations Act 1957*[2]

Head of Global Scientific & Regulatory Affairs, PureCircle Limited, 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 200 W Jackson Blvd, 8th Floor, Chicago, Illinois, 60606, USA this 14 of March 2019

Declared before me

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

**Checklist for General Requirements (3.1.1)**

| Check | Page No. | Mandatory Requirements |
|---|---|---|
| ✓ | 5 | A. Form of application<br>✓ Application in English<br>✓ Executive Summary (separated from main application electronically)<br>✓ Relevant sections of Part 3 clearly identified<br>✓ Pages sequentially numbered<br>✓ Electronic copy (searchable)<br>✓ All references provided |
| ✓ | 5 | B. Applicant Details |
| ✓ | 6 | C. Purpose of the Application |
| ✓ | 6 - 7 | D. Justification for the application<br>✓ Regulatory impact information<br>✓ Impact on international trade |
| ✓ | 7 | E. Information to support the application<br>✓ Data requirements |
| ✓ | 8 | F. Assessment procedure<br>✓ General |
| ✓ | 8;<br>Appendix A | G. Confidential commercial information<br>✓ CCI material separated from other application material<br>✓ Formal request including reasons<br>✓ Non-confidential summary provided |
| ✓ | 8 | H. Other confidential information<br>✓ Confidential material separated from other application material<br>✓ Formal request including reasons |
| ✓ | 8 | I. Exclusive capturable commercial benefit<br>✓ Justification provided |
| ✓ | 9 - 10 | J. International and other national standards<br>✓ International standards<br>✓ Other national standards |
| ✓ | 11;<br>Appendix B | K. Statutory Declaration |
| ✓ | 11;<br>Appendix C | L. Checklists provided with application<br>✓ 3.1.1 Checklist<br>✓ All page number references from application included<br>✓ Any other relevant checklists for Chapters 3.2-3.7 |

**Checklist for Food Additives (3.3.1)**

| Check | Page No. | Mandatory Requirements |
|---|---|---|
| ✓ | 13 - 16 | A.1 Nature and technological purpose information |
| ✓ | 17 - 20 | A.2 Identification information |
| ✓ | 20 | A.3 Chemical and physical properties |
| ✓ | 21 - 22 | A.4 Impurity profile |
| ✓ | 22 - 25 | A.5 Manufacturing process |
| ✓ | 32 - 33 | A.6 Specifications |
| ✓ | 34 | A.7 Food Labelling |
| ✓ | 34 | A.8 Analytical detection method |
| ✓ | 34 | A.9 Additional functions |
| ✓ | 36 - 45 | B.1 Toxicokinetics and metabolism information |
| ✓ | 45 - 49 | B.2 Toxicity information |
| ✓ | 49 - 52 | B.3 Safety assessments from international agencies |
| ✓ | 54 | C.1 List of foods likely to contain the food additive |
| ✓ | 54 | C.2 Proposed use levels |
| ✓ | 55 | C.3 Likely level of consumption |
| N/A | | C.4 Percentage of food group to contain the food additive |
| ✓ | 55 | 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 |
|---|---|---|
| ✓ | 26 | A.1 Type of processing aid |
| ✓ | 26 | A.2 Identification information |
| ✓ | 27 | A.3 Chemical and physical properties |
| ✓ | 27; Appendix A | A.4 Manufacturing process |
| ✓ | 28 | A.5 Specification information |
| N/A | | A.6 Analytical method for detection |
| ✓ | 29 | C.1 Information on enzyme use in other countries |
| ✓ | 29 - 30; Appendix A | C.2 Toxicity information of enzyme |
| ✓ | 30; Appendix A | C.3 Allergenicity information of enzyme |
| N/A | | C.4 Overseas safety assessment reports |
| ✓ | 30 - 31 | D.1 Information on source organism |
| ✓ | 31 | D.2 Pathogenicity and toxicity of source microorganism |
| ✓ | 31; Appendix A | D.3 Genetic stability of source organism |
| ✓ | 31; Appendix A | E.1 Nature of genetic modification of source organism |

N/A, not applicable