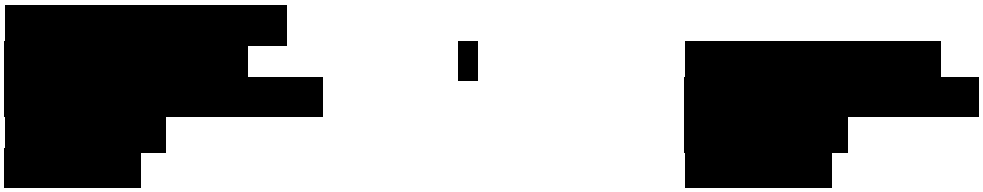
# APPLICATION FOR THE APPROVAL OF STEVIOL GLYCOSIDES FROM *YARROWIA LIPOLYTICA* UNDER THE AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE – STANDARD 1.3.1 – FOOD ADDITIVES



**WHICH COMPRISE THE GENERAL PARTNERSHIP:**

**DATE:**
18 December 2020

# Application for the Approval of Steviol Glycosides from *Yarrowia lipolytica* under the *Australia New Zealand Food Standards Code* – Standard 1.3.1 – Food Additives

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# Application for the Approval of Steviol Glycosides from *Yarrowia lipolytica* under the *Australia New Zealand Food Standards Code* – Standard 1.3.1 – Food Additives

## A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), the following general information must be provided:

1. Format of the application
2. Applicant details
3. Purpose of the application
4. Justification for the application
5. Information to support the application.
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7. Confidential commercial information
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Each point is addressed in the following subsections.

# A.1 Format of the Application

## A.1.1 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 *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), 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

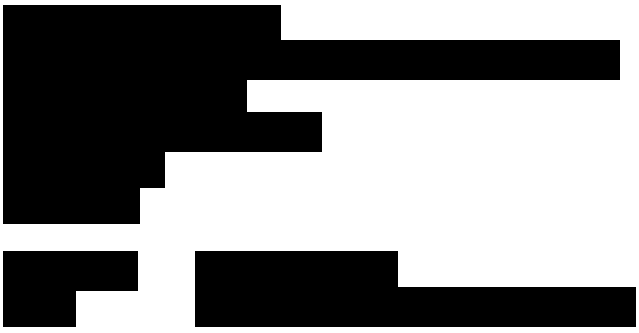
C. Information on the safety of the food additive

D. Information on dietary exposure to the food additive

The application is presented in this format. At the start of each section (A to D), the information that must be addressed therein is specified in more detail. Additionally, an executive summary for the application has been provided as a separate electronic document to this application. The application has been prepared in English and submitted electronically, as required within the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a).

# A.2 Applicant Details

Avansya V.O.F. (Avansya) is a general partnership under the laws of The Netherlands ("**vennootschap onder firma**") between Cargill Sweeteners Holding B.V. (Cargill) and DSM Food Specialties Stevia B.V. (DSM), and is a manufacturer of fermentation-derived sweeteners used in food, beverage, flavours, and fragrances applications to retailers, foodservice providers, and food, beverage, flavour, and fragrances manufacturers throughout the globe. The application is therefore made jointly by Cargill and DSM as the legal entities that comprise the Avansya V.O.F. partnership. The contact details for the person responsible for this application are listed below.



In addition, Dr. Alexandra Lobach, Senior Manager of Toxicology, Chemistry & Regulatory Affairs, Food & Nutrition, at Intertek Health Sciences, Inc., assisted in the preparation of this application and will be involved in the submission and stewardship of this application. Her contact details are listed below:

## A.3 Purpose of the Application

This application is being submitted to Food Standards Australia New Zealand (FSANZ), and the applicant is seeking approval for a purified steviol glycoside mixture (Reb MD) for use as a sweetener that is produced by fermentation of simple sugars using a *Yarrowia lipolytica* production strain. Reb MD is primarily comprised of rebaudioside M and may contain a mixture of the following additional glycosides in various concentrations, which are present in the leaves of the *Stevia rebaudiana* plant: rebaudiosides A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and dulcoside A. The distribution of individual steviol glycosides present in Reb MD will vary depending on the production process and final product formulation. Reb MD contains not less than 95% total steviol glycosides, determined as the sum of the aforementioned steviol glycosides.

Currently, as listed in *Schedule 3 – Purity and Identity* of the *Australia New Zealand Food Standards Code*, "steviol glycosides from fermentation" (S3—39) are steviol glycoside preparations that contain a prescribed steviol glycoside obtained from a defined source by fermentation (FSANZ, 2020a). The currently listed prescribed steviol glycoside in S3—39 is rebaudioside MD that is obtained from "*Saccharomyces cerevisiae strain CD15407 containing novel genes for the production of rebaudiosides*". The general purity parameter for steviol glycosides from fermentation is not less than 95% of steviol glycosides on the dried basis. On this basis, this steviol glycoside mixture (Reb MD) produced from a *Y. lipolytica* production strain is chemically and substantially equivalent to rebaudioside MD obtained from *Saccharomyces cerevisiae* strain CD15407 that was the subject of Application A1170 previously submitted to FSANZ by Cargill. As such, Reb MD from a *Y. lipolytica* production strain meets the general specification parameters for steviol glycosides from fermentation as defined in S3—39. However, *Y. lipolytica* is not listed as a source organism for Reb MD. Therefore, the purpose of this application is to amend S3—39 to add this *Y. lipolytica* strain as a source for Reb MD: "*Yarrowia lipolytica strain VRM containing pathway genes for the production of steviol glycosides*".

## A.4 Justification of the Application

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

Reb MD from a *Y. lipolytica* production strain, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, such as Reb MD from *S. cerevisiae* strain CD15407 (Application A1170)[1], would be used as high-intensity sweeteners in reduced-calorie or no-sugar-added products. While steviol glycoside preparations are already available for use in food as sweeteners throughout Australia and New Zealand and many other parts of the world, the use of Reb MD as an alternative to parent steviol glycosides presents an improved sensory profile, and therefore, a better sweetness quality for consumers, as discussed in detail in Section B.1.

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

Minor steviol glycosides present in the leaves of *S. rebaudiana* Bertoni, such as rebaudioside M, are associated with improved sweetness quality when compared to major steviol glycosides, such as rebaudioside A and stevioside. Therefore, it is of great interest to industry to produce minor steviol glycosides using alternative manufacturing processes that are more efficient than the traditional leaf extraction processes, which yield very low levels of minor glycosides. The manufacturing process for steviol glycoside mixtures that uses a *Y. lipolytica* production strain can yield much higher levels of minor glycosides, such as rebaudioside M, and it is therefore anticipated that the availability of sweeteners such as Reb MD generated using this technology will benefit the food industry in Australia and New Zealand and globally. Reb MD provides improved sensory characteristics over major steviol glycosides such as rebaudioside A and stevioside while having similar stability, making it suitable for a wide variety of applications, functioning as a multi-purpose and zero-calorie sweetener.

The benefits to the consumer for the use of Reb MD would be similar to those for steviol glycoside mixtures currently permitted for use in Australia and New Zealand. Reb MD will replace sugar in foods for the benefit of consumers who are seeking foods and beverages with reduced calories from sugar to maintain a reduced-calorie diet. The use of Reb MD in various products would also benefit individuals with specific medical conditions that require reduced sugar intakes, such as diabetics, as steviol glycosides do not interfere with glucose homeostasis (EFSA, 2010).

Considering that the applicant intends to market Reb MD in the same approved food uses and at the same use levels as other steviol glycosides that are already approved for many food applications within Australia and New Zealand, there is no perceived benefit or added cost to government (FSANZ, 2017). This application for the use of Reb MD in Australia and New Zealand is part of a global regulatory strategy with equivalent submissions prepared and submitted in the United States (U.S.) [Generally Recognized as Safe (GRAS), GRN 000882 – U.S. FDA, 2020a], Mexico (approved food additive – Cofepris, 2018), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (positive opinion issued and steviol glycoside framework including new specification for Steviol Glycosides from Fermentation has been established).

[1] Note that the steviol glycoside preparation that was the subject of Application A1170, and that is referred to as rebaudioside MD in Schedule S3—39, is a mixture of rebaudioside M and rebaudioside D with similar a distribution of steviol glycosides as the rebaudioside M preparation that is the subject of this application. Both preparations primarily contain rebaudioside M and are considered chemically equivalent.

## A.5 Information to Support the Application

Detailed technical information regarding the manufacture of steviol glycoside mixtures such as Reb MD produced *via* fermentation of simple sugars using a *Y. lipolytica* production strain is presented in Section B of this application. Information to support the safety of the ingredient is presented in Section C and is based on the fact that since steviol glycosides produced by fermentation are chemically equivalent to steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni*,* the extensive safety database that exists for steviol glycosides extracted from *S. rebaudiana* Bertoni may be applied to establish the safety of Reb MD. As such, the numerous reviews and opinions by scientific bodies and regulatory authorities on the safety of steviol glycosides are summarised, along with any new safety data published in the scientific literature since the approved application submitted by Cargill for steviol glycosides produced by *S. cerevisiae* (A1170) (FSANZ, 2019b).

## A.6 Assessment Procedure

The applicant considers the most appropriate assessment procedure for the application herein is related to *Standard 1.3.1 – Food Additives* of the *Australia New Zealand Food Standards Code* in order to amend S3—39 to add the *Y. lipolytica* strain as a source for Reb MD: "*Yarrowia lipolytica strain VRM containing pathway genes for the production of steviol glycosides*". Considering the shared metabolic fate of steviol glycosides produced by fermentation with steviol glycosides extracted from *S. rebaudiana* Bertoni and the extensive safety database that exists for steviol glycosides extracted from the leaf, this addition is expected to fall under the General Procedure (Subdivision D of the *Food Standards Australia New Zealand Act*), Cost Category Level 2.

## A.7 Confidential Commercial Information (CCI)

The applicant requests that the following specific information related to the construction of the production organism, *Y. lipolytica*, and the final Reb MD product be considered confidential commercial information (CCI) and informs FSANZ in writing as follows:

- Details regarding the specific genetic modifications and construction of the production organism are considered trade secrets related to the manufacturing process and are provided in Appendix C-1 (Information on the Production Organism).

- The genomic sequences of the genes added to the production organism are considered trade secrets related to the manufacturing process and are included in Appendix C-2 (Bioinformatic Assessments for Toxigenicity and Allergenicity).

- Analytical details of some of the detection methods included in the product specifications are internal Standard Operating Procedures, are considered trade secrets, and are included in Appendix C-3 (Analytical Methods).

- An *in vitro* microbial hydrolysis study on Reb MD has been conducted that has not been published and is therefore considered a trade secret and included in Appendix C-4 (Reb MD Microbial Hydrolysis Study).

- Reports detailing the results of residual protein and recombinant DNA analyses for Reb MD have not been published and are considered trade secrets and are included in Appendix C-5 (Reb MD Residual Protein Analysis Report, Reb MD Recombinant DNA Analytical Report).

As such, the applicant requests that the above data and information be considered CCI by FSANZ due to its proprietary nature that is of significant commercial value to the company. Non-confidential descriptions of the CCI are provided in the respective sections of this application.

## A.8 Other Confidential Information

No other confidential information is contained within this application.

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

Currently, the applicant is not the only manufacturer of steviol glycosides; however, due to the nature of the yeast strain technology, it is assumed that only the applicant will be able to commercially benefit from of the production of Reb MD from this specific *Y. lipolytica* strain for use in Australia and New Zealand upon approval of this application. Therefore, the application would confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the *Food Standards Australia New Zealand Act*.

## A.10 International and Other National Standards

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

In June 2019, at the 87th meeting of the JECFA Committee, a framework for developing specifications for steviol glycosides produced by 4 different manufacturing methods was established and adopted. The 4 manufacturing technologies are defined as (a) extraction; (b) fermentation; (c) enzymatic modification; and (d) enzymatic glucosylation (JECFA, 2019). The framework and the specifications for each production method have been published in the latest *Compendium of Food Additive Specifications* (JECFA, 2020), listing the separate specification for each as follows: (a) Steviol Glycosides from *Stevia rebaudiana* Bertoni; (b) Steviol Glycosides from Fermentation; (c) Enzyme Modified Steviol Glycosides; and (d) Enzyme Modified Glucosylated Steviol Glycosides. (b) Steviol Glycosides from Fermentation are defined as follows:

> "*Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used. Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of Yarrowia lipolytica and Saccharomyces cerevisiae that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides* […] *Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present*" (JECFA, 2020).

The specification parameters and limits defined for Steviol Glycosides from Fermentation are identical to those for Steviol Glycosides from *Stevia rebaudiana* Bertoni, such that Steviol Glycosides from Fermentation must contain not less than 95% of total of steviol glycosides, on the dried basis. Steviol glycoside mixtures produced by fermentation of simple sugars, Reb MD from *Y. lipolytica* as well as Reb MD from *S. cerevisiae,* meet the JECFA specification for Steviol Glycosides from Fermentation.

## A.10.2 United States

In the U.S., Reb MD produced by *Y. lipolytica* has GRAS status for use as a general-purpose sweetener in a variety of foods and beverages, excluding infant formula and products under the United States Department of Agriculture's jurisdiction, at use levels determined by Good Manufacturing Practice (GMP) and also is GRAS for use as a table top sweetener. Reb MD produced by *Y. lipolytica* has been GRAS-notified to the U.S. Food and Drug Administration (FDA) under GRN 000882, which received a "no questions" letter from the Agency (Cargill, 2019; U.S. FDA, 2020a). Reb MD produced by *Y. lipolytica* is substantially equivalent to "RebMD", a steviol glycoside preparation produced by *S. cerevisiae* that received a "no questions" letter from the U.S. FDA regarding its use as a general-purpose sweetener (GRN 000626, U.S. FDA, 2016a). Likewise, 2 other GRAS Notices for rebaudioside A and rebaudioside M produced by a similar strain of *Y. lipolytica* (GRN 000632 and GRN 000759) have also received "no questions" letters from the U.S. FDA (U.S. FDA, 2016b, 2018).

In general, several types of steviol glycoside preparations, including purified steviol glycosides (≥95% purity) extracted from *S. rebaudiana*, enzyme-modified steviol glycosides, and purified steviol glycosides produced *via* microbial fermentation or enzymatic bioconversion, have GRAS status in the U.S. for use as general-purpose sweeteners in a variety of foods and beverages. With the exception of the notifications currently undergoing review, all submitted notifications have received a "no questions" letter from the U.S. FDA. A summary of the GRAS Notices submitted to the U.S. FDA to date for steviol glycosides and the Agency's corresponding response, where available, is presented in Table A.10.2-1 (U.S. FDA, 2020b).

The use of powdered stevia leaves and its leaf-refined extracts in dietary supplement products have been notified to the U.S. FDA under the New Dietary Ingredient Notification requirements of the *Dietary Supplement Health and Education Act of 1994* (DSHEA, 1994; Geuns, 2003; Schoenhals, 2003). As such, stevia is used in a variety of energy bars and beverages that have been labelled and are marketed as dietary supplements (Schoenhals, 2003).

**Table A.10.2-1 Summary of GRAS Notices Submitted To-Date to the U.S. FDA for Steviol Glycosides (U.S. FDA, 2020b)**

| Company | Substance | FDA Response | GRAS Notice No. |
|---|---|---|---|
| Whole Earth Sweetener Company LLC (subsidiary of Merisant) | Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000252 |
| Cargill, Inc. | Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000253 |
| McNeil Nutritionals | Purified steviol glycosides with rebaudioside A as the principal component | No questions | GRN 000275 |
| Blue California | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000278 |
| Sweet Green Fields, LLC | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000282 |
| Wisdom Natural Brands | Purified steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000287 |
| Sunwin USA, LLC and Wild Flavors | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000303 |
| Sunwin USA, LLC and Wild Flavors | Purified steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000304 |
| Pyure Brands, LLC | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000318 |
| PureCircle USA, Inc. | Purified steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000323 |

**Table A.10.2-1 Summary of GRAS Notices Submitted To-Date to the U.S. FDA for Steviol Glycosides (U.S. FDA, 2020b)**

| Company | Substance | FDA Response | GRAS Notice No. |
|---|---|---|---|
| GLG Life Tech, Ltd. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000329 |
| NOW Foods | Enzyme modified steviol glycoside preparation (EMSGP) | No questions | GRN 000337 |
| GLG Life Tech, Ltd. | Stevioside purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (stevioside) | No questions | GRN 000348 |
| GLG Life Tech, Ltd. | Purified steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000349 |
| Guilin Layn Natural Ingredients, Corp. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000354 |
| BrazTek International Inc. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000365 |
| Sinochem Qingdao Co., Ltd. | Purified steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000367 |
| Zhucheng Haotian Pharm Co., Ltd. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000369 |
| Toyo Sugar Refining Co., Ltd. and Nippon Paper Chemicals Co., Ltd. | Enzyme modified steviol glycosides | No questions | GRN 000375 |
| GLG Life Tech Corporation | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000380 |
| Chengdu Wagott Pharmaceutical | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000388 |
| Chengdu Wagott Pharmaceutical | Steviol glycosides with stevioside as the principal component | No questions | GRN 000389 |
| Daepyung Co., Ltd. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000393 |
| Daepyung Co., Ltd. | Steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000395 |
| MiniStar International, Inc. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000418 |
| Daepyung Co., Ltd. | Enzyme-modified steviol glycosides | No questions | GRN 000448 |
| Daepyung Co., Ltd. | Enzyme-modified steviol glycosides | No questions | GRN 000452 |
| PureCircle USA, Inc. | Rebaudioside D purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside D) | No questions | GRN 000456 |
| Almendra Limited | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A) | No questions | GRN 000461 |
| Qufu Xiangzhou Stevia Products Co., Ltd. | Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni | No questions | GRN 000467 |
| PureCircle, Ltd. | Purified steviol glycosides with rebaudioside X as the principal component | No questions | GRN 000473 |
| GLG Life Tech Corporation | High purity steviol glycosides (minimum purity 95%) | No questions | GRN 000493 |
| GLG Life Tech Corporation | High purity rebaudioside M | No questions | GRN 000512 |

**Table A.10.2-1 Summary of GRAS Notices Submitted To-Date to the U.S. FDA for Steviol Glycosides (U.S. FDA, 2020b)**

| Company | Substance | FDA Response | GRAS Notice No. |
|---|---|---|---|
| Almenda (Thailand) Limited | Steviol glycosides with rebaudioside A and stevioside as the principal components | No questions | GRN 000516 |
| GLG Life Tech Corporation | High purity rebaudioside C | No questions | GRN 000536 |
| GLG Life Tech Corporation | High purity rebaudioside D | No questions | GRN 000548 |
| Procuctora Alysa SpA | High purity steviol glycosides (minimum purity 95%) consisting primarily of rebaudioside A | No questions | GRN 000555 |
| PureCircle Limited | Glucosylated steviol glycosides (minimum purity 80%) | No questions | GRN 000607 |
| PureCircle Limited | Purified steviol glycosides | No questions | GRN 000619 |
| Cargill, Inc. | Steviol glycosides produced in *Saccharomyces cerevisiae* | No questions | GRN 000626 |
| DSM Nutritional Products, LLC | Rebaudioside A from *Yarrowia lipolytica* | No questions | GRN 000632 |
| Hunan Huacheng Biotech Inc. | High purity steviol glycosides (minimum purity 97%) consisting primarily of rebaudioside A | No questions | GRN 000638 |
| GLG Life Tech Corporation | Enzyme-modified steviol glycosides | No questions | GRN 000656 |
| PureCircle USA | Glucosylated steviol glycosides (minimum purity 95%) | No questions | GRN 000662 |
| Blue California | Rebaudioside M | No questions | GRN 000667 |
| Xinghua GL Stevia Co., Ltd. | Purified steviol glycosides | No questions | GRN 000702 |
| Blue California | Rebaudioside D | No questions | GRN 000715 |
| Shangdong Shengxiangyuan Biotechnology | Purified steviol glycosides | No questions | GRN 000733 |
| PureCircle Limited | Steviol glycosides consisting primarily of rebaudioside M | No questions | GRN 000744 |
| PureCircle Limited | Steviol glycosides consisting primarily of rebaudioside M | No questions | GRN 000745 |
| DSM Food Specialties | Steviol glycosides consisting primarily of rebaudioside M produced in *Yarrowia lipolytica* | No questions | GRN 000759 |
| Sichuan Ingia Biosynthetic Co., Ltd. | Rebaudioside D | No questions | GRN 000764 |
| Cargill, Inc. | Stevia leaf extract | No questions | GRN 000768 |
| Tate and Lyle | Rebaudioside M | No questions | GRN 000780 |
| GLG Life Tech Corporation | Steviol glycosides (minimum purity 95%) | No questions | GRN 000790 |
| Steviana Bioscience (Suzhou) Inc. | Purified steviol glycosides | No questions | GRN 000795 |
| Sichuan Ingia Biosynthetic Co., Ltd. | Rebaudioside M | No questions | GRN 000799 |
| Amyris, Inc. | Rebaudioside M | No questions | GRN 000812 |
| Haigen-BGG Natural Ingredients Limited | Glucosylated steviol glycosides | No questions | GRN 000821 |
| Blue California | Rebaudioside E | No questions | GRN 000823 |
| Jiang Su Svetia Biotechnology Co., Ltd. | Purified steviol glycosides | No questions | GRN 000838 |
| Sinochem Health Company Ltd. | Purified steviol glycosides | No questions | GRN 000839 |
| GLG Life Tech Corporation | Rebaudioside M | No questions | GRN 000846 |
| Qufu Shengren Pharmaceutical Co., Ltd | Glucosylated steviol glycosides | No questions | GRN 000858 |
| Cargill, Inc. | Rebaudioside M | Withdrawn | GRN 000867 |
| Daepyung Co., Ltd. | Glucosylated steviol glycosides | Pending | GRN 000878 |
| Cargill, Inc. | Rebaudioside M | No questions | GRN 000882 |

FDA = Food and Drug Administration; GRAS = Generally Recognized as Safe; U.S. = United States.

### A.10.3 European Union

Steviol glycosides (E 960) are approved for use in the European Union (EU) as sweetening agents in a number of food and beverage categories. Steviol glycoside preparations available in the EU must comply with the specifications for steviol glycosides that were adopted by the European Commission in 2012 and updated in 2016 (EU, 2016). The steviol glycoside specifications presently stipulate that steviol glycoside products from *S. rebaudiana* must contain no less than 95% of 11 named steviol glycosides, including dulcoside, rebaudiosides A, B, C, D, E, F, and M, rubusoside, steviolbioside, and stevioside. The European Food Safety Authority (EFSA) recently published a scientific opinion on the safety of a proposed amendment to the steviol glycoside specification in the EU to expand the list of steviol glycosides to all those identified in the leaves of *S. rebaudiana* Bertoni (EFSA, 2020). The proposed change is to include all 60 steviol glycosides in the same limit value of 95% and is currently under review by the European Commission. In 2019, EFSA issued a scientific opinion on the safety of rebaudioside M produced *via* enzyme-catalysed bioconversion of purified stevia leaf extract and concluded that there are no safety concerns with this steviol glycoside preparation (EFSA, 2019). The EFSA Panel recommended that the European Commission consider establishing a separate specification for rebaudioside M produced *via* enzyme-catalysed bioconversion of purified stevia leaf extract. This recommendation is currently under review by the Commission.

### A.10.4 Canada

Steviol glycosides are approved for use in Canada as sweeteners in a variety of food categories. Health Canada has authorised the use of all steviol glycosides extracted from the *S. rebaudiana* Bertoni plant and has extended the approval to include those that are produced alternatively *via* *S. cerevisiae* production strains CD15380, CD15407, and Y63348 (Health Canada, 2020a,b). The Canadian purity standards of steviol glycosides state that all steviol glycosides, in combination, must reach the total of at least 95% purity in finished preparations (Health Canada, 2017). It is also understood that the inclusion of *S. rebaudiana* Bertoni as a permitted source of steviol glycosides also extends to include steviol glycosides extracted from the leaf that are then converted enzymatically to generate steviol glycosides.

### A.10.5 Asia

Steviol glycosides are approved for use in several countries located in the South and North Asia and Asia-Pacific regions (PureCircle Stevia Institute, 2020). For example, the Ministry of Health and Welfare in Japan has authorised the use of 3 types of stevia extracts: α-glucosyltransferase-treated stevia, powdered stevia, and stevia extract (Japan Food Chemical Research Foundation, 2014). Purified stevioside (crude extract, 50% purity, and ≥90% purity) and *S. rebaudiana* leaf extracts are also accepted for general use as sweeteners in foods and beverages in Japan (Marie, 1991; Das *et al.*, 1992; Ferlow, 2005). Likewise, the Food Safety and Standards Authority of India has approved the use of steviol glycosides in a variety of food and beverage categories (FSSAI, 2015; MOHFW, 2016).

### A.10.6 Central/South America

Stevioside, *S. rebaudiana* leaves, and highly refined extracts are permitted for use as low-calorie sweeteners in several countries in central and south America (PureCircle Stevia Institute, 2020). Of relevance, in Mexico, steviol glycosides produced by *S. cerevisiae* (*i.e.*, Reb MD) that are considered chemically equivalent to steviol glycosides produced by *Y. lipolytica,* and rebaudioside A and rebaudioside M from multiple gene donors expressed in *Y. lipolytica* (*i.e.,* the Reb MD that is the subject of this application) are approved food additives (Cofepris, 2018).

## A.11 Statutory Declaration

A signed Statutory Declaration for Australia is provided as Appendix A.

## A.12 Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix B.

# B. TECHNICAL INFORMATION ON THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a) the following technical information must be provided:

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

Each point is addressed in the following subsections.

## B.1 Nature and Technological Purpose of Reb MD

In Australia and New Zealand, food additives must comply with a monograph published from a specified list of sources, such as JECFA (FSANZ, 2020a). The JECFA Committee recently expanded the steviol glycoside monograph to include separate specifications for 4 different manufacturing technologies, namely (a) Steviol Glycosides from *Stevia rebaudiana* Bertoni; (b) Steviol Glycosides from Fermentation; (c) Enzyme Modified Steviol Glycosides; and (d) Enzyme Modified Glucosylated Steviol Glycosides (JECFA, 2020). (b) Steviol Glycosides from Fermentation are defined as follows:

> "*Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used. Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of Yarrowia lipolytica and Saccharomyces cerevisiae that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides* […] *Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present*" (JECFA, 2020).

Reb MD produced by *Y. lipolytica* meets the JECFA specification for Steviol Glycosides from Fermentation.

Reb MD, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand such as Reb MD from *S. cerevisiae* strain CD15407 (Application A1170), would be used as a high-intensity sweetener in reduced-calorie or no-sugar-added products. While steviol glycoside preparations are already available for use in food as sweeteners throughout Australia and New Zealand and many other parts of the world, the use of Reb MD as an alternative to the major individual steviol glycosides, such as stevioside and rebaudioside A, presents an improved sensory profile and therefore a better sweetness quality for consumers, as discussed in the following Section B.1.1.

### B.1.1 Taste Attributes

Preparations of steviol glycosides are used primarily as sweeteners in various food products and are generally 200 to 350 times sweeter than sucrose. The sweetness intensity of individual steviol glycosides vary. For instance, the sweetness potency of rebaudioside A is often quoted as 200 to 300 times sweeter than sucrose (DuBois *et al*., 1991), whereas rebaudioside M has been shown to up to 350 times as sweet as sugar (Prakash *et al*., 2014). Sweetness potency, however, depends strongly on concentration for all high potency sweeteners (HPS). For accuracy, it is usual to state the sucrose equivalence at which a particular potency has been measured, as well as the measuring medium or matrix. For comparison of different HPS, the most common medium is water and where the medium is not specified, it is always presumed to be water. However, there is no industry-wide agreement on a common sucrose equivalency at which to quote sweetness potency values. Realistic use levels of HPS are generally in the range of 4 to 8% sucrose equivalence. For instance, at 6% sucrose equivalence, the potency of rebaudioside A is 200 (DuBois *et al*., 1991).

Recent investigations into the sensory characteristics of additional steviol glycosides (*i.e.*, rebaudioside M) were published by Prakash *et al.* (2014). Using the Beidler Model, it was estimated that rebaudioside M is 200 to 350 times sweeter than sucrose. Sensory testing of rebaudioside M compared to rebaudioside A (in water) indicated that the 2 steviol glycosides had a similar sweetness intensity; however, rebaudioside M had a reduced perception of bitterness, astringency, and bitter lingering. Overall, rebaudioside M was found to have a clean, sweet taste with a slightly bitter or liquorice aftertaste and demonstrated functionality in a range of beverages and foods either on its own or blended with other non-caloric or carbohydrate sweeteners. These results are consistent with the generally recognised sweetness of steviol glycosides as 100 to 300 times sweeter than sucrose as specified by JECFA (JECFA, 2020).

## B.1.2 Stability

### *B.1.2.1 General Stability of Steviol Glycosides*

The stability of steviol glycosides is discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999) and has been reviewed by several scientific advisory bodies and regulatory agencies involved in the evaluation of steviol glycoside safety (JECFA, EFSA, and FSANZ). Specifically, at the 68$^{th}$ meeting of the JECFA, the Committee evaluated the stability of steviol glycosides under conditions simulating their use in foods (JECFA, 2007a). JECFA noted that steviol glycosides do not undergo browning or caramelisation when heated and are reasonably stable under elevated temperatures used in food processing. Based on the findings from the studies submitted for review, 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. In particular, steviol glycosides with purities between 90 to 94% are stable for at least 180 days when stored at temperatures up to 24°C in acidic solutions (pH 2 to 4). Conversely, when water solutions of steviol glycosides were heated to 80°C for 8 hours at pH 4.0 and 3.0, 4 and 8% decomposition, respectively, was observed. When the temperature was increased further to 100°C, increased rates of steviol glycoside decomposition, equivalent to 10 and 40% at pH 4.0 and 3.0, respectively, were observed. These results indicate that the stability of steviol glycosides is pH and temperature dependent.

The stability of high purity rebaudioside A (≥97%) under conditions simulating the proposed conditions of use was described in detail in GRN 000253 for rebaudioside A purified from *S. rebaudiana* (Bertoni) Bertoni (GRN 000253 – U.S. FDA, 2008). Studies assessing the bulk stability of the rebaudioside A product (dry), as well as the stability of the ingredient in representative food matrices (real food/beverages at both room and elevated temperatures), were summarised within the Notice. The photostability of rebaudioside A also was examined under dry and aqueous conditions. Collectively, the results from stability studies conducted with rebaudioside A demonstrate its stability in foods representing a broad spectrum of pH and temperature conditions, corroborating the findings by JECFA at their 68$^{th}$ meeting. Given the structural similarities of steviol glycosides as described in Sections B.2 and B.3 that follow, it is expected that the stability characteristics of the Reb MD product would be very similar to those observed for rebaudioside A.

### *B.1.2.2 Stability of Reb MD*

The first study is a conventional shelf-life stability study with 3 non-consecutive lots (Lot No. 200304-01, 200306-02, and 200115-03) of Reb MD. Samples are being analysed in duplicate for moisture content, loss on drying, steviol glycoside content, and microbial parameters (aerobic plate count, yeast, and mould). The study is being conducted in a controlled environment at 25°C and 60% relative humidity. Testing has occurred at baseline and 3 months, with continued time point testing currently underway. Available results are provided in Table B.1.2.2-1 and support the stability of steviol glycosides produced by *Y. lipolytica* (*i.e.,* Reb MD) under conventional shelf-life stability conditions for up to 3 months.

The second study is an accelerated shelf-life stability study with 3 non-consecutive lots (Lot No. 200304-01, 200306-02, and 200115-03) of Reb MD. Samples are being analysed in duplicate for moisture content, loss on drying, and steviol glycoside content. The study is being conducted in a controlled environment at 40°C and 75% relative humidity. Testing has occurred at baseline, 1, 2, and 3 months, with continued time point testing currently underway. Available results are provided in Table B.1.2.2-2 and support the stability of steviol glycosides produced by *Y. lipolytica* (*i.e.,* Reb MD) under accelerated shelf-life stability conditions for up to 3 months.

**Table B.1.2.2-1 Results from a Conventional Stability Study on 3 Commercial Lots of Reb MD**

| Parameter | Lot No. 200304-01 | | Lot No. 200306-02 | | Lot No. 200115-03 | |
|---|---|---|---|---|---|---|
| | Initial | 3 m | Initial | 3 m | Initial | 3 m |
| Reb M (%) | 91.80 | 92.45 | 90.75 | 90.20 | 92.80 | 92.70 |
| Reb A (%) | 1.50 | 1.47 | 1.43 | 1.41 | 1.37 | 1.35 |
| Reb B (%) | 0.89 | 0.95 | 0.95 | 1.02 | 0.76 | 0.83 |
| Reb C (%) | ND | ND | ND | ND | ND | ND |
| Reb D (%) | 4.67 | 4.66 | 5.52 | 5.47 | 4.66 | 4.60 |
| Reb F (%) | ND | ND | ND | ND | ND | ND |
| Rubusoside (%) | ND | ND | ND | ND | ND | ND |
| Stevioside (%) | ND | ND | ND | ND | ND | ND |
| Steviolbioside (%) | ND | ND | ND | ND | ND | ND |
| Dulcoside A (%) | ND | ND | ND | ND | ND | ND |
| TSG (%) | 98.85 | 99.55 | 98.65 | 98.10 | 99.60 | 99.55 |
| Moisture initial (%) | NA | NA | NA | NA | 5.21 | 4.20 |
| Moisture after equilibration (%) | 5.98 | 8.98 | 6.60 | 9.21 | 7.67 | 8.64 |
| LOD (%) | 4.70 | 4.80 | 5.80 | 5.50 | 4.00 | 4.00 |
| Aerobic plate count (CFU/g) | <10 | <10 | <10 | <10 | <10 | ≈110 |
| Yeast (CFU/g) | <10 | <10 | <10 | <10 | <10 | <10 |
| Mould (CFU/g) | <10 | <10 | <10 | <10 | <10 | <10 |

CFU = colony forming units; LOD = loss on drying; m = month(s); NA = not applicable; ND = not detected; Reb = rebaudioside; TSG = total steviol glycosides.

**Table B.1.2.2-2 Results from an Accelerated Stability Study on 3 Commercial Lots of Reb MD**

| Parameter | Lot No. 200304-01 | | | | Lot No. 200306-02 | | | | Lot No. 200115-03 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | 1 m | 2 m | 3 m | Initial | 1 m | 2 m | 3 m | Initial | 1 m | 2 m | 3 m |
| Reb M (%) | 91.80 | 92.90 | 91.45 | 92.00 | 90.75 | 91.00 | 90.25 | 90.85 | 92.80 | 91.45 | 91.75 | 91.25 |
| Reb A (%) | 1.50 | 1.50 | 1.48 | 1.46 | 1.43 | 1.43 | 1.44 | 1.42 | 1.37 | 1.34 | 1.35 | 1.31 |
| Reb B (%) | 0.89 | 1.07 | 1.18 | 1.23 | 0.95 | 1.22 | 1.35 | 1.42 | 0.76 | 0.87 | 1.01 | 1.06 |
| Reb C (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Reb D (%) | 4.67 | 4.65 | 4.60 | 4.65 | 5.52 | 5.43 | 5.60 | 5.56 | 4.66 | 4.57 | 4.58 | 4.50 |
| Reb F (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Rubusoside (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Stevioside (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Steviolbioside (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Dulcoside A (%) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| TSG (%) | 98.85 | 100.00 | 98.85 | 99.30 | 98.65 | 99.10 | 98.65 | 96.20 | 99.60 | 98.25 | 98.65 | 98.15 |
| Moisture initial (%) | NA | 5.52 | NA | NA | NA | 6.61 | NA | NA | 5.21 | NA | NA | NA |
| Moisture after equilibration (%) | 5.98 | 7.89 | 6.68 | 8.57 | 6.60 | 8.25 | 7.26 | 11.30 | 7.67 | 7.67 | 7.58 | 8.15 |
| LOD (%) | 4.70 | 4.60 | 4.90 | 4.60 | 5.80 | 5.40 | 5.70 | 5.40 | 4.00 | 4.00 | 4.10 | 4.10 |

CFU = colony forming units; LOD = loss on drying; m = month(s); NA = not applicable; ND = not detected; Reb = rebaudioside; TSG = total steviol glycosides.

## B.2 Information to Enable Identification of Reb MD

Information to enable the identification of Reb MD, including the chemical structure, the chemical name, the molecular weight and formula, and the common name, is presented below.

### B.2.1 Identity of Substance

Reb MD is obtained from a *Y. lipolytica* production strain *via* fermentation of simple sugars and is primarily comprised of rebaudioside M and may contain a mixture of the following additional glycosides in various concentrations, which are naturally present in the leaves of the *S. rebaudiana* Bertoni plant, such that the total steviol glycoside content is no less than 95%: rebaudiosides A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and/or dulcoside A. Steviol glycosides produced *via* fermentation of simple sugars using a *Y. lipolytica* production strain are identical to steviol glycosides extracted from the leaves of *S. rebaudiana* and conform to the current JECFA purity criteria of ≥95% steviol glycosides (INS No. 960). Steviol glycosides produced by *Y. lipolytica* meet the JECFA definition for Steviol Glycosides from Fermentation as being, "*a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose)*" (JECFA, 2020) and are chemically and substantially equivalent to rebaudioside MD that is obtained from *S. cerevisiae* strain CD15407 (S3—39) (the subject of Application A1170). Reb MD will be sold under the proposed trade name of 'EverSweet'.

The chemical names for the individual steviol glycosides that may be present in Reb MD are listed below.

| | |
|---|---|
| Rebaudioside A | 13-[(2-O-β-D-glucopyranosyl-3-O-β-Dglucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester |
| Rebaudioside B | 13-[(2-O-β–D-glucopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid |
| Rebaudioside C | 13-[(2-O-α–L-rhamnopyranosyl-3-O-β–D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester |
| Rebaudioside D | 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 |
| Rebaudioside E | 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 |
| Rebaudioside F | 13-[(2-O-β-D-xylofurananosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester |
| Rebaudioside M | 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-3-O-β-D-glucopyranosyl -β-D-glucopyranosyl ester |
| Stevioside | 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester |
| Steviolbioside | 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid |

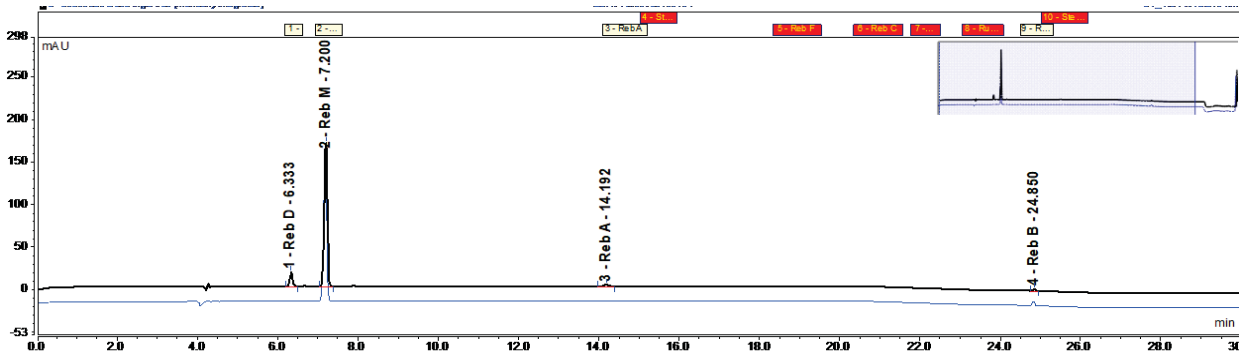
| | |
|---|---|
| Rubusoside | 13-β-D-glucopyranosyloxykaur-16-en-18-oic acid, β-D-glucopyranosyl ester |
| Dulcoside A | 13-[(2-O-α–L-rhamnopyranosyl-β–D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, β-D-glucopyranosyl ester |

Steviol glycosides extracted from *S. rebaudiana* Bertoni are identified based on the high-performance liquid chromatography (HPLC) method published by JECFA that previously identified only 9 principal glycosides (JECFA, 2010), but has been updated to include all steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2017a, 2020). Cargill has developed an HPLC method to detect steviol glycosides based on the JECFA (2010) method, by optimizing the technique to detect steviol glycosides with higher degrees of glycosylation (*e.g.*, rebaudiosides M and D), similar to the new JECFA HPLC method. Further details on Cargill's HPLC method are provided in Section B.6.4 and Appendix D.

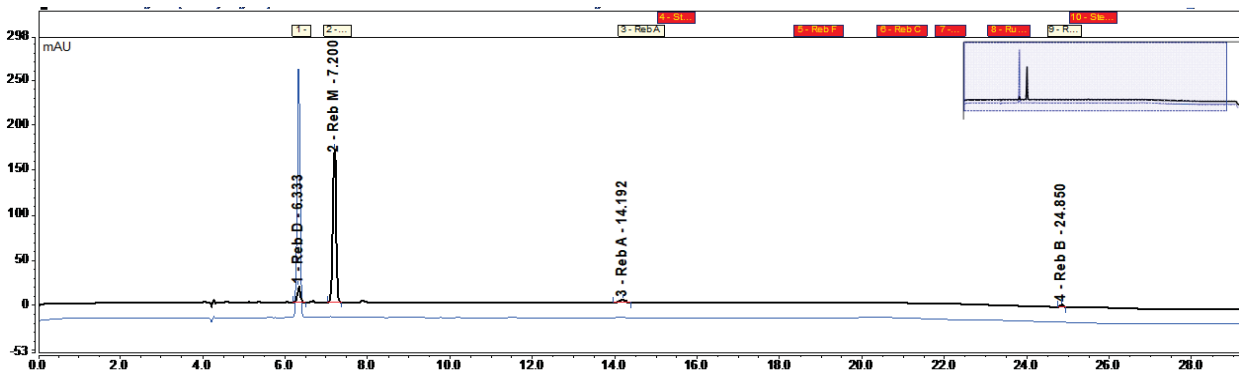
An example chromatogram of a purified steviol glycoside product from *Y. lipolytica* (Reb MD Lot No. 200306-B1) obtained using Cargill's HPLC method is presented in Figure B.2.1-1. The 2 primary glycosides present in Reb MD, rebaudiosides M and D, were identified in the chromatogram at retention times of 7.200 and 6.333 minutes, based on the retention times of the rebaudioside M and rebaudioside D standards that are overlaid on the chromatograms in Figure B.2.1-1 (blue traces). The rebaudioside M and D standards are derived from *S. rebaudiana* Bertoni. Therefore, these data demonstrate that steviol glycosides produced by *Y. lipolytica* production strains have the same HPLC retention times as steviol glycosides from *S. rebaudiana* Bertoni and establish that steviol glycosides from these 2 sources are chemically identical. The same applies to steviol glycosides produced by *S. cerevisiae* production strains, as previously presented in Cargill's approved FSANZ application A1170 (FSANZ, 2019b).

**Figure B.2.1-1 Example High-Performance Liquid Chromatography Chromatogram of Reb MD (Lot No. 200306-B1; black trace)**

951) Overlay with Rebaudioside M Standard (blue trace)




b) Overlay with Rebaudioside D Standard (blue trace)




Reb = rebaudioside.

### B.2.2 Composition

The fermentation product, Reb MD, is primarily comprised of rebaudioside M and may contain a mixture of the following additional glycosides in various concentrations, such that the total steviol glycoside content is no less than 95%: rebaudiosides A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and/or dulcoside A. The composition of Reb MD produced by *Y. lipolytica* is chemically and substantially equivalent to rebaudioside MD that is obtained from *S. cerevisiae* strain CD15407 (S3—39) (the subject of Application A1170). The distribution of steviol glycosides present in Reb MD will vary depending on the production process and final product formulation, as described in Section B.5. Purified Reb MD meets or exceeds the ≥95% steviol glycoside purity definition for Steviol Glycosides from Fermentation established by JECFA (JECFA, 2020). The Chemical Abstract Service numbers, empirical formulae, molecular weights, and $R_1$ and $R_2$ groups for the individual steviol glycosides that may be present in the final Reb MD product, as well as the aglycone steviol, are summarised in Table B.2.2-1.

**Table B.2.2-1 Molecular Weight and Formula, and R-Groups in Backbone Structure (see Figure B.3-1)**

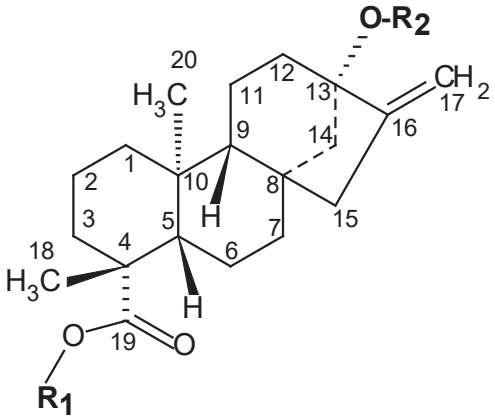
| Steviol Glycoside | CAS Number | Molecular Weight | Molecular Formula | R-Groups in Backbone Structure | |
|---|---|---|---|---|---|
| | | | | $R_1$ | $R_2$ |
| Rebaudioside A | 58543-16-1 | 967.01 | $C_{44}H_{70}O_{23}$ | β-Glc | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- |
| Rebaudioside B | 58543-17-2 | 804.88 | $C_{38}H_{60}O_{18}$ | H | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- |
| Rebaudioside C | 63550-99-2 | 951.02 | $C_{44}H_{70}O_{22}$ | β-Glc | Rhaα(1-2)[Glcβ(1-3)]Glcβ1- |
| Rebaudioside D | 63279-13-0 | 1,129.15 | $C_{50}H_{80}O_{28}$ | B-Glc-β-Glc(2-1) | Glcβ(1-2)[Glcβ(1-3)]Glcβ1 |
| Rebaudioside E | 63279-14-1 | 967.01 | $C_{44}H_{70}O_{23}$ | Glcβ(1-2)Glcβ1- | Glcβ(1-2)Glcβ1- |
| Rebaudioside F | 438045-89-7 | 936.99 | $C_{43}H_{68}O_{22}$ | β-Glc | β-Glc-β-Xyl(2-1) |
| Rebaudioside M | 1220616-44-3 | 1,291.3 | $C_{56}H_{90}O_{33}$ | Glcβ(1-2)[Glcβ (1-3)]Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- |
| Stevioside | 57817-89-7 | 804.88 | $C_{38}H_{60}O_{18}$ | β-Glc | β-Glc-β-Glc(2-1) |
| Steviolbioside | 41093-60-1 | 642.73 | $C_{32}H_{50}O_{13}$ | H | β-Glc-β-Glc(2-1) |
| Rubusoside | 64849-39-4 | 642.73 | $C_{32}H_{50}O_{13}$ | β-Glc | β-Glc |
| Dulcoside A | 64432-06-0 | 788.88 | $C_{38}H_{60}O_{17}$ | β-Glc | β-Glc-α-Rha(2-1) |
| Steviol | 471-80-7 | 318.46 | $C_{20}H_{30}O_3$ | H | H |

CAS = Chemical Abstracts Service; Glc = Glucose; Rha = Rhamnose; Xyl = Xylose.

## B.3 Information on the Chemical and Physical Properties of Reb MD

Steviol glycosides produced by *Y. lipolytica* production strains (*i.e.,* Reb MD) is a white to off-white powder with a characteristic sweet taste, consistent with the description of commercial steviol glycoside preparations in the most recent Chemical and Technical Assessment (CTA) published by JECFA (FAO, 2016). All steviol glycosides, either extracted from the *S. rebaudiana* Bertoni leaf or produced by *S. cerevisiae* or *Y. lipolytica* production strains, share the same backbone structure (see Figure B.3-1) and individual glycosides differ only with respect to the type and number of sugar moieties at positions $R_1$ and $R_2$. On this basis, Reb MD produced by *Y. lipolytica* is chemically and substantially equivalent to rebaudioside MD that is obtained from *S. cerevisiae* strain CD15407 (S3—39) (the subject of Application A1170). Based on the structural similarities among steviol glycosides, it is expected that the physiochemical properties of steviol glycosides produced from *Y. lipolytica* will be identical to those of steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni.

**Figure B.3-1 Backbone Structure for Steviol Glycosides**




## B.4 Information on the Impurity Profile of Reb MD

Reb MD is a high purity steviol glycoside product containing no less than 95% steviol glycosides, and all specification parameters and limits for Reb MD are consistent with those defined by FSANZ for steviol glycosides from fermentation (S3—39), by JECFA for Steviol Glycosides from Fermentation and by the European Commission for Steviol Glycosides from *S. rebaudiana* Bertoni. As such, microbiological and heavy metal specification parameters that have been established for steviol glycosides to ensure safe use in food are applied to Reb MD. Batch samples of Reb MD are routinely tested to verify compliance with the set chemical and microbiological specification parameters. Additionally, since Reb MD is produced from *Y. lipolytica*, the absence of protein following Reb MD purification has been confirmed using the bicinchoninic acid (BCA) protein assay (limit of detection of 25 ppm) and the absence of residual DNA has been confirmed using polymerase chain reaction (PCR) analysis (limit of detection of 10 ng/g Reb MD). The details of these analyses are provided in Section B.6.4.

## B.5 Manufacturing Process of Reb MD

### B.5.1 Overview

Reb MD is a purified steviol glycoside mixture that is produced *via* fermentation of simple sugars (including dextrose and sucrose) using a *Y. lipolytica* production strain that has been engineered to produce steviol glycosides. The manufacturing process for Reb MD produced by *Y. lipolytica* is similar to the manufacturing process for rebaudioside MD from *S. cerevisiae* strain CD15407 (S3—39) that was evaluated by FSANZ under Application A1170. Reb MD is manufactured in accordance with current Good Manufacturing Practice (cGMP). Following the fermentation, Reb MD is purified in accordance with the methodologies outlined in the CTA published by Food and Agriculture Organization of the United Nations/JECFA for steviol glycosides (FAO, 2016). A schematic overview of the manufacturing process for Reb MD is presented in Figure B.5.1-1 below.

To note, Reb MD is currently manufactured in the U.S. and it will not itself be manufactured in Australia or New Zealand, thus the fermentation substrates, production organism, and processing aids used for its manufacture will not enter the territory.

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

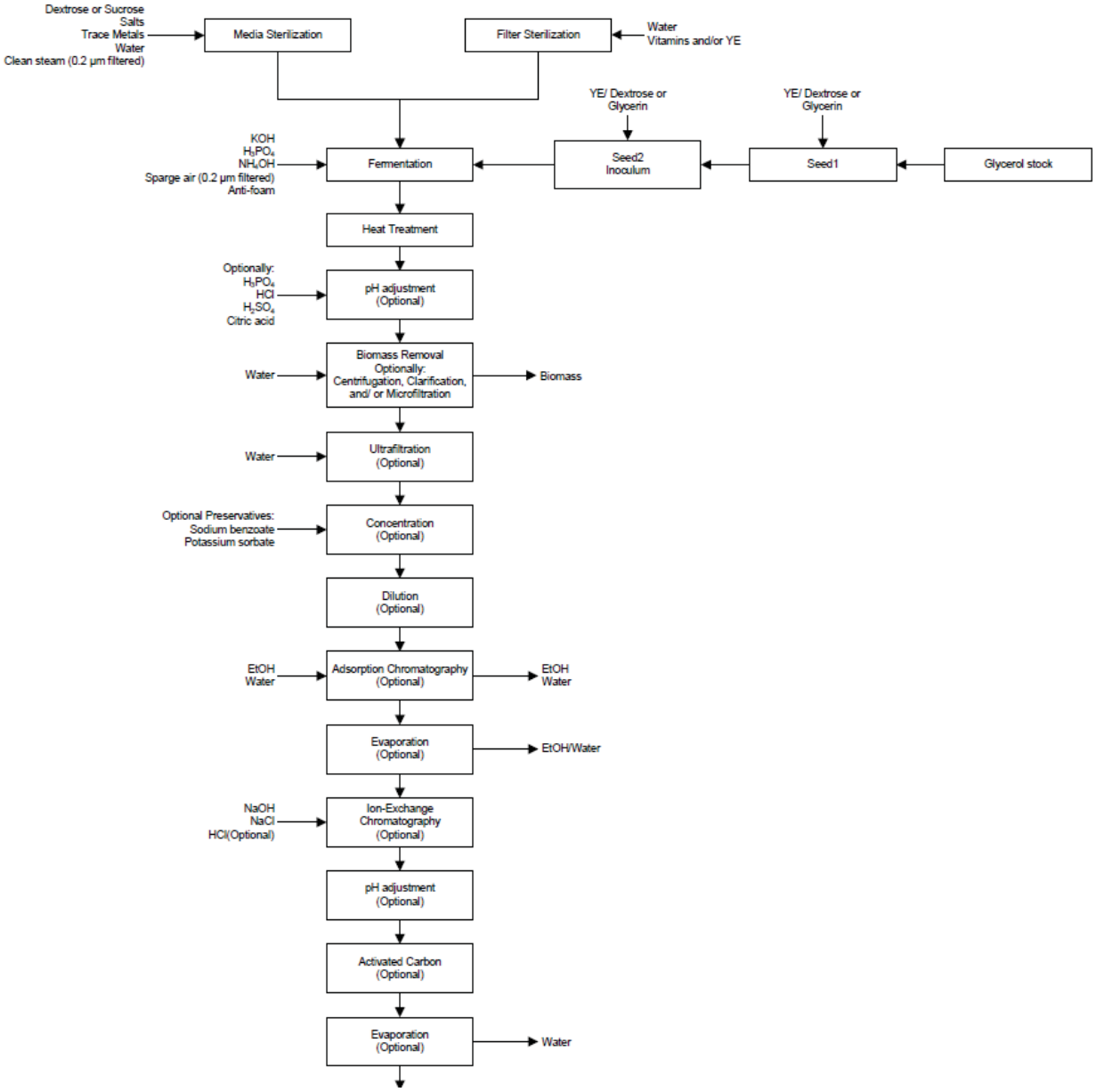
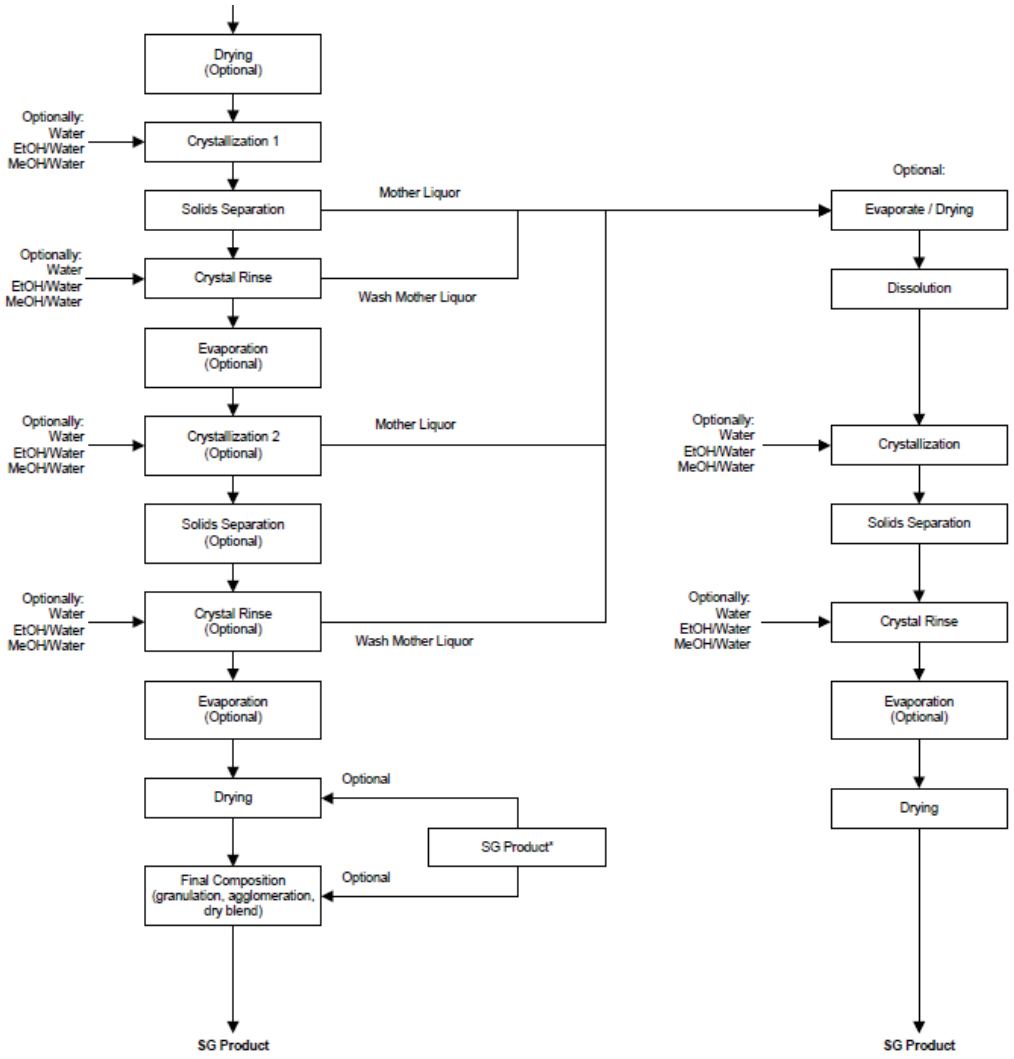


**Figure B.5.1-1 Schematic Overview of the Manufacturing Process for Reb MD**




Reb = rebaudioside; SG = steviol glycoside; YE = yeast extract.
* Represents the optional blending with additional Reb MD product that meets product specifications.

## B.5.2 Raw Materials, Processing Aids, and Equipment Specifications

Information regarding the raw materials, processing aids, and equipment used during the manufacture of Reb MD is provided in Table B.5.2-1. All raw materials, processing aids, and equipment listed below are food-grade quality and comply with relevant *Food and Chemicals Codex* or other internationally recognised standards. To note, the purification and filtration aids used in the second part of the manufacturing process to purify Reb MD are already recognised for use in the manufacture of steviol glycosides. Of note, none of the fermentation medium raw materials listed in Table B.5.2-1 have been derived from major allergens. Moreover, the nature of the crystallisation step in the manufacture of Reb MD (as described in Section B.5.3 below) removes any trace of culture medium from the final product.

**Table B.5.2-1 Raw Materials, Processing Aids, and Equipment Used in the Manufacture of Reb MD**

| Raw Material/Processing Aid/Equipment | Use |
|---|---|
| Dextrose | Fermentation medium |
| Sucrose | Fermentation medium |
| Glycerine | Fermentation medium |
| Ammonium sulphate | Fermentation medium |
| Potassium phosphate | Fermentation medium |
| Magnesium sulphate | Fermentation medium |
| Potassium sulphate | Fermentation medium |
| Sodium sulphate | Fermentation medium |
| Biotin | Fermentation medium |
| Calcium pantothenate | Fermentation medium |
| Niacin (nicotinic acid) | Fermentation medium |
| Thiamine | Fermentation medium |
| Pyridoxine | Fermentation medium |
| para-Aminobenzoic acid | Fermentation medium |
| Myo-inositol | Fermentation medium |
| Sodium Hydroxide | Fermentation medium; ion-exchange regeneration |
| Sodium EDTA | Fermentation medium |
| Zinc sulphate | Fermentation medium |
| Manganese chloride | Fermentation medium |
| Manganese sulphate | Fermentation medium |
| Copper sulphate | Fermentation medium |
| Calcium chloride | Fermentation medium |
| Ferrous sulphate | Fermentation medium |
| Potassium iodide | Fermentation medium |
| Ammonium hydroxide | Fermentation medium |
| Citric acid | Fermentation medium |
| Phosphoric acid | Fermentation medium |
| Potassium hydroxide | Fermentation medium |
| Sulfuric acid | Fermentation medium |
| Antifoam | Fermentation medium |
| Boiler chemicals | Fermentation medium |
| Yeast extract | Seed cultures |
| Potassium sorbate | Preservative |
| Sodium benzoate | Preservative |
| Microfiltration/ Ultrafiltration | Purification |
| Adsorption resin | Purification |
| Ion-exchange resin | Purification |
| Hydrochloric acid | Ion-exchange regeneration |
| Activated carbon | Decolorizing agent |
| Ethanol[a] | Elution solvent crystallisation |
| Methanol[b] | Crystallisation |

EDTA = ethylenediaminetetraacetic acid; Reb = rebaudioside.

[a] Joint FAO/WHO Expert Committee on Food Additives (JECFA) specifications for steviol glycosides specify a level of not more than 5,000 ppm for ethanol residues.

[b] JECFA specifications for steviol glycosides specify a level of not more than 200 ppm for methanol residues.

### B.5.3 Manufacturing Process

Dextrose or sucrose, salts, trace metals, and water are steam-sterilised (121°C for 30 minutes) and mixed with the filter-sterilised vitamins, yeast extract, and filtered deionised water to create the fermentation medium. The final medium is mixed with the yeast inoculum, which has been grown sequentially from the original glycerol stock solution using dextrose or glycerine and yeast extract as nutrition sources and allowed to ferment under aerobic conditions. The pH of the fermentation process is maintained at pH 4.5 to 6.5 using potassium hydroxide, phosphoric acid, or ammonium hydroxide. The fermentation broth undergoes heat treatment (75 to 95°C, 5 minutes to 1 hour) to stop fermentation and kill the yeast cells. Optionally, the pH of the broth is adjusted to <4.5 with phosphoric acid, hydrochloric acid, sulfuric acid, or citric acid and the yeast biomass is subsequently removed from the dissolved product by any combination of centrifugation, microfiltration, or clarification. The product stream may be purified by ultrafiltration to further remove dissolved proteins and other nitrogen-containing compounds. Preservatives such as potassium sorbate and sodium benzoate may be added to the filtrate and the pH of the filtrate may be lowered to <4.2 to minimise microbial contamination downstream. The filtrate may undergo typical purification processes used for steviol glycosides extracted from *S. rebaudiana* Bertoni leaves. Additionally, the optional drying steps described below can be utilised to vary the percentages of the individual steviol glycosides in the final product.

The filtrate may be passed through an adsorption resin, retaining steviol glycosides, thus separating them from other constituents that may be present in the filtrate. The resin is subsequently washed with ethanol or water to elute steviol glycosides. The eluate undergoes evaporation to remove ethanol or water. The steviol glycoside-rich eluate may be further treated through ion-exchange resins and optional pH adjustment and activated carbon treatment to remove additional impurities and coloured substances from the eluate. The eluate is concentrated by evaporation to initiate crystallisation in water or mixed with aqueous ethanol or methanol to start crystallisation. A second crystallisation may be conducted depending upon the desired steviol glycoside composition in final product. Optionally, the eluate may be dried prior to crystallisation in the presence of aqueous ethanol or methanol. The mother liquor is separated from the solids and retained for further processing. The crystals are rinsed with water or ethanol (wash added to mother liquor) and optionally evaporated prior to drying. The product is then checked for its final composition using HPLC and released as the final steviol glycoside product (Reb MD). Additionally, the final product may be blended with Reb MD from other production lots that meet the specifications outlined in Section B.6.

As mentioned above, the mother liquor separated from the steviol glycoside crystals is further dried or the liquid concentrated by evaporation to isolate any remaining steviol glycosides. The concentrate or solids are re-dissolved in water or aqueous ethanol or methanol to crystallise steviol glycosides. The crystals are separated, rinsed with water or ethanol, optionally evaporated, and dried. Following drying, the steviol glycosides may be designated as the final product or mixed with Reb MD produced previously.

## B.6 Specification for Identity and Purity of Reb MD

### B.6.1 Existing Specifications for Steviol Glycosides

Specifications for 4 types of steviol glycoside products are defined in Schedule 3 of the *Australia New Zealand Food Standards Code*, including “rebaudioside M” (S3—31), “steviol glycoside mixtures containing rebaudioside M” (S3—32), “steviol glycosides from *Stevia rebaudiana* Bertoni” (S3-35), and “steviol glycosides from fermentation” (S3—39) (FSANZ, 2020a). Of relevance to this application, the specification for steviol glycosides from fermentation (S3—39) relates to steviol glycoside preparations that (a) are obtained from fermentation; (b) are not obtained from the leaves of the *S. rebaudiana* Bertoni plant; and (c) contain a prescribed steviol glycoside. Currently, the only listed prescribed steviol glycoside in S3—39 is rebaudioside MD that is sourced from “*S. cerevisiae strain CD15407 containing novel genes for the production of rebaudiosides*”. Reb MD produced by *Y. lipolytica* is chemically and

substantially equivalent to rebaudioside MD from *S. cerevisiae* strain CD15407. The current specification for Steviol Glycosides from Fermentation in the *Australia New Zealand Food Standards Code* is presented in Table B.6.1-1.

JECFA recently established and adopted a framework for developing specifications for steviol glycosides produced by different technologies. The current framework for steviol glycosides encompasses 4 different manufacturing technologies defined as: (a) extraction; (b) fermentation; (c) enzymatic modification; and (d) enzymatic glucosylation (JECFA, 2019). Separate specifications for each technology have been established. Of relevance to this application, the specification for Steviol Glycosides from Fermentation is defined as follows:

> "*Steviol glycosides from fermentation consist of a mixture of compounds containing a steviol backbone conjugated to various sugar moieties (e.g. glucose or sucrose) depending on the specific production organism and fermentation conditions used. Steviol glycosides from fermentation are obtained from the fermentation of non-toxigenic non-pathogenic strains of Yarrowia lipolytica and Saccharomyces cerevisiae that have been genetically modified with heterologous genes from multiple donor organisms to overexpress steviol glycosides* […] *Commercial products are primarily composed of either rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D; additional minor steviol glycosides may be present*" (JECFA, 2020).

The current JECFA specification for Steviol Glycosides from Fermentation is presented in Table B.6.1-1.

**Table B.6.1-1 Existing Specifications for Steviol Glycosides from Fermentation**

| Specification Parameter | | FSANZ Specification for Steviol Glycosides from Fermentation (FSANZ, 2020a) | JECFA Specification for Steviol Glycosides from Fermentation (JECFA, 2020) |
|---|---|---|---|
| Description | | White to light yellow powder, approximately 200 to 300 times sweeter than sucrose. | White to light yellow powder, odourless or having a slight characteristic odour. About 200 to 300 times sweeter than sucrose. |
| Assay | | NLT 95% steviol glycosides on the dried basis | NLT 95% total steviol glycosides, on the dried basis |
| Solubility | | Freely soluble in water | Freely soluble in a mixture of ethanol and water (50:50), sparingly soluble in water and sparingly soluble in ethanol |
| pH | | Between 4.5 and 7.0 (1% solution) | Between 4.5 and 7.0 (1 in 100 solution) |
| Ash | | NMT 1% | NMT 1% |
| Loss on drying | | NMT 6% (105°C, 2 hour) | NMT 6% (105°, 2 h) |
| Residual solvents: | Methanol | NMT 200 mg/kg | NMT 200 mg/kg |
| | Ethanol | NMT 5,000 mg/kg | NMT 5,000 mg/kg |
| Arsenic | | NMT 1 mg/kg | NMT 1 mg/kg |
| Lead | | NMT 1 mg/kg | NMT 1 mg/kg |
| Cadmium | | NMT 1 mg/kg | NS |
| Mercury | | NMT 1 mg/kg | NS |
| Total (aerobic) plate count | | NS | NMT 1,000 CFU/g |
| Yeasts and moulds | | NS | NMT 200 CFU/g |
| *Escherichia coli* | | NS | Negative in 1 g |
| *Salmonella* | | NS | Negative in 25 g |

CFU = colony forming units; FSANZ = Food Standards Australia New Zealand; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NLT = not less than; NMT = not more than; NS = not specified.

## B.6.2 Proposed Specifications for Reb MD

The steviol glycoside mixture (Reb MD) produced from a *Y. lipolytica* production strain meets the general specification parameters for Steviol Glycosides from Fermentation as defined in S3—39 and outlined in Table B.6.1-1 above. Reb MD is listed as a prescribed steviol glycoside in the specification, but the only source is defined as *S. cerevisiae* strain CD15407. Therefore, it is proposed that the list of prescribed steviol glycosides in S3—39 be amended to include *Y. lipolytica* VRM as a source for Reb MD as follows in Table B.6.2-1 (amended text is italicised).

**Table B.6.2-1 Prescribed Steviol Glycosides (S3—39) (FSANZ, 2020a)**

| Steviol Glycoside | Source |
|---|---|
| Rebaudioside MD | *Saccharomyces cerevisiae* strain CD15407 containing novel genes for the production of rebaudiosides |
| | *Yarrowia lipolytica* strain VRM containing novel genes for the production of steviol glycosides |

## B.6.3 Product Analysis

### *B.6.3.1 Physical and Chemical Analysis of Reb MD*

Analysis of 5 non-consecutive lots of Reb MD verified that the manufacturing process as described in Section B.5.3 produces a consistent product that meets the specifications for steviol glycosides from fermentation (S3—39) (FSANZ, 2020a). A summary of the physical and chemical analyses for the 5 lots is presented in Table B.6.3.1-1 (see Appendix E for Certificates of Analysis). All Cargill internal analytical methods (*i.e.,* "ERT-xxx-x") are provided in Appendix C-3.

**Table B.6.3.1-1 Summary of Physical and Chemical Analyses for 5 Lots of Reb MD**

| Specification Parameter | FSANZ Specification[a] | Analytical Method | Manufacturing Lot | | | | |
|---|---|---|---|---|---|---|---|
| | | | 200124-B1 | 200221-B1 | 200226-B1 | 200302-B1 | 200306-B1 |
| Description | White to light yellow powder, approximately 200 to 300 times sweeter than sucrose | ERT-039-4 | Conforms | Conforms | Conforms | Conforms | Conforms |
| Assay | NLT 95% steviol glycosides on the dried basis | ERT-017-3 | 97.2 | 100.2 | 99.7 | 97.5 | 99.4 |
| Solubility | Freely soluble in water | ERT-047-1 | Conforms | Conforms | Conforms | Conforms | Conforms |
| pH (1% solution) | 4.5 to 7.0 | ERT-006-1 | 5.4 | 4.8 | 5.2 | 4.9 | 5.0 |
| Ash (%) | NMT 1 | AOAC 945.46 | <1.0 | 0.06 | <0.04 | <0.04 | 0.06 |
| Loss on drying (%) | NMT 6 | ERT-027-1 | 0.5 | 0.85 | 3.0 | 2.7 | 1.3 |
| ***Residual Solvents*** | | | | | | | |
| Methanol (mg/kg) | NMT 200 | ERT-046-1 | <200 | <200 | <200 | <200 | <200 |
| Ethanol (mg/kg) | NMT 5,000 | ERT-046-1 | 0.45 | 0.08 | 0.24 | 0.39 | 0.33 |
| ***Heavy Metals*** | | | | | | | |
| Arsenic (mg/kg) | NMT 1 | AOAC 2015.01 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 |
| Lead (mg/kg) | NMT 1 | AOAC 2015.01 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 |
| Cadmium (mg/kg) | NMT 1 | AOAC 2015.01 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 |

**Table B.6.3.1-1 Summary of Physical and Chemical Analyses for 5 Lots of Reb MD**

| Specification Parameter | FSANZ Specification[a] | Analytical Method | Manufacturing Lot | | | | |
|---|---|---|---|---|---|---|---|
| | | | 200124-B1 | 200221-B1 | 200226-B1 | 200302-B1 | 200306-B1 |
| Mercury (mg/kg) | NMT 1 | AOAC 2015.01 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 |

AOAC = Association of Analytical Communities; FSANZ = Food Standards Australia New Zealand; NLT = not less than; NMT = not more than; Reb = rebaudioside.
[a] FSANZ Specification for Steviol Glycosides from Fermentation (FSANZ, 2020a).

### *B.6.3.2 Microbiological Analysis of Reb MD*

The analyses from 5 non-consecutive lots of Reb MD shows that the product is consistently free of microbial contaminants. A summary of the microbial analysis for the 5 lots of Reb MD is presented in Table B.6.3.2-1 (see Appendix E for Certificates of Analysis).

**Table B.6.3.2-1 Summary of the Microbiological Analyses for 5 Lots of Reb MD**

| Specification Parameter | JECFA Specification[a] | Analytical Method | Manufacturing Lot | | | | |
|---|---|---|---|---|---|---|---|
| | | | 200124-B1 | 200221-B1 | 200226-B1 | 200302-B1 | 200306-B1 |
| Aerobic plate count (CFU/g) | NMT 1,000 | AOAC 966.23 | <10 | <10 | <10 | <10 | <10 |
| Yeast (CFU/g) | NMT 200 | AOAC 997.02 | <10 | <10 | <10 | <10 | <10 |
| Mould (CFU/g) | | | <10 | <10 | <10 | <10 | <10 |
| *Escherichia coli* | Negative in 1 g | USP 62 | Negative | Negative | Negative | Negative | Negative |
| Salmonella | Negative in 25 g | AOAC RI100201 | Negative | Negative | Negative | Negative | Negative |

AOAC = Association of Analytical Communities; CFU = colony forming units; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NMT = not more than; USP = United States Pharmacopeia.
[a] JECFA Specification for Steviol Glycosides from Fermentation (JECFA, 2020).

## B.6.4 Other Chemical Analysis

### *B.6.4.1 Steviol Glycoside Composition*

Reb MD is primarily comprised of rebaudioside M and may contain a mixture of the following additional glycosides in various concentrations, such that the total steviol glycoside content is no less than 95%; rebaudiosides A, B, C, D, E, F, stevioside, steviolbioside, rubusoside, and dulcoside A. The distribution of steviol glycosides present in Reb MD will vary depending on the production process and final product formulation, as described in Section B.5.3. Reb MD from *Y. lipolytica* is chemically and substantially equivalent to rebaudioside MD that is obtained from *S. cerevisiae* strain CD15407 (S3—39) (the subject of Application A1170). In order to determine the steviol glycoside composition, Cargill developed an ultra-high performance liquid chromatography (UHPLC) method utilizing gradient elution with ultraviolet light detection, similar to the updated HPLC method described in the JECFA specifications for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2020). Similar to the JECFA HPLC method, Cargill's method allows for the improved separation of steviol glycosides, especially rebaudioside M and rebaudioside D, in comparison to the isocratic elution method previously utilised by JECFA (JECFA, 2010), and thereby, improves the quantification of steviol glycosides with similar run times. This is the same method that was outlined in the Application A1170 for Steviol Glycosides from *S. cerevisiae* submitted by Cargill to FSANZ. Details of Cargill's UHPLC method are provided in Appendix C-3.

The steviol glycoside distribution of 5 non-consecutive commercial lots of Reb MD (Lot No. 200124-B1, 200221-B1, 200226-B1, 200302-B1, and 200306-B1) was determined using UHPLC. As presented in Table B.6.4.1-1, Reb MD consists primarily of rebaudiosides M (85.4 to 96.3%) and rebaudioside D (3.1 to 10.7%), with small amounts of rebaudioside A (0.43 to 0.65%) and rebaudioside B (0.14 to 0.68%). Total steviol glycoside content in these 5 commercial lots of Reb MD was between 97.2 and 100.2%. Corresponding HPLC chromatograms are provided in Appendix D.

**Table B.6.4.1-1 Steviol Glycoside Composition of Reb MD**

| Steviol Glycosides (% dry weight) | Manufacturing Lot | | | | |
|---|---|---|---|---|---|
| | 200124-B1 | 200221-B1 | 200226-B1 | 200302-B1 | 200306-B1 |
| Rebaudioside A | 0.589 | 0.447 | 0.428 | 0.640 | 0.650 |
| Rebaudioside B | 0.683 | 0.421 | 0.142 | 0.671 | 0.505 |
| Stevioside | 0 | 0 | 0 | 0 | 0 |
| Rebaudioside C | 0 | 0 | 0 | 0 | 0 |
| Rebaudioside D | 9.440 | 3.103 | 3.404 | 10.7445 | 8.7515 |
| Rebaudioside F | 0 | 0 | 0 | 0 | 0 |
| Rebaudioside M | 86.519 | 96.262 | 95.690 | 85.436 | 89.530 |
| Rubusoside | 0 | 0 | 0 | 0 | 0 |
| Dulcoside A | 0 | 0 | 0 | 0 | 0 |
| Steviolbioside | 0 | 0 | 0 | 0 | 0 |
| Total steviol glycosides | 97.231 | 100.232 | 99.664 | 97.491 | 99.435 |

Reb = rebaudioside.

### *B.6.4.2 Protein Analysis*

To confirm the success of the purification steps in the manufacturing process (*e.g.,* ion exchange chromatography, adsorption chromatography, and crystallisation) and to confirm the absence of residual protein in Reb MD, samples from the same 5 non-consecutive lots of final product (Lot No. 200124-B1, 200221-B1, 200226-B1, 200302-B1, and 200306-B1) were assayed by the BCA protein assay. An analytical standard of high purity (>95%) rebaudioside M was also tested. All samples were prepared at a concentration of 1 mg/mL and protein content in the solution was measured against a standard curve of bovine serum albumin (BSA): BSA 5 µg/mL to 250 µg/mL. All assessments were carried out in triplicate. Full details of the analytical method are described in the study report that is provided in Appendix C-6. Overall, no residual protein was detected in any of the Reb MD test samples above the limit of detection of 25 ppm for the assay.

### *B.6.4.3 Residual DNA Analysis*

To confirm the absence of residual recombinant DNA in Reb MD, samples from the same 5 non-consecutive lots of final product (Lot No. 200124-B1, 200221-B1, 200226-B1, 200302-B1, and 200306-B1) were assayed by PCR. Primer design and PCR conditions were designed to amplify a 0.679 kb fragment of one of the uridine diphosphate-glucosyltransferase genes (UGT2) inserted in the production strain. Genomic DNA extracted from the production strain was used as a positive control. Reb MD samples were prepared in water and to determine the matrix-dependent limit of detection, samples were spiked with positive control genomic DNA from the production strain (7 ng to 7 fg). Each Reb MD test sample was also assayed without a DNA spike. Genomic DNA was extracted from the test samples and the PCR reaction targeting the UGT2 DNA sequence was carried out. In the spiked Reb MD samples, positive control genomic DNA down to 0.062 ng/g product was detected (*i.e.*, matrix-dependent limit of detection). This assay was developed according to the EFSA guidance on the characterisation of microorganisms as production organisms that recommends the utilisation of an upper limit of 10 ng DNA/g of product (EFSA, 2018a). In all Reb MD samples tested without a DNA spike, no genomic DNA was detected above 10 ng/g detection parameters. These results confirm the absence

of residual genomic DNA in the Reb MD final product above the EFSA recommended analytical limit of detection of 10 ng/g. The full study report is provided in Appendix C-5.

## B.7 Information for Food Labelling

Reb MD is a mixture of steviol glycosides and therefore will follow the same food labelling as already established for steviol glycosides. Steviol glycosides, including Reb MD, are considered high-intensity sweeteners and flavour enhancers when added to various food products, and have been assigned the INS number 960. Therefore, Reb MD will be labelled under the functional class, sweetener, as "sweetener (960)" or "sweetener (steviol glycosides)".

## B.8 Analytical Method for Detection

An internal HPLC method is used to quantify the total amount of steviol glycosides and the proportion of individual steviol glycosides present in Reb MD to meet established, internationally recognised, specifications for steviol glycosides (*e.g.*, FSANZ, JECFA) (Section B.6.1). The assay is based on the JECFA (2010) HPLC method for steviol glycosides and was optimised by Cargill to account for the steviol glycosides with a higher degree of glycosylation (*e.g.*, rebaudiosides M and D) present in the final product that are not captured in the JECFA (2010) HPLC methodology. Cargill considers this optimised HPLC method to be confidential commercial information. Briefly, experimentally determined correction factors for rebaudiosides M and D are used instead of using molecular weight correction factors, as in the case of the JECFA (2010) HPLC method. The method used by Cargill employs standard solutions of purified rebaudiosides M and D that are used to compare the response factors (area/standard concentration) for the 2 glycosides to the analytical standard (rebaudioside A), allowing for the experimental determination of the correction factors (response factor [rebaudioside A]/response factor [rebaudioside D or M]). Further proprietary description of Cargill's HPLC method is provided in Appendix C-3.

## B.9 Potential Additional Purposes of Reb MD when Added to Food

To decrease the sugar content of foods and beverages, Reb MD as a high-intensity sweetener may be used as an alternative sweetening agent to sucrose with the benefit of reducing the caloric content of these products. Therefore, consumers maintaining reduced-calorie diets may seek out such foods and beverages containing steviol glycoside products, such as Reb MD, as they will contain reduced calories from sugar. Additionally, individuals with specific medical conditions that require reduced sugar intakes, for instance diabetics, may also use steviol glycoside products such as Reb MD for this purpose.

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

In accordance with Section 3.3.1 – Food Additives of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), the safety information outlined must be provided for new food additives.

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

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

These points are addressed in the section that follows.

Section 3.3.1 – Food Additives of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a) 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 are outlined in the following section.

Furthermore, based on a pre-submission consultation, FSANZ considers that the guideline that best meets the assessment needs of the *Y. lipolytica* production strain/organism is Guideline 3.3.2 Processing Aids, but only parts C2, C3, D1, D2, D3, and E1. As such, the following information on the production strain/organism is presented:

1. Information related to the safety of an enzyme processing aid (Sections C2 and C3)

2. Additional information related to the safety of an enzyme processing aid derived from a microorganism (Section D1, D2, and D3)

3. Additional information related the safety of an enzyme processing aid derived from a genetically-modified microorganism (Section E1)

# C.1 Introduction

The safety of steviol glycosides has been extensively evaluated and is supported by conclusions from several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, FSANZ, European Commission's Scientific Committee on Food, EFSA, and Health Canada. The data available for these evaluations included a comprehensive examination on the comparative metabolism and pharmacokinetics of steviol glycosides in animals and humans, acute, short-term, and long-term toxicity and carcinogenicity studies, reproductive and developmental toxicology studies, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies. Although many earlier studies examining the safety of steviol glycosides were conducted with stevioside of various purities, the database pertaining to the safety of steviol glycosides was expanded following the completion of additional short-term toxicity, reproductive toxicity, *in vitro* and *in vivo* mutagenicity/genotoxicity studies, and human studies with high purity rebaudioside A. Several studies available in the public domain conducted with stevia extracts have demonstrated the shared metabolic fate of all steviol glycosides. Following ingestion, steviol glycosides are hydrolysed to steviol by members of the *Bacteroidaceae* family residing in the colon. The common metabolite steviol is absorbed from the lower gastrointestinal tract, conjugated to glucuronic acid, and excreted primarily *via* the urine in humans. Because of this shared metabolic fate, the safety database that exists for individual steviol glycosides can therefore be extended to include all glycosylated derivates of the aglycone steviol.

Steviol glycosides, whether produced by fermentation or extracted from the *S. rebaudiana* plant, are metabolised and biologically handled in an identical manner following oral administration. A discussion on the metabolic fate of steviol glycosides, including a study demonstrating that steviol glycosides produced by fermentation are metabolised by human faecal homogenates in the same manner as steviol glycosides extracted from *S. rebaudiana* Bertoni, is provided in Section C.2. Since the steviol glycosides produced by *Y. lipolytica* are identical and therefore substantially equivalent to those steviol glycosides extracted from *S. rebaudiana* Bertoni*,* the extensive safety database that exists for steviol glycosides extracted from the plant may be applied to establish the safety of Reb MD. To identify any new studies in the public domain related to steviol glycoside safety, an updated search of the scientific literature was conducted subsequent to the previous application submitted by Cargill for steviol glycosides produced by *S. cerevisiae* (A1170) (FSANZ, 2019b). Summaries of the conclusions from authoritative scientific and regulatory bodies on the safety of steviol glycosides are provided in Section C.3.

# C.2 Steviol Glycoside Safety Data

## C.2.1 Metabolic Fate of Steviol Glycosides

### *C.2.1.1 Microbial Degradation*

*In vitro* and *ex vivo* studies have confirmed that steviol glycosides are not hydrolysed by digestive enzymes of the upper gastrointestinal tract and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea *et al*., 1997; Geuns *et al*., 2003, 2007; Koyama *et al*., 2003a). 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 (Renwick and Tarka, 2008). Several *in vitro* studies mimicking the anaerobic conditions of the colon have confirmed the ability of the gut microbiota 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*., 2003b; Nikiforov *et al.*, 2013; Purkayastha *et al.*, 2014, 2015, 2016). Specifically, Koyama *et al*. (2003b) investigated the degradation of a stevia mixture containing rebaudioside A, stevioside, rebaudioside C, and dulcoside A (purities not reported) in the presence of human faecal homogenates under anaerobic conditions. Similar to studies conducted with individual steviol glycosides (*e.g.*, stevioside or

rebaudioside A), the stevia mixture was degraded completely to steviol within 24 hours of incubation. Nikiforov *et al.* (2013) conducted a similar *in vitro* study using rat cecal contents and reported that rebaudioside D was hydrolysed to stevioside and steviol over a 90-minute period, which was comparable to the hydrolysis of rebaudioside A. In addition, Prakash Chaturvedula and Prakash (2013) observed that incubation of rebaudioside E with crude pectinase (from *Aspergillus niger*) resulted in the generation of steviol; pectinolytic bacteria are known to reside in the human intestine (Jensen and Canale-Parola, 1985), further establishing the intestinal metabolism of steviol glycosides.

Given the large collection of *in vitro* steviol glycoside metabolism studies with human faecal homogenates, Purkayastha *et al.* (2016) re-assessed the existing data to allow for improved comparison of results between different studies. Published studies that compared individual steviol glycoside metabolism (dulcoside A, rebaudiosides B, C, D, E, F, M, and steviolbioside) to that of rebaudioside A at similar test concentrations (0.2 or 2.0 mg/mL, depending on solubility) and incubation times (up to 48 hours) were collected and compared. Assessment of the data in parallel demonstrated that steviol glycosides, irrespective of the type of sugar moiety (*e.g.,* glucose, rhamnose, xylose) or the number of sugar moieties attached to the steviol backbone, were metabolised to steviol at generally similar hydrolysis rates. The authors noted that while subtle differences may exist in the degradation rates of individual glycosides, it is unlikely that the absorption rate of steviol *in vivo* would be significantly impacted, particularly when compared to rebaudioside A (Purkayastha *et al.,* 2016). Since steviol glycosides produced by fermentation are identical in their chemical structure to those extracted from the *S. rebaudiana* Bertoni plant, they will also be metabolised by human faecal homogenates to steviol at generally similar hydrolysis rates, as demonstrated in Section C.2.1.2.

#### C.2.1.1.1 Microbial Degradation of Reb MD

As described above, to demonstrate that different individual steviol glycosides share the same metabolic fate as the major steviol glycoside rebaudioside A, several microbial degradation studies have been conducted *in vitro* with human faecal homogenates. A summary of the *in vitro* microbial metabolism study conducted with Reb MD produced by *Y. lipolytica* is presented below and the full report is provided in Appendix C-4.

Human faecal homogenate samples were prepared based on the pooling of faecal samples from 2 healthy male and 2 healthy female volunteers. Reb MD produced by *Y. lipolytica* (Lot No. 200124-B1) was mixed and incubated in 3 (n=2 pooled) adult male and adult female pooled faecal homogenate samples at concentrations of 0.2 mg/mL under anaerobic conditions at 37±5°C for 4 to 48 hours in triplicate. To demonstrate the complete metabolic hydrolysis of steviol glycosides from *Y. lipolytica*, the formation of steviol, the final stable metabolite, was assayed at each timepoint using an established liquid chromatography-mass spectrometry (LC-MS) method. The mean steviol metabolite concentration was used to determine the percent steviol metabolite formed based on the chemical composition of the study material and the molar equivalent metabolic conversion of steviol glycosides to steviol. Rebaudioside 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 to ensure that the experimental incubation conditions were satisfactory, and to allow for comparison of the hydrolysis rate and degree between the 2 materials. Steviol was also included as a stability control.

A summary of the mean steviol metabolite concentrations formed and the percent steviol glycoside metabolised to steviol in adult male and adult female faecal homogenates is presented in Table C.2.1.1.1-1, and data for rebaudioside A from *S. rebaudiana* Bertoni are provided in Table C.2.1.1.1-2 for comparison. These data indicate that near complete deglycosylation of steviol glycosides from *Y. lipolytica* occurred within an incubation period of 24 to 48 hours in pooled faecal homogenates. At 48 hours the mean percent steviol glycoside metabolised to steviol was 94.3% in male samples and 102.5% in female samples. The positive control rebaudioside A from *S. rebaudiana* Bertoni had a similar rate and overall degree of hydrolysis as steviol glycosides produced by fermentation. These data demonstrate that steviol glycosides from *Y. lipolytica* (Reb MD), comprised of primarily rebaudioside M and D, in the presence of human faecal homogenates is metabolised nearly completely to steviol within 48 hours and confirms that these glycosides share the same metabolic fate as steviol glycosides, such as rebaudioside A, from *S. rebaudiana* Bertoni.

**Table C.2.1.1.1-1 Hydrolysis of Steviol Glycosides from *Y. lipolytica* (Reb MD) in Human Faecal Homogenates**

| Gender | Time Point (hour) | Mean Steviol Concentration (µg/mL) | Standard Deviation | % Steviol Glycoside Metabolised to Steviol |
|---|---|---|---|---|
| Male | 0 | <LLOQ | NA | NA |
| | | <LLOQ | NA | NA |
| | 4 | 9.91 | 1.21 | 19.8 |
| | | <LLOQ | NA | NA |
| | 8 | 43.0 | 0.456 | 85.9 |
| | | 9.90 | 0.694 | 19.9 |
| | 12 | 50.8 | 2.14 | 102 |
| | | 33.1 | 2.02 | 66.2 |
| | 16 | 49.0 | 1.09 | 98.1 |
| | | 46.5 | 2.50 | 93.0 |
| | 24 | 49.8 | 0.517 | 99.5 |
| | | 48.6 | 3.19 | 97.1 |
| | 48 | 46.4 | 0.9 | 92.8 |
| | | 47.9 | 0.914 | 95.8 |
| Female | 0 | <LLOQ | NA | NA |
| | | <LLOQ | NA | NA |
| | 4 | <LLOQ | NA | NA |
| | | <LLOQ | NA | NA |
| | 8 | 32.1 | 1.00 | 46.3 |
| | | 38.3 | 0.762 | 76.6 |
| | 12 | 32.1 | 2.32 | 64.2 |
| | | 49.1 | 1.46 | 98.2 |
| | 16 | 47.1 | 1.97 | 94.2 |
| | | 48.2 | 3.67 | 96.4 |
| | 24 | 49.6 | 0.779 | 99.2 |
| | | 50.6 | 3.86 | 101 |
| | 48 | 49.0 | 2.77 | 98.0 |
| | | 53.7 | 0.519 | 107 |

LLOQ = lower limit of quantification, 0.2 µg/mL; NA = not applicable.

**Table C.2.1.1.1-2 Hydrolysis of Rebaudioside A from *S. rebaudiana* Bertoni in Human Faecal Homogenates**

| Gender | Time Point (hour) | Mean Steviol Concentration (µg/mL) | Standard Deviation | Mean % Molar Equivalent Reb A Metabolised to Steviol | Standard Deviation |
|---|---|---|---|---|---|
| Male | 0 | <LLOQ | NA | NA | NA |
| | | <LLOQ | NA | NA | NA |
| | 4 | 14.5 | 1.05 | 21.1 | 0.190 |
| | | <LLOQ | NA | NA | NA |
| | 8 | 53.6 | 1.52 | 81.4 | 2.31 |
| | | 14.3 | 1.30 | 21.8 | 1.97 |
| | 12 | 61.9 | 3.27 | 94.1 | 4.96 |
| | | 30.9 | 2.34 | 47.0 | 3.55 |
| | 16 | 58.6 | 4.26 | 89.0 | 6.47 |
| | | 38.7 | 2.72 | 58.7 | 4.12 |
| | 24 | 63.5 | 3.69 | 96.5 | 5.61 |
| | | 61.8 | 10.19 | 93.9 | 15.5 |
| | 48 | 56.8 | 4.84 | 86.2 | 7.35 |
| | | 62.1 | 10.65 | 94.2 | 16.2 |
| Female | 0 | <LLOQ | NA | NA | NA |
| | | <LLOQ | NA | NA | NA |
| | 4 | <LLOQ | NA | NA | NA |
| | | <LLOQ | NA | NA | NA |
| | 8 | 9.66 | 6.58 | 14.7 | 10.0 |
| | | 31.9 | 2.49 | 48.4 | 3.79 |
| | 12 | 14.1 | 4.50 | 21.5 | 6.83 |
| | | 51.5 | 3.21 | 78.2 | 4.88 |
| | 16 | 22.5 | 1.58 | 34.1 | 2.40 |
| | | 57.9 | 5.89 | 87.8 | 8.95 |
| | 24 | 38.7 | 2.72 | 58.8 | 4.11 |
| | | 59.2 | 8.89 | 89.9 | 13.5 |
| | 48 | 60.0 | 2.90 | 91.1 | 4.40 |
| | | 60.2 | 3.65 | 91.4 | 5.54 |

LLOQ = lower limit of quantification, 0.2 µg/mL; NA = not applicable; Reb = rebaudioside.

### *C.2.1.2 Absorption, Distribution, Metabolism, and Elimination*

Following hydrolysis to steviol, the aglycone is absorbed systemically *via* the portal vein and distributed to a number of organs and tissues, including the liver for additional metabolism, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; Sung, 2002; Koyama *et al*., 2003a; Wang *et al*., 2004; Roberts and Renwick, 2008). Peak concentrations of steviol were detected in the plasma of Sprague-Dawley rats within 15 to 30 minutes of oral steviol administration, whereas following oral administration of a mixture of rebaudioside A (28.8%), rebaudioside C (25.2%), stevioside (17.0%), and dulcoside A (10.2%), peak steviol concentrations were attained at approximately 8 hours (Nakayama *et al*., 1986; Koyama *et al*., 2003a; Roberts and Renwick, 2008). As confirmed by high levels of radioactivity in the lower gastrointestinal tract for up to several hours after oral administration of radiolabelled steviol glycosides, the delay between the detection of radioactivity/steviol levels in the plasma and the time of administration of steviol glycosides is due to the fact that glycosides must be cleaved to steviol by the colonic microbiota before absorption can occur (Koyama *et al*., 2003a).

Following absorption from the colon, steviol primarily undergoes conjugation with glucuronic acid to steviol glucuronide in the liver. 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 of radiolabelled rebaudioside A or stevioside (Roberts and Renwick, 2008). Consistent with this, free- and glucuronide-conjugated steviol were primarily observed in the plasma of rats administered rebaudioside D indicating that systemic absorption of steviol glycosides is low (Nikiforov *et al.*, 2013). Similarly, steviol glucuronide was detected in the plasma following ingestion of stevioside or rebaudioside A in humans, with maximal concentrations detected 8 and 12 hours after administration, respectively (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The differences in the time to reach maximum steviol glucuronide plasma concentrations between stevioside and rebaudioside A are due to the simpler structure and faster bacterial degradation of stevioside (Wheeler *et al.,* 2008). Moreover, significant inter-individual variability in maximum plasma steviol glucuronide levels, and in the time required to reach peak plasma levels, was noted in study participants following stevioside ingestion (Geuns *et al*., 2007). Such variations can likely be attributed to differences in the time required to release steviol from the glycoside in the gut as a result of inter-individual variability in the microflora composition and/or gastric emptying rates.

In rats, free and conjugated steviol, as well as any unhydrolysed fraction of the administered glycosides, are excreted primarily in the faeces (generally within 48 hours), with smaller amounts of free and conjugated steviol appearing in the urine (less than 3%) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Sung, 2002; Roberts and Renwick, 2008). Two steviol conjugates were identified by Nakayama *et al*. (1986) in the bile of Wistar rats, one that was hydrolysed by a weak acid and another that was hydrolysed by a weak acid and β-glucuronidase; therefore, following the elimination of steviol glucuronide in the bile, steviol may be released from its conjugated form by the microflora and may enter enterohepatic circulation. In humans, elimination of steviol glycosides, primarily as steviol glucuronide with very small amounts of the unchanged glycoside or steviol, occurs *via* the urine (Kraemer and Maurer, 1994; Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008). 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) also were eliminated in the faeces of humans (Geuns and Pietta, 2004; Simonetti *et al*., 2004; Geuns *et al*., 2007; Wheeler *et al*., 2008). The difference in the route of elimination of systemically absorbed steviol as steviol glucuronide in rats and humans (*via* the bile and in the urine, respectively) 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). Although the primary routes of elimination of steviol glucuronide differ between rats and humans, the difference 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.

To characterise the pharmacokinetic/toxicokinetic differences more accurately in the production of steviol/steviol glucuronide following oral consumption of steviol glycosides between rats and humans, Roberts *et al.* (2016) conducted comparative studies in rats and humans. Male Sprague-Dawley rats and healthy male human volunteers were orally administered a single dose of stevioside (40 mg/kg body weight; equivalent to 16 mg steviol equivalents/kg body weight) and plasma samples collected over the following 72 hours were analysed for steviol and steviol glucuronide by liquid chromatography-tandem mass spectrometry. Although peak plasma concentrations ($C_{max}$) of steviol and steviol glucuronide occurred slightly later in humans in comparison to rats, $C_{max}$ values of plasma steviol were similar between rats and humans (~72 to 77 ng/mL). $C_{max}$ values for steviol glucuronide, however, were approximately 25-fold higher in humans than rats (~4,400 ng/mL *vs.* 180 ng/mL). Systemic exposure was determined based on the area-under-the-curve of the concentration *vs.* time data, and steviol and steviol glucuronide exposure were measured to be 2.8-fold higher (~1,650 ng*h/mL *vs.* 590 ng*h/mL) and 57-fold higher (~136,000 ng*h/mL *vs.* 2,400 ng*h/mL), respectively, in humans compared to rats.

#### *C.2.1.3 Summary and Conclusions*

Collectively, the degradation and pharmacokinetic studies on steviol glycosides confirm the common metabolic pathway for all steviol glycosides as previously noted: steviol glycosides are rapidly hydrolysed to steviol, steviol is absorbed and conjugated with glucuronic acid, and steviol glucuronide is excreted primarily *via* the urine in humans. Steviol glycosides, whether produced by fermentation or extracted from the *S. rebaudiana* Bertoni plant, share this same metabolic fate. This is consistent with the fact that except for having different numbers and types of sugar moieties, steviol glycosides, regardless of source, share the same structural backbone steviol. Considering the common pathway of metabolism, and the fact that systemically, exposure only occurs to steviol following consumption of steviol glycosides, the safety data and conclusions drawn for individual steviol glycosides from *S. rebaudiana* Bertoni, therefore, can be extended to include all steviol glycosides including those derived from fermentation of yeast, such as *Y. lipolytica* and *S. cerevisiae* production strains.

### C.2.2 Recent Toxicological and Human Studies with Steviol Glycosides

An updated search of the publicly available scientific literature identified additional toxicological and safety studies on steviol glycosides that were conducted subsequent to the previous application submitted by Cargill for steviol glycosides produced by *S. cerevisiae* (A1170) (FSANZ, 2019b). The literature search was completed using ProQuest and included searches of the following databases for pertinent literature on the safety of steviol glycosides: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Due to the purity criteria laid down in several specifications, studies were excluded if the test article investigated had a purity of less than 95% steviol glycosides. In general, these additional studies corroborate the safety of steviol glycosides.

#### *C.2.2.1 Repeated-Dose Studies in Animals*

Barrios-Correa *et al*. (2018) investigated the brain of mice for changes in the JAK2/STAT3 signalling pathway and changes in appetite and body composition following chronic intake of commercial sweeteners. Adult BALB/c mice (9/sex/group) were provided with one of the following drinking water formulations for 6 weeks: sucrose (10% dilution of sucrose per 100 mL purified water), sucralose (one 1 g packet of commercial sucralose sweetener Splenda®, equivalent to 0.012 g sucralose, per 100 mL water), or steviol glycosides (one 1 g packet of commercial steviol glycoside sweetener Svetia®[2], equivalent to 0.025 g rebaudioside A, per 100 mL water; approximately 15 mg/kg body weight/day steviol equivalents). The control mice were given purified water and all the animals were provided food and water *ad libitum*. Food and water intakes were measured daily throughout the study period. Body weight was measured at study start and weekly thereafter, and energy intake was determined at study end. Utilizing these data, an approximate exposure to rebaudioside A present in the Svetia® sweetener was calculated to be 15 mg/kg body weight/day steviol equivalents, which is about 4 times higher than the upper limit of the steviol glycoside acceptable daily intake (ADI). Body composition and expression of total and phosphorylated JAK2, STAT3, and Akt, as well as SOCS3 and ObRb in the brain tissue were measured. Male mice provided with steviol glycosides showed significantly decreased energy intake, adiposity, downregulation of feeding behaviour, and decreased weight gain, compared with controls. Increased expression of pJAK2 and pSTAT3 in the brain were also observed in male mice supplemented with steviol glycosides when compared to the controls. On the other hand, JAK2 and pJAK2 expression was upregulated in female mice supplemented with steviol glycosides, compared to the controls. The authors concluded that alterations in brain activity with regards to signalling pathways that control appetite and energy balance occurred following repeated steviol glycoside intake; however, since the

[2] Svetia® is a co-crystallised blend of cane sugar and high purity rebaudioside A and is twice as sweet as sugar. The blend is formulated with 2.5% rebaudioside A (http://www.svetia.us/home/#nutrimental-table).

administered dose was calculated to be about 4 times higher than the upper limit of the ADI for steviol glycosides, the relevance of these data to human exposure to steviol glycosides in food is limited.

Han *et al.* (2019) investigated the effects of stevioside (97% purity) on feed intake and digestibility in goats in a replicated 3 x 3 Latin square design. Male Xiangdong Black goats (n=3/group) were provided a diet containing 0, 400, or 800 mg stevioside/kg forage (rice straw) for 20 days (approximately 3.7 and 7.2 mg/kg body weight/day steviol equivalents). The forage was provided with a feed concentrate twice daily and consumed *ad libitum*. Faecal samples collected on Days 12 to 17 were analysed for nutrient digestibility and chemical composition, and total tract digestibility was calculated. On Day 18, feeding behaviour (including eating, ruminating, and resting) was analysed over a 24-hour period by visual examination. Serum metabolites, glucose, total protein, albumin, globulin, triglyceride, and total cholesterol were examined from blood samples taken from each animal on Day 19. Rumen fluid was analysed for pH, volatile fatty acid concentrations, and ammoniacal nitrogen ($NH_3$-N) concentration from samples collected on Days 19 and 20. The following statistically significant results were reported following exposure to stevioside in the forage: a linear increase in dry matter intake of forage and total diet; a quadratic increase in rumen pH; a quadratic decrease in total volatile fatty acids; a quadratic response of stevioside on rumen isobutyrate and isovalerate, with an increase from 0 to 400 mg/kg stevioside and a decrease from 400 to 800 mg/kg stevioside; and linear and quadratic increases in neutral and acid detergent fibre digestibility, with an increase in digestibility from 0 to 400 mg/kg stevioside but a decrease in digestibly from 400 to 800 mg/kg stevioside. The authors suggested that the addition of stevioside may have increased the palatability of the forage. The addition of stevioside in the forage had no significant effects on parameters measured in the serum. The authors concluded that the addition of stevioside to the feed of goats increased dry matter intake and increased the digestibility of neutral and acid detergent fibre.

Nettleton *et al.* (2019) investigated the possible effects of rebaudioside A on the gut microbiota in rats. Male Sprague-Dawley rats (8/group) received 0 (control) or 2 to 3 mg rebaudioside A/kg in drinking water, in conjunction with 0 (prebiotic control) or 10% prebiotic oligofructose-enriched inulin in the diet for 9 weeks. Body weight and faeces were collected weekly and food and fluid intake biweekly, while oral glucose and insulin tolerance tests, gut permeability tests, and tissue harvest were performed at the end of the 9-week study period. Collected cecal matter underwent microbiota sequencing, and the concentration of short-chain fatty acids was also analysed. Compared to the control group, rebaudioside A consumption alone did not alter weight gain or glucose tolerance and adding rebaudioside A did not interfere with the benefits of the prebiotic except for a significant reduction in cecal weight. Rebaudioside A alone altered the following in the gut microbiota when compared to the control group: significant reduction in *Bifidobacteriaceae*, lower abundance of Clostridiales family XIII and *Ruminococcaceae UGG 005*, increased relative abundance of *Akkermansia muciniphila* and *Akkermansiaceae*, and increased abundance of *Bacteroides goldsteinii and Bacteroides thetaiotaomicron*. Furthermore, rebaudioside A significantly reduced tyrosine hydroxylase and dopamine transporter mRNA levels in the nucleus accumbens when compared to the control group ($p=0.044$). The authors concluded that the effects of long-term low-dose rebaudioside A on the rat gut microbiota should be further investigated. The relevance of these findings to the human gut have not been evaluated.

In a study conducted by Sánchez-Tapia *et al*. (2019), the effects of steviol glycosides on the functionality of adipocytes in conjunction with a normal or high fat diet were investigated. Male Wistar rats (n=6/group) received either a control or high-fat diet, and 0 or 2.5% steviol glycosides (purity and mg/kg exposure not specified) *ad libitum* in drinking water for 4 months. Body weight, serum glucose, white and brown tissue gene expression, and histological examination of adipose tissue were measured after 4 months of steviol glycoside exposure. The following statistically significant effects were reported in the high-fat diet/steviol glycosides group when compared to the respective high-fat diet control group: decrease in body weight, decrease in serum leptin, and an increase in serum glucose. For rats receiving steviol glycosides with the control diet, a statistically significant increase in serum glucose and leptin was

reported in comparison to the associated control diet group. Neither steviol glycoside groups reported significant changes in serum insulin compared to controls. Tongue taste receptor T1R2 was statistically significantly induced in rats receiving steviol glycosides with the control diet when compared to the associated control group, while the addition of the high-fat diet reduced the abundance of T1R2. Relative expression of TNFα in white adipose tissue was significantly increased in both groups that received steviol glycosides, when compared to the respected diet control groups. The authors stated that the biological significance of the results of the study will need to be investigated through additional studies in humans.

The effects of several sweeteners, including rebaudioside A were evaluated in mice for effects on endothelial progenitor cells, inflammation, and behaviour (Schiano *et al*., 2019). Male C57BL/6 mice were randomly assigned to receive 2.8 (n=6) or 5.6 (n not reported) mg/kg body weight/day rebaudioside A (Sigma-Aldrich) in drinking water for 8 weeks. Control animals were provided drinking water without any sweeteners added. Several other sweeteners were separately evaluated; however, they will not be discussed herein. Body weights were measured at baseline, Day 21 of the test period, and on the final day of the study (Day 56). Blood pressure was measured before and after the treatment period using the tail-cuff method. Blood was collected at the end of the study and analysed for levels of endothelial progenitor cells, aspartate aminotransferase, alanine aminotransferase (ALT), blood urea nitrogen, total cholesterol, high-density lipoprotein (HDL), triglycerides total bilirubin, alkaline phosphatase, and glucose levels. Peripheral inflammatory reaction was measured in the left hind paw *via* injection of carrageenan and mechanical allodynia was measured using the Von Frey filament test. Following the treatment period, animals in the high-dose group were subject to behavioural testing, including depressive-like behaviour (tail suspension test, forced swimming test, and muscle strength *via* wire hanging) and obsessive-compulsive and anxiety behaviour (marble burying test, and memory test using a maze). Both doses of rebaudioside A were reported to significantly reduce the relative percent value of endothelial progenitor cells in the blood compared to the control ($p<0.05$ and $p<0.01$ for low- and high-dose groups, respectively). A significant increase in body weight ($p<0.001$) and blood glucose ($p<0.05$) was reported in both rebaudioside A groups in comparison to the control. Total cholesterol was significantly decreased ($p<0.05$) in the low-dose group in comparison to the control. HDL was significantly decreased in both rebaudioside A groups ($p <0.05$ and $p<0.01$ for low- and high-dose groups respectively), whereas low-density lipoprotein (LDL) was significantly increased in the high-dose group ($p<0.01$). ALT was significantly lower in both treatment groups ($p<0.05$ and $p<0.01$ for low- and high-dose groups respectively). No adverse effects on behavioural changes were reported. It was concluded that rebaudioside A decreased the amount of endothelial progenitor cells in the blood, however the inflammatory response was not compromised.

One older 90-day repeat-dose oral toxicity study in male and female Sprague-Dawley rats was included in this summary of the published safety data on steviol glycosides since it assessed the toxicity of a steviol glycoside preparation produced by fermentation (Rumelhard *et al*., 2016). In this 90-day oral toxicity study, Rumelhard *et al*. (2016) evaluated the safety of rebaudioside A (>95% purity) produced *via* fermentation by *Y. lipolytica* genetically engineered to express the *S. rebaudiana* metabolic pathway. This study was conducted in accordance with U.S. FDA Redbook 2000 and Organisation for Economic Co-operation and Development 408 guidelines for repeat-dose toxicity studies (OECD, 1998; U.S. FDA, 2000). Male and female Sprague-Dawley rats (20/sex/group) were administered rebaudioside A in the diet at dose levels of 0 (basal diet), 500, 1,000, or 2,000 mg/kg body weight/day for 90 days. No deaths or clinical signs of toxicity were observed throughout the study. Significantly lower changes in body weights, body weight gain, and cumulative body weight gain were observed among males in the 2,000 mg/kg body weight/day dose group in comparison to the control group. These decreases were not associated with changes in food consumption and were not observed at study completion among female rats. The authors associated the changes in body weights with the lower caloric value of the diet containing rebaudioside A in comparison to the basal diet alone and did not consider this finding to be adverse. No other test article related effects in haematology, coagulation, serum chemistry, and urinalysis parameters, or upon gross pathological and histopathological examinations were observed.

Based on the above findings, the authors determined a no-observed-adverse-effect level (NOAEL) for rebaudioside A produced *via* fermentation of simple sugars using a *Y. lipolytica* production strain to be 2,000 mg/kg body weight/day, equivalent to 2,057 and 2,021 mg/kg body weight/day for males and females, respectively. Furthermore, the authors concluded that the safety profile of rebaudioside A from a genetically engineered yeast is similar to that of plant-derived steviol glycosides.

The results of these repeat-dose studies therefore corroborate the safety of steviol glycosides and the study by Rumelhard *et al*. (2016) also confirms that steviol glycosides from *Y. lipolytica* are no different in their safety profile than steviol glycosides extracted from *S. rebaudiana* Bertoni.

#### *C.2.2.2 Genotoxicity*

In a chromosome aberration test and micronucleus test, the genotoxic potential of stevia in human lymphocytes obtained from the venous blood of healthy adult donors (2 males and 2 females) was investigated by Uçar *et al*. (2018). The lymphocytes were cultured for 24 and 48 hours at 37°C and exposed to 0, 1, 2, 4, 8, and 16 µg/mL stevia (steviol glycoside purity of 99%) in duplicate. Mitomycin C (0.2 µg/mL) was used as the positive control. The cells were cultured for a total of 72 hours and then collected, fixed onto slides, and assessed by scoring 400 metaphases for each treatment for chromosome aberrations and assessed by scoring a total of 4,000 binucleated cells per concentration for micronuclei formations. The micronucleus test was conducted with the same test concentrations, culture conditions, and times as the chromosome aberration assay. There were no significant differences in the number of chromosome aberrations or micronucleations at any test concentration compared to the negative control. The authors concluded a lack of genotoxicity of steviol glycosides in human lymphocytes.

Yılmaz *et al*. (2020) investigated the potential oxidative and genotoxic capabilities of steviol glycosides in mice. BALB/c mice (4/sex/group) received 0, 470, 620, 940, or 1,880 mg steviol glycosides (Reb A; 98.65% purity)/kg body weight/day dissolved in water *via* gavage daily for 28 days (doses equivalent to 155, 205, 310, or 620 mg steviol/kg body weight/day, respectively). Clinical observations were made daily, while body weight and food consumption were measured weekly. At sacrifice bone marrow samples were taken to evaluate for chromosomal aberrations and mitotic activity. Mitosis was arrested by intraperitoneal injection of 5 mg colchicine/kg body weight 2 hours prior to exsanguination. Bone marrow slides were prepared and for each animal 100 well-spread metaphases were analysed, the number of abnormal cells was quantified, and the mitotic index was determined based on scoring 1,000 cells. Terminal blood samples were collected to measure total oxidant status (TOS), total antioxidant status (TAS), paraoxonase-1 (PON-1), and high- and low-density lipoprotein-cholesterol levels (HDL-C and LDL-C). The number of chromosomal aberrations/cell was unchanged in the 470 mg/kg group and was significantly increased ($p<0.05$) in the 620, 940, and 1,880 mg/kg groups compared to the control. The mitotic index was significantly increased in all dose groups when compared to the control ($p<0.05$) according to the tabulated results; however, the text of the article contraindicated this as follows:

> "*The lowest MI* [mitotic index] *was observed in the control group and*, *although the results were not statistically significant*, *the SG* [steviol glycoside] *doses increased the MI in all experimental groups compared with the control*".

Based on the tabulated data, TAS, TOS, HDL-C, and LDL-C values were not significantly different from the control in all dose groups and PON-1 was significantly decreased in the 620 mg/kg group compared to the control. In contrast, the text of the article states, "*The LDL-C value was statistically higher in the 620 mg/kg dose groups than in the control (p<0.05)*". The authors concluded, "*Our findings indicated that the SG doses used apparently did not have in vivo genotoxic effects but did exerted weak genotoxicity which is most probably related to the increased oxidative damage*". Given the inconsistencies noted between the tabulated data and the text of the article, the relevance of the

findings reported in this study with regard to genotoxicity and oxidative damage of rebaudioside A are unclear.

One older study was included in this summary of the published safety data on steviol glycosides since it assessed the genotoxicity of a steviol glycoside preparation produced by fermentation (Rumelhard *et al*., 2016). Rebaudioside A (>95% purity) produced *via* fermentation of a genetically engineered yeast (*Y. lipolytica*) to express the *S. rebaudiana* metabolic pathway was not mutagenic in the Ames reverse mutation assay when tested at concentrations of up to 5,000 µg/plate in the presence or absence of metabolic activation (Rumelhard *et al*., 2016). Additionally, fermentative rebaudioside A was not cytotoxic and did not include micronuclei formation in cultured peripheral human lymphocytes when incubated for up to 3 hours in the presence or absence of metabolic activation or up to 24 hours in the absence of metabolic activation at concentrations of up to 5,000 µg/plate as part of an *in vitro* micronucleus assay. These findings corroborate the previous conclusions by JECFA (2010) that steviol glycosides are not genotoxic.

### *C.2.2.3 Long-term Toxicity and Carcinogenicity*

The chronic toxicity and carcinogenicity of steviol glycosides has been previously addressed in the safety evaluations by the scientific bodies and regulatory agencies described in Section C.3. No new data were identified in relation to this endpoint.

### *C.2.2.4 Reproductive and Developmental Toxicity*

The effects of *S. rebaudiana* extract (purity not reported) on reproductive function in diabetes-induced healthy adult male rats (albino Wistar) were examined by Ghaheri *et al.* (2018). Diabetes mellitus was induced with an intraperitoneal injection of 50 mg streptozotocin/kg body weight. The rats that reached fasting glucose levels greater than 250 mg/dL after 72 hours were selected for the study. The animals (7/group) were administered *via* gavage 5, 50, or 100 mg stevia extract/kg body weight daily for 28 days. The non-commercial stevia extract was prepared by hot water extraction, but the purity of the final extract was not reported. A diabetic and non-diabetic control group received 2 mL of distilled water only. Sexual behaviours 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. Serum testosterone concentration was measured at the end of the study period. Histological examination was carried out on the right testis and epididymis. In diabetic low-dose animals, significantly increased frequency of intromission was observed compared to diabetic controls, along with significantly increased frequency of ejaculation when compared to diabetic controls and high-dose animals. Significantly decreased latency in ejaculation was observed in low-dose animals compared to high-dose animals; this effect was not significantly different between the treated animals and the controls. No statistically significant differences in the other sexual behaviour parameters were observed. Significantly reduced numbers of Leydig cells were noted in high-dose animals *versus* the non-diabetic control group; however, this effect was not statistically significantly different compared to the diabetic controls. It is also likely that the high-dose exposure to stevia is above the upper limit of the steviol glycoside ADI, limiting the relevance of this finding with respect to human exposure. No changes in organ weights and serum testosterone levels were reported between groups. The results of this study demonstrate that a non-commercial aqueous stevia extract may reduce some of the adverse reproductive effects reported in rats with streptozotocin-induced diabetes.

Jiang *et al.* (2018) evaluated the effects of daily consumption of rebaudioside A (obtained from Aladdin Co., Ltd., China) on the ovarian cycle and steroidogenesis in weanling rats. Female weanling Sprague-Dawley rats (body weight 42.3±4.1 g; 6/group) received 0.5 or 2.5 mM rebaudioside A in drinking water for 48 consecutive days (equivalent to approximately 76 and 486 mg/kg body weight/day steviol equivalents, assuming 100% purity). The control rats received normal water, and all animals were provided with rat chow and water *ad libitum*. Food and water intake, and body weight were measured every third day in the morning. The day of vaginal opening was recorded (from tightly closed to open) and vaginal smears were taken daily to monitor the oestrous cycle. Following the study period, blood samples and ovaries on diestrus-2 were collected. Serum progesterone levels were detected using a radioimmunoassay. The ovaries were examined *via* H&E staining, Western blot, and immunohistochemistry. A significant decrease in body weight was observed in high-dose animals from Day 18 until Day 30, when body weights returned to similar weights as that found in the control group. Water intake during the first 3 weeks of the study was significantly increased in high-dose rebaudioside A-treated animals compared to the controls. During the last 3 weeks of the study, water intake was significantly higher in the high-dose animals compared to the low-dose animals. Serum progesterone levels were significantly decreased in treated rats compared to controls. Increased expression of taste receptor type 2 subunit 38 (T2R38) was observed in low- and high-dose groups, while lower expression of other proteins (T1R3, Gα, StAR, CYP11A1, 3β-HSD, CYP17A1, 17β-HSD, and CYP19A1) in the ovaries was observed, compared to controls. In addition, a lower expression of T1R3 and Gα proteins in rebaudioside A-treated groups was observed. Given that the doses of rebaudioside A utilised in this study on a steviol equivalent basis are well above the upper limit of the steviol glycoside ADI (about 19 to 120 times higher), the relevance of these data to human exposure to steviol glycosides in food is limited.

Gholizadeh *et al.* (2019) conducted a study investigating the effects of Stevia extract on testicular steroidogenesis, spermatogenesis, stereological characteristics, and reproductive function in diabetic rats. Male Wistar rats (12/group) received an injection of nicotinamide followed by streptozotocin to chronically induce diabetes. Diabetic rats were administered *via* gavage 0 (water) or 400 mg/kg Stevia extract (purity not reported) per day for 28 days. An additional group of diabetic rats received 500 mg/kg metformin per day, and an additional control group of non-diabetic rats received only water. At the end of the study rats were weighed, blood was drawn, and following exsanguination the testicles were excised. Serum concentrations of luteinizing hormone (LH) and testosterone were measured and changes in teste histology were evaluated. Results in the diabetic control group were compared to the non-diabetic controls, and results in the Stevia extract group were compared to the diabetic control group, and statistically significant changes are summarised below, unless otherwise stated. Testosterone and LH levels were significantly decreased (LH not statistically) in diabetic control rats and consumption of Stevia extract significantly increased LH levels. While testes weight and volume were significantly decreased (not statistically) in the diabetic control rats, only weight was significantly increased with exposure to Stevia extract. While seminiferous tubule and germinal epithelium volumes were significantly decreased in diabetic control rats, consumption of Stevia extract significantly increased germinal epithelium volume. The number of sexual lineage cells (spermatogonia, spermatocytes, round spermatids, long spermatids, Sertoli cells, and Leydig cells) were significantly decreased in diabetic control rats, and Stevia extract exposure significantly increased these numbers (except for Leydig cells), returning the cell counts to non-diabetic control levels, except for round spermatids and Sertoli cells. Diabetic control rats had a significant decrease in percentage of rapid progressive sperm, and sperm count, and a significant increase in the percentage of non-progressive and immotile sperms. While exposure to Stevia extract significantly ameliorated these results, rapid progressive sperm and immotile sperms did not return to non-diabetic control levels. In this rat model of diabetes, Stevia extract was concluded to possibly reduce reproductive adverse effects and improve infertility.

The effects of rebaudioside A on the expression of guinea pig uterine taste receptors was investigated by Li *et al.* (2020). In this study, female Harley-white guinea pigs (6/group) were randomly assigned to receive water (control), 0.5 mM, or 2.5 mM rebaudioside A solution (calculated to be approximately be 44 and 172 mg steviol/kg body weight/day respectively) *ad libitum* for 28 days. Food and water intake were recorded daily, and body weight was measured once weekly. The day of vaginal opening was recorded, and the oestrus cycle was monitored daily by vaginal smears. At the end of the study period, the animals were euthanised, and blood samples were collected and measured for serum progesterone and oestradiol levels. Samples of the ovaries and uteruses were collected and subject to histological and immunohistochemical analyses, including antral follicle count, corpus luteum count, and measurement of sweet taste receptor T1R2 and T1R3 expression. Ovary weight was also recorded. The following results are reported in comparison to the control group, unless otherwise stated. Food intake in the low-dose rebaudioside A group was significantly higher during the first week of treatment ($p<0.05$) but there was no significant difference during Weeks 3 and 4. Water consumption was significantly lower in the high-dose rebaudioside A group from Weeks 2 to 4 ($p<0.05$) with no change reported in the low-dose group. Body weight was significantly higher in both rebaudioside A groups at Day 14 ($p<0.05$) but was not reported to be significantly different from control at any other timepoint. Serum progesterone levels were significantly higher in the low-dose rebaudioside A group ($p<0.05$) and expression of T1R2 in the uterus was significantly increased in the high-dose rebaudioside A group ($p<0.05$). Increased numbers of atretic follicles were reported in both rebaudioside A groups ($p<0.05$) and corpus luteum count in the ovaries was also significantly higher in the high-dose rebaudioside A group ($p<0.05$). In both treatment groups, T1R3 expression was increased significantly in lutein cells of the corpus luteum and in the high-dose group elevated expression of T1R2 in stromal and epithelial cells of the uterus was also reported. The authors concluded more attention is needed towards the potential adverse reproductive effects of non-nutritive sweeteners. However, as the doses administered in this study were in excess of the ADI, human dietary relevance of these findings is limited.

### *C.2.2.5 Human Studies*

In a randomised single-blinded, crossover, placebo-controlled clinical study, Ahmad *et al.* (2018) investigated the effects of a single dose of stevia leaf powder (prepared from dried stevia leaves; steviol glycoside content not reported) on blood glucose and related parameters in healthy subjects. 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 a single dose of either a placebo cookie (made from 100% wheat flour) or cookie containing stevia leaf powder (3% w/w; approximately equivalent to 4.2 g stevia) in the morning. A 1- to 2-week washout period was carried out before and after each treatment period. The subjects were instructed to avoid vigorous physical activity prior to each study visit and to maintain 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. Palatability, blood pressure, weight, height, and BMI were also measured. A decrease in appetite was observed in the stevia group compared to the control group, and the 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 statistically significant differences were observed in the 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 authors concluded that consumption of stevia leaf powder in cookies decreased hunger when compared to cookies without stevia leaf powder.

Rizwan *et al.* (2018) conducted a prospective, interventional, randomised, single-blind, placebo-controlled trial to investigate the beneficial effect of stevioside along with the conventional antihypertensive and anti-diabetic medications in chronic kidney disease patients. A total of 97 patients with Stage I to Stage III chronic kidney disease were split into 2 groups, 43 (mean age 53.60±11.27 years) were assigned to the placebo group, and 44 (mean age 55±11.75 years) were assigned to the treatment group and received 500 mg stevioside/day (purity not reported; 250 mg twice a day) for 3 months. An additional 10 subjects without chronic kidney disease were included as a healthy control group. The following parameters were assessed at baseline and after 3 months of treatment: blood pressure, blood biochemistry, and urinalysis. At baseline, diastolic blood pressure and several blood biochemistry parameters were reported to be significantly different ($p<0.05$) between the stevioside and placebo groups. After 3 months, the only uniquely different parameter was a significant increase in urinary protein:creatinine ($p<0.05$) in the stevioside group. When comparing the data within each group obtained at 3 months to the initial baseline measurements, statistically significant changes were reported in both stevioside and placebo groups for systolic and diastolic blood pressure, and serum uric acid ($p<0.05$). Unique changes in the stevioside group included decreases in fasting and postprandial blood sugar ($p=0.041$ and p 0.013, respectively), an increase in serum creatinine ($p=0.027$), a decrease in serum uric acid ($p=0.009$), and a decrease in microalbumin ($p=0.041$). The authors concluded that the oral consumption of stevioside for 3 months (500 mg/day) has the potential to improve select biochemical parameters in the blood and urine of chronic kidney disease patients undergoing conventional treatment regimens.

In a double-blind randomised controlled clinical study, Cocco *et al.* (2019) investigated the effects of repeat-consumption of a snack containing stevia on the development of dental caries in children located in Porto Torres, Italy. 264 schoolchildren (6 to 9 years of age; 61.53% females and 38.47% males) at risk of developing caries were randomised to receive twice-daily (once in the morning, and afternoon) cookies containing sugar (n=88), maltitol (n=87), or stevia (n=89; dose and purity not reported) as the only sweetener in the cookie for 42 days. The following clinical parameters were measured at baseline, after 42 days of snack consumption, and 120 days after completion of the study: side effects, carious lesion scores, interproximal plaque pH, and mutans streptococci and lactobacilli counts in the saliva. Tolerance and side effects to the sweetener was assessed shortly after receiving the snack, and 28 days later *via* a questionnaire. No side effects were reported by any of the children, based on the questionnaire. Levels of dental caries did not differ significantly between the 3 groups, and all had similar incidences of dental caries. Bleeding scores in all 3 groups were statistically and significantly lowered when compared to the associated baseline score. Concentration of mutans streptococci and lactobacilli in the stevia group were statistically and significantly decreased 120 days after the treatment period when compared to baseline. No statistically significant changes in bacterial counts were observed in the control group. Furthermore, the minimum and maximum interproximal plaque pH, and pH drop were significantly increased 120 days after consumption of the stevia-containing cookies when compared to baseline ($p<0.05$). At baseline, no differences were observed between the 3 groups regarding Cariogram, but after 42 days of snack consumption the number of subjects who have a low probability of developing new dental caries was significantly higher in the stevia group when compared to baseline ($p<0.01$). The authors concluded that stevia-based snacks positively modified parameters (mutans streptococci, lactobacilli, plaque pH) related to the developmental of dental caries.

In a parallel-arm randomised control trial, Higgins and Mattes (2019) investigated the effects of low-calorie sweeteners on body weight, ingestive behaviour, and glucose tolerance in overweight or obese adults (BMI between 25 and 40 kg/m$^2$). Subjects (39 in the control, 28.2±9.5 age, 54% female; 28 in the treatment group, 27.1±9.6 age, 64% female) consumed daily a beverage (1.25, 1.5, or 1.75 L; volume determined based on body weight) sweetened with sucrose (control; 100, 200, or 140 g, respectively) or rebaudioside A (0.66 g/beverage; average daily exposure 2.6 mg/kg steviol equivalents) for 12 weeks. Participants were told not to consume any food or drink other than water for ≥2 hours before drinking the test beverage. Ingestive frequency was determined based on the participants self-reported number of eating events and the sensory characteristics of the beverages were recorded

using a survey. Body weight, total body water, and glucose tolerance were assessed at baseline and Week 12. Food and energy intakes were measured at baseline, Week 4, 8, and 12. The beverages were not found to be significantly different in terms of perceived intensity of sweet, salty, sour, bitter, and aftertaste. When compared to the control group at Week 12, consumption of rebaudioside A did not have a significant effect on body weight, body composition, ingestive frequency, or glucose tolerance. Energy intake was significantly higher in the sucrose control group than the rebaudioside A group at 4, 8, and 12 weeks ($p<0.01$). The authors concluded that based on the results of this study, consumption of rebaudioside A at about 2.6 mg/kg/day steviol equivalents for 12 weeks had no effect on body weight in overweight or obese adults.

In a 3-arm single-blinded randomised crossover trial, the effect of stevia consumption on glucose levels, food consumption, and appetite was investigated and compared to water and sugar (Farhat *et al.*, 2019). Participants (10 males and 10 females, 26.1±10.56 years old, BMI 23.44±3.42 kg/m$^2$) consumed 1 of 3 different preloads, 300 mL of water mixed with citric acid, sugar (60 g), or stevia (1 g, purity not reported), separated by a 4- to 5-day washout period. Three hours prior to the administration of the preload participants consumed a 360-kcal breakfast, and 30 minutes after the preload received an *ad libitum* pizza lunch. Energy intake from this meal was determined based on the weight of pizza consumed. Using the 100 mm Visual Analogue Scale (VAS), participants were asked to rate their hunger, desire to eat, fullness, and satisfaction 180 minutes prior to lunch and at 30-minute intervals until 120 minutes after lunch. Blood glucose samples were obtained *via* a finger prick test before preload and lunch, and at 30-minute intervals until 120 minutes after lunch. The consumption of the different preloads did not have a significant effect on energy intake at lunch. The sugar preload resulted in a statistically significant increase in area under the curve for glucose ($p<0.0001$) when compared to the other 2 groups, but there was no difference between water and stevia. After adjusting for the calorie content and sugar preload, postprandial glucose levels did not differ significantly among groups. Regarding VAS scores, a statistically significant increase in hunger scores ($p<0.05$) and a desire to eat ($p=0.001$) for the water preload were reported compared to sugar and stevia groups, with no differences between sugar and stevia. Stevia was concluded to lower appetite sensation and to not increase postprandial glucose levels and food consumption.

In a parallel-arm, double blind, randomised controlled trial, an investigation was conducted on the glycaemic and lipid profile in adults with type 2 diabetes in response to daily consumption of stevia extract or sucralose in tea (Ajami *et al*., 2020). For 8 weeks, 15 subjects (5 males and 10 females, 55.3±7.4 years old, BMI 30.87±6.32 kg/m$^2$) drank a daily cup of black tea sweetened with 2% stevia extract (mg/kg dose and purity not reported), and 19 subjects (7 males and 12 females, 52.1±7.6 years old, BMI 27.51±3.04 kg/m$^2$) drank a daily cup of black tea sweetened with a sucralose tablet (sucralose amount not reported). To obtain baseline measurements, all subjects fasted for 12 hours followed by consumption of a standardised breakfast as well as the respective tea sweetened with either stevia extract or sucralose. Blood samples were obtained and analysed for fasting blood sugar (FBS), glycosylated haemoglobin (HbA1c), and glycaemic and lipid profiles. Postprandial glucose (PPG) was measured by taking a blood sample 2 hours after the meal. These measurements were recorded again after 4 and 8 weeks, and FBS and PPG were also measured 3 times weekly throughout the study by the subjects using portable glucometers. Dietary intakes were recorded at the beginning and end of the study 3 times weekly, using 24-hour recall questionnaires. After 8 weeks, there was no significant difference between groups in the measured blood parameters of insulin, FBS, HbA1c, 2-hour PPG, triglycerides, total cholesterol, LDL, and HDL. While total energy intakes were not significantly different, a significant reduction in saturated fatty acid intake ($p=0.01$) was reported in the stevia group compared to the sucralose group. It was concluded that compared to sucralose, stevia did not have any negative effects on blood glucose and lipid levels in diabetic subjects.

## C.3. Summary of Steviol Glycoside Safety Opinions

### C.3.1 The Joint FAO/WHO Expert Committee on Food Additives (JECFA)

The safety of steviol glycosides has been extensively reviewed by JECFA at several meetings (JECFA, 1999, 2006, 2007b, 2009, 2017b). JECFA concluded that the metabolic fate of steviol glycosides is similar in humans and rats, such that steviol glycosides are converted to steviol through the successive removal of glucose units by intestinal bacteria. Steviol is then absorbed from the colon, rapidly converted to steviol glucuronide, and excreted *via* the urine in humans. JECFA also concluded that steviol glycosides are not mutagenic and that steviol is not mutagenic *in vivo*. Studies conducted in humans demonstrated that steviol glycosides, meeting the established purity specifications, did not cause any adverse effects when consumed at doses of up to 4 mg steviol equivalents/kg body weight/day by individuals with type-2 diabetes mellitus for up to 16 weeks and individuals with normal or low-normal blood pressure for 4 weeks. Based on the above findings, JECFA calculated an ADI for steviol glycosides of 0 to 4 mg/kg body weight, expressed as steviol equivalents. The ADI was determined by applying a 100-fold safety factor for inter-and intra-species differences to the NOAEL of 970 mg stevioside/kg body weight/day (equivalent to 383 mg steviol equivalents/kg body weight/day) determined from a carcinogenicity study conducted with stevioside in rats (Toyoda *et al*., 1997). Initial specifications established by JECFA (2010) stipulated that the purity of steviol glycoside preparations was to be not less than 95% of the 9 named steviol glycosides (stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside). JECFA most recently re-assessed the safety of steviol glycosides at the 82nd meeting by reviewing all new data which had become available since the previous evaluation, and the ADI for steviol glycosides was confirmed. Based on the new data, a tentative specification was established for "Steviol glycosides from *Stevia rebaudiana* Bertoni", which defined steviol glycosides as:

> "*All compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni, including glucose, rhamnose, xylose, fructose, and deoxyglucose*"[3] (JECFA, 2017a).

The inclusion of all steviol glycosides within JECFA's purity specification further confirms that the safety of steviol glycosides is based on the general recognition that all glycosides are hydrolysed to the aglycone steviol and that the safety demonstrated for 1 glycoside is relevant to all glycosides in general. The Committee also evaluated data on a novel yeast-derived steviol glycoside product resulting in the issuance of new specifications for "Rebaudioside A from multiple gene donors expressed in *Yarrowia lipolytica*", with a purity definition of no less than 95% rebaudioside A (JECFA, 2016).

In June 2019, at the 87th meeting of JECFA, a framework for developing specifications for steviol glycosides by method of production was established and adopted. The 4 manufacturing technologies included in the framework are defined as (a) extraction: a process of hot water extraction from the leaves of *S. rebaudiana* Bertoni; (b) fermentation: a process in which a genetically modified microorganism is used to produce specific steviol glycosides; (c) enzymatic modification: a process in which steviol glycosides that have been extracted from the leaves of *S. rebaudiana* Bertoni undergo enzymatic conversion of major steviol glycosides to minor ones; and (d) enzymatic glucosylation: a process in which steviol glycosides that have been extracted from the leaves of *S. rebaudiana* Bertoni undergo enzyme-catalysed reactions to add glucose units to the steviol glycosides *via* α-(1-4) linkages (JECFA, 2019). During its evaluation, the Committee reviewed data on these new methods of manufacture and the identity and purity of steviol glycosides produced by these technologies. With

[3] The Committee reviewed a validated HPLC-ultraviolet method for the assay at the 84th meeting and based on these data the 2 additional saccharides (galactose and arabinose) were included in the definition and the tentative status was removed from the specification (JECFA, 2017a).

respect to the fermentation process, the Committee noted that the microorganisms used in the fermentation process are of safe lineage (*e.g., Y. lipolytica, S. cerevisiae*), the inserted genes are derived from sources that are non-toxigenic and non-pathogenic, and the residues arising from the fermentation process do not introduce any concerns related to toxicity or allergenicity (JECFA, 2019). The Committee concluded that the current ADI of 0-4 mg/kg body weight established at the 69th meeting of JECFA for steviol glycosides applies to steviol glycosides produced by the 4 defined manufacturing technologies and that "*no safety issues exist for steviol glycosides produced by any one of these methods resulting in products with ≥95% steviol glycosides as per existing specifications*" (JECFA, 2020).

### C.3.2 Food Standards Australia New Zealand (FSANZ)

FSANZ conducted their own evaluation of the safety of steviol glycosides in 2008 based upon the data previously reviewed by JECFA, in addition to supplementary published and unpublished safety data (FSANZ, 2008). FSANZ also established an ADI of 4 mg/kg body weight/day as steviol equivalents. FSANZ recently approved a request to amend the definition of steviol glycosides in the *Australia New Zealand Food Standards Code* to include "*all minor steviol glycosides*" extracted from the *S. rebaudiana* Bertoni leaf in addition to the 10 steviol glycosides (stevioside, rebaudioside A, B, C, D, F, and M, dulcoside A, rubusoside, and steviolbioside) which were approved previously (FSANZ, 2008, 2015, 2017). As part of the approval process, FSANZ performed a risk assessment in which it considered *in vitro* biotransformation studies of several steviol glycosides, the results of which demonstrated that steviosides, rebaudiosides, and dulcosides are biotransformed to steviol and are consistent with previously-approved steviol glycosides. Based on the outcome of the safety assessment, FSANZ concluded that the ADI for steviol glycosides from *S. rebaudiana* Bertoni leaf of 0 to 4 mg/kg body weight (as steviol) is "*applicable to all steviol glycosides in stevia leaf*", of which FSANZ recognises includes at least 40 different steviol glycosides (FSANZ, 2017). FSANZ issued specifications for steviol glycosides from *S. rebaudiana* Bertoni with a total steviol glycoside content of no less than 95% on the dried basis, which expands the definition to include all individual steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni. Most recently, FSANZ conducted risk assessments on alternative manufacturing processes to typical extraction from the *S. rebaudiana* Bertoni leaf, including fermentation and enzymatic conversion of stevia leaf extract. Following its assessment, FSANZ determined that these alternative manufacturing processes do not pose a public health or safety concern. Consequently, the *Australia New Zealand Food Standards Code* has been recently amended to include specifications for steviol glycosides from fermentation (S3—39) (*i.e.,* Cargill's steviol glycosides produced by *S. cerevisiae* production strains) and the specification for Steviol Glycosides from *Stevia rebaudiana* Bertoni (S3—35) has been updated to include enzymatic conversion of stevia leaf extract as an acceptable method to manufacture (a) rebaudiosides D and M using enzymes derived from strains of *Pichia pastoris*; and (b) rebaudiosides D, M, and AM using enzymes derived from strains of *Escherichia coli* (FSANZ, 2020a). To note, Reb MD produced from a *Y. lipolytica* production strain that is the subject of this current application is chemically and substantially equivalent to rebaudioside MD obtained from *S. cerevisiae* strain CD15407 that was the subject of Application A1170 previously submitted to FSANZ by Cargill.

### C.3.3 European Food Safety Authority (EFSA)

EFSA (2010) evaluated the safety of steviol glycosides[4] for use in food in the EU at the request of the European Commission as part of the authorisation process for food additives. EFSA evaluated the available data and allocated an ADI of 4 mg/kg body weight, expressed as steviol equivalents, for steviol glycosides. Following this safety opinion, the European Commission permitted the use of steviol glycosides as a sweetening agent under Commission Regulation (EU) No 1131/2011 (EU, 2011). In a subsequent scientific opinion, EFSA expanded the definition of steviol glycosides to include rebaudiosides D and M and concluded that "*extending the current specifications to include* [2 additional

[4] Consisting of stevioside, rebaudioside A, B, C, D, and F, dulcoside A, rubusoside, and steviolbioside (EFSA, 2010)

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 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*" (EFSA, 2015). EFSA concluded in a recent evaluation of glucosylated steviol glycosides that the data provided was not sufficient to assess the safety of these glycosides due to the limited evidence on the complete hydrolysis of glucosylated steviol glycosides to steviol and responded that metabolic fate data from parent steviol glycosides cannot be used in a read-across approach (EFSA, 2018b). In 2019, EFSA issued a scientific opinion on the safety of rebaudioside M produced *via* enzyme-catalysed bioconversion of purified stevia leaf extract (EFSA, 2019). Following its assessment, the Panel concluded:

> "*There is no safety concern for Rebaudioside M produced via enzymatic bioconversion of purified stevia leaf extract using UDP-glucosyltransferase and sucrose synthase enzymes produced by the genetically modified yeast K. phaffii UGT-a and K. phaffii UGT-b, to be used as a food additive*".

EFSA most recently evaluated the safety of a proposed amendment to the steviol glycoside specification in the EU to expand the list of steviol glycosides to all those identified in the leaves of *S. rebaudiana* Bertoni (EFSA, 2020). The proposed change is to include all 60 steviol glycosides identified in the leaves of *S. rebaudiana* Bertoni, including both 'major' and 'minor' steviol glycosides, in the same limit value of 95% total steviol glycosides. This amendment would bring the EU specification for steviol glycosides in line with the current FSANZ and JECFA specifications for Steviol Glycosides from *Stevia rebaudiana* Bertoni. In its evaluation, the EFSA Panel acknowledged that all steviol glycosides share the same metabolic fate and therefore concluded that the safety of the 60 identified steviol glycosides presented in the application can be established based on read-across from the toxicology database that exists for steviol glycosides from *S. rebaudiana* Bertoni previously evaluated by EFSA. Furthermore, it was concluded that the ADI of 4 mg/kg body weight expressed as steviol equivalents applies to all 60 steviol glycosides.

### C.3.4 Health Canada

Health Canada reviewed the safety of steviol glycosides and similar to other scientific and regulatory authorities, established an ADI of 4 mg steviol equivalents/kg body weight (Health Canada, 2012a). Steviol glycosides as initially defined by JECFA were approved by Health Canada for use as sweetening agents at levels of up to 0.35% calculated as steviol equivalents (Health Canada, 2012b). In addition to the 9 steviol glycosides initially considered by JECFA, Health Canada expanded the purity definition of steviol glycosides to include rebaudioside M as being 1 of the 10 steviol glycosides that may be present alone or in combination in finished preparations to reach the total steviol glycoside content of at least 95% purity (Health Canada, 2016). Health Canada has since received a request to expand the use of the food additive 'steviol glycosides' to include all steviol glycosides in the *S. rebaudiana* Bertoni plant (Health Canada, 2017). Following a safety assessment in which no safety concerns were identified, Health Canada expanded the steviol glycoside food additive description as requested. It is understood that this definition also extends to include steviol glycosides extracted from the leaf that are then converted enzymatically to generate steviol glycosides, such as rebaudioside M, with improved sensory profiles. Most recently, Health Canada conducted a premarket safety assessment on steviol glycosides derived from *S. cerevisiae* production strains for use in a variety of foods (*i.e.,* Cargill's steviol glycosides produced by *S. cerevisiae* production strains) (Health Canada, 2019, 2020). Since no safety concerns were identified, the List of Permitted Sweeteners was updated to include fermentation processes as an alternative source to *S. rebaudiana* Bertoni for steviol glycosides, enabling the use of steviol glycosides from *S. cerevisiae* production strains in all foods where steviol glycosides from *S. rebaudiana* Bertoni are permitted and at the same use levels (Health Canada, 2020b).

# C4. Safety Assessment of the Production Strain/Organism

The safety of the production strain/organism was assessed in accordance with Guideline 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a), Parts C2, C3, D1, D2, D3, and E1.

## C.4.1 Information Related to the Safety of a Processing Aid

### *C.4.1.1 Information on the Potential Toxicity of the Processing Aid*

To confirm that the proteins expressed in the *Y. lipolytica* production strain are not associated with any toxic potential, the Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information was used to conduct sequence alignment queries of the full-length FASTA protein sequences of the inserted gene sequences against curated databases maintained by UniProt containing (a) venom proteins and toxins (UniProtKB/Swiss-Prot Tox-Prot[5]); and (b) virulence factors (UniProtKB/Swiss-Prot/TrEMBL[6]). The full search report is considered confidential and is provided in Appendix C.

The BLAST searches identified sequence matches with 17 to 68% identity with various animal venom proteins and toxins and virulence factors, and associated E-values ranging from $3x10^{-60}$ to 10.0. E-values greater than $1x10^{-7}$ suggest that proteins are unlikely to share structural homology (Hileman *et al.*, 2002). The sequence alignments with low E-values (in the range of $10^{-9}$ to $10^{-60}$) shared approximately 21 to 31% identity with cytochrome P450 monooxygenases. It should be noted that cytochrome P450 monooxygenase is a native enzyme involved in endogenous reactions in humans. Based on the bioinformatic searches conducted, it is anticipated that the inserted genes do not encode for proteins that are 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 the inserted gene sequences with known animal venom proteins and toxins and virulence factors (*i.e.,* sequence identities of no more than 68% and associated E- values of greater than $1x10^{-7}$), 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 the proteins encoded by the inserted genes, no toxic or pathogenic potential is anticipated with Reb MD produced by *Y. lipolytica*.

The safety of the heterologous proteins expressed by the production strain are further supported by the history of safe use of these proteins and their sources within the food supply. As described below in Section C.4.3., most of the heterologous proteins originated from the plant *S. rebaudiana* Bertoni (*i.e.,* the current botanical source of steviol glycosides). The safe use of proteins from *S. rebaudiana* Bertoni are supported by other applications that have been previously reviewed by FSANZ, such as; Cargill's *S. cerevisiae* production strain application for Reb MD (A1170) and Amyris's more recent *S. cerevisiae* production strain application for steviol glycosides (A1207). The safety of the heterologous proteins from *Y. lipolytica* (*i.e.,* derived from native genes that were overexpressed or heterologous genes for reactions native to the yeast) is supported by the QPS status of *Y. lipolytica* (see

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

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

Section C.4.2.2) (EFSA, 2018c). Furthermore, several heterologous proteins expressed by the production strain were derived from edible plants with a long history of safe use in the food supply (see Appendix C for identity of the sources).

#### *C.4.1.2 Information on the Potential Allergenicity of the Processing Aid*

An allergenicity screen of the heterologous gene sequences inserted in the *Y. lipolytica* production strain was conducted according to the approach outlined by FAO/WHO (2001) and the Codex Alimentarius (2009) in order to confirm the lack of potential for allergenic cross-reactivity of the inserted gene sequences in the production strain. This screen for relevant matches to known putative allergens was carried out using the AllergenOnline database version 19 (available at http://www.allergenonline.org; updated 10 February 2019) that is maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2019). A FASTA 35.04 overall search of AllergenOnline was conducted using default settings (E-value/score cut-off = 1 and maximum alignments of 20). Searches were conducted using the full-length amino acid sequence and an 80-amino acid 'sliding window' (segments 1–80, 2–81, 3–82, *etc*.) 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 (Codex Alimentarius, 2003, 2009). Significant homology is defined as an identity match of greater than 35% (Codex Alimentarius, 2009), and in such instances, cross-reactivity with the known allergen must be considered a possibility. Using this search strategy, no identity matches of greater than 35% were identified. The full search report is considered confidential and is provided in Appendix C.

Additionally, the raw fermentation medium materials listed in Table B.5.2-1 that are used in the manufacturing process for the *Y. lipolytica* production strain are not derived from any major allergens.

### C.4.2 Additional Information Related to the Safety of a Processing Aid Derived from a Microorganism

#### *C.4.2.1 Information on the Source Microorganism*

The production organism has been designated as *Y. lipolytica* VRM. The production organism is derived from a *Y. lipolytica* parent line that is non-pathogenic, non-toxigenic, and is well characterized. Widespread in nature, the primary habitat of *Y. lipolytica* is currently unknown; however, the species is often found in hyper-saline marine environments (Hagler and Mendonça-Hagler 1979; Butinar *et al.*, 2005) and was first isolated from milled corn (maize) fiber tailings by L.J. Wickerham (Kurtzman, 2011). The identity of the production organism as *Y. lipolytica* has been confirmed *via* 18s rRNA sequencing and the report is provided in Appendix C.

*Y. lipolytica* was previously classified as *Candida lipolytica* (van der Walt and von Arx, 1980) and other names that have been used for this yeast include *Endomycopsis lipolytica, Saccharomycopsis lipolytica, Mycotorula lipolytica,* and *Yallowia lipolytica. Y. lipolytica* belongs to the Dipodascaceae family and the taxonomic identity is presented in the table below.

**Table C.4.2.1-1 Taxonomic identity of *Yarrowia lipolytica***

| | |
|---|---|
| **Kingdom** | Fungi |
| **Phylum** | Ascomycota |
| **Class** | Saccharomycetes |
| **Order** | Saccharomycetales |
| **Family** | Dipodascaceae |
| **Genus** | *Yarrowia* |
| **Species** | *Yarrowia lipolytica* |

#### *C.4.2.2 Information on the Pathogenicity and Toxicity of the Source Microorganism*

*Y. lipolytica* has been extensively studied and is customarily classified as a biosafety class 1 microorganism (Groenewald *et al*., 2014). It has a long history of safe use in the production of food (*e.g.*, cheese ripening) and food ingredients (*e.g.*, citric acid, γ-decalactone). Under Schedule 15, yeast and yeast products are permitted as food additives and colourings with no limitations other than cGMP (FSANZ, 2020b). EFSA has granted Qualified Presumption of Safety (QPS) status for *Y. lipolytica* and therefore has deemed it safe to derive genetically modified strain lineages to use in the production of food additives and enzymes (EFSA, 2018c). The JECFA Committee has deemed *Y. lipolytica* to be an acceptable production organism for commercial products that are primarily composed of rebaudioside A, rebaudioside M, or a combination of rebaudioside M and rebaudioside D (not less than 95% total steviol glycosides) (JECFA, 2020). Reb MD produced by *Y. lipolytica* has GRAS status for a variety of food and beverage uses and has been GRAS-notified to the U.S. FDA under GRN 000882 that has recently received a "no questions" letter from the Agency (Cargill, 2019; U.S. FDA, 2020a). Other steviol glycoside preparations (*e.g.,* rebaudioside A and rebaudioside M) obtained from *Y. lipolytica* expressing steviol glycoside biosynthesis pathway genes, similar to the production organism that is described in this application for Reb MD, are GRAS for use as table top sweeteners and as general-purpose non-nutritive sweeteners in foods in the U.S. (GRN 000632 and 000759 – U.S. FDA, 2016b, 2018). In addition, under 21 CFR §173.165, *Y. lipolytica* (identified by its prior classification of *Candida lipolytica*) is permitted for use as a secondary direct food additive for fermentation production of citric acid in the U.S. (U.S. FDA, 2020c). In 2011, the U.S. FDA received 2 GRAS Notices for the production of an eicosapentaenoic acid (EPA)-rich triglyceride by *Y. lipolytica* (GRN 355) and for erythritol produced *via* biotransformation by a strain of *Y. lipolytica* (GRN 382) and responded with "no questions" letters regarding the GRAS status of both ingredients (U.S. FDA, 2011a,b).

#### *C.4.2.3 Information on the Genetic Stability of the Source Organism*

The identity of the production strain is confirmed through whole genome sequencing of the production strain. As homologous recombination is used for the genetic transformation of the yeast, the genetic elements introduced are stable. The cell line stability is demonstrated by using secondary and tertiary cell banks and comparing productivities to primary cell banks. Extended seed trains also are typically tested to ensure retention of phenotype over many generations.

The introduced genes encoding the enzymes for the pathway are required for production of steviol glycosides. The production of steviol glycosides over the course of the fermentation by the production organism additionally validate the genetic stability of the host. In order to maintain steviol glycoside production, the genes encoding the enzymes need to be transcribed and translated from the genome and the resulting steviol glycoside production is evidence of this. Production strain performance has been shown to be consistent over a number of fermentations. Data supporting the genetic stability of the production strain and the inserted genes is provided in Appendix C.

### C.4.3 Additional Information Related to the Safety of a Processing Aid Derived from a Genetically Modified Microorganism

#### *C.4.3.1 Information on the Methods used in the Genetic Modification of the Source Organism*

*a) Full description of the gene construct, including information on the size, source and function of all genetic components, including marker genes*

A full description of the gene constructs is considered confidential information and is provided in Appendix C.

*b) Full details of any modifications to the DNA or amino acid sequence of the enzymes*

Full details of modifications to the inserted gene sequences are considered confidential information and are provided in Appendix C.

*c) Full description of the final production strain, including the steps and methods used to construct it, the integration site (plasmid or chromosome) of the introduced gene and organisation of all inserted genetic material*

*Y. lipolytica* does not produce steviol glycosides and therefore, its metabolism needed to be redirected to allow maximal flux towards the precursors of steviol glycosides. Heterologous genes were introduced into the genome to allow production of steviol glycosides from these precursors. The incorporated DNA is either produced by gene synthesis or sourced from biosafety level 1 organisms and is not associated with any known allergens or toxins. Most of the genes originated from the plant *S. rebaudiana* Bertoni (*i.e.,* the current botanical source of steviol glycosides) or other edible plants with a long history of safe consumption (see Appendix C for identity) but were produced by gene synthesis. In addition to enzymes specific to the steviol glycoside pathway, native genes from *Yarrowia* were overexpressed or heterologous genes for reactions native to the yeast were introduced to increase the flow of carbon into the steviol glycoside pathway and the transport of steviol glycosides.

The steps and methods used to construct the final production strain are described as follows:

**1) <u>Parental Strain</u>**

Three parental strains of *Y. lipolytica* (strains ATCC 76861, ATCC 76982, and ATCC 201249) were obtained directly from the American Type Culture Collection (ATCC) and used to generate 2 starting strains. Strain construction initiated with 2 strains (strains ML326 and ML350) that had opposite mating types to allow for subsequent mating and natural polymorphic variation.

**2) <u>Production Strain</u>**

Both starting strains (ML326 and ML350) were engineered with the steviol glycoside production pathways according to general transformation procedures, as described in further in the next section. After several modifications to each strain, the strains were mated to produce diploids, and said diploids were sporulated to produce haploid progeny. A single haploid progeny was further modified by transformation to improve production. The spores were screened for high steviol glycoside production and the production strain was derived from one of these spores. Antibiotic resistance markers (kanamycin, hygromycin, and nourseothricin) were transiently used in the process. Marker systems were rendered non-functional restoring antibiotic sensitivity to the strain, which was confirmed using PCR analysis and by verifying that the strain was sensitive to the relevant antibiotics.

**3) <u>Construction of the Production Strain</u>**

The genes used to generate the production strain code for enzymes required to synthesize, transport, and improve the overall production efficiency of steviol glycosides. The parental strains of *Y. lipolytica* were initially modified to over-express the genes responsible for the production of steviol glycosides (*i.e.,* Reb M, Reb D). Most of the genes originated from the plant *S. rebaudiana* Bertoni or other edible plants but were produced by gene synthesis and adapted with respect to codon usage for optimal expression in the yeast. *S. rebaudiana* Bertoni is the current botanical source of steviol glycosides. In addition to enzymes specific to the steviol glycoside pathway, native genes from *Yarrowia* were overexpressed to increase the flow of carbon into the steviol glycoside pathway and transport of steviol glycosides.

*Yarrowia* strains of both mating types were engineered for steviol glycoside production. These strains were mated, the diploid sporulated, and spores with steviol glycoside production were selected. One of these spores was further developed for the production of steviol glycosides. Strain ML10371 (MAT - A, lysl -, ura3 -, leu2 -) was transformed with defined DNA fragments using a lithium acetate/PEG fungal transformation protocol method and transformants were selected on minimal medium. Antibiotic resistance markers nourseothricin and hygromycin (HPH hygromycin resistance gene) were used during integrations and were rendered non-functional in commercial strains. The *Y. lipolytica* strains are transformed with expression cassettes, containing promoters, open reading frames and terminators encoding the genes in the rebaudioside M pathway. These expression cassettes are transformed with standard transformation techniques and the resulting strain is marker free, as confirmed by sequencing. The cassettes are integrated into the genome, next to random integrations, and precision genome engineering techniques were also used. Besides transformation, classical techniques like mutagenesis and selection are used to improve the strain even further. The introduced DNA sequences are integrated partly in predefined loci (targeted integration) but mostly randomly integrated. Strains were also subjected to classical strain improvement techniques. The yeast *Y. lipolytica* is not known to harbor any genes encoding for toxins or otherwise harmful sequences, therefore both random and targeted introduction of DNA sequences will not lead to an increased risk due to unintended pleiotropic effects. The final production strain is sensitive to kanamycin, nourseothricin, and hygromycin. The production strain is not toxigenic or pathogenic and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens. The incorporated DNA is either synthetic or sourced from biosafety level 1 organisms and is not associated with any known allergens or toxins.

The integration sites of the introduced genes and organisation of all inserted genetic material is provided in the form of a lineage map, which is provided in Appendix C and is confidential informaton.

*d) Information on the stability of the inserted genes*

Information on the stability of the inserted genes is provided in Section C.4.2.3 above and in Appendix C and is confidential information.

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

In accordance with Section 3.3.1 – Food Additives of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019a) the following dietary exposure information must be provided:

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

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

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

Each point is addressed in the following subsections.

## D.1 Proposed Food Uses and Use Levels of Reb MD

Reb MD is intended for use as an intense sweetener in the same approved food uses and at the same use levels as other steviol glycosides currently in the Australian/New Zealand marketplace. Table D.1-1 presents the currently approved food uses and use levels for steviol glycosides in Australia and New Zealand as per Schedule 15 (FSANZ, 2020b).

**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

Reb MD is intended for use as an intense sweetener in Australia and New Zealand under the same conditions of use as those presently authorised for steviol glycosides. Considering that Reb MD is intended to be a direct replacement for other steviol glycosides, the expected intakes of Reb MD would be similar to the intakes from other steviol glycosides that are currently on the market in Australia and New Zealand. Based on the foregoing, a separate intake assessment for Reb MD was not performed for the purposes of this application. In addition, since steviol glycoside use levels are expressed as steviol equivalents, specific use levels for each individual glycoside are not required. The use levels encompass all individual glycosides and are based on the total content of steviol in the final food or beverage product resulting from the addition of any steviol glycoside preparation meeting the appropriate specifications.

## D.3 Global Use of Reb MD

In the U.S., Reb MD produced by *Y. lipolytica* has GRAS status for use as a general-purpose sweetener in a variety of food and beverage uses (excluding infant formula products) at levels determined by GMP and also for use as a table top sweetener (GRN 000882 – U.S. FDA, 2020b). Reb MD produced by *Y. lipolytica* has been GRAS-notified to the U.S. FDA under GRN 000882, which recently received a "no questions" letter from the Agency (Cargill, 2019; U.S. FDA, 2020a). In Mexico, rebaudioside M from multiple gene donors expressed in *Y. lipolytica* (*i.e.,* Reb MD that is the subject of this application) is an approved food additive (Cofepris, 2018).

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