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# APPLICATION

## MAY 2020 [REVISED]

TO:

FOOD STANDARDS AUSTRALIA NEW ZEALAND

IN RELATION TO:

Application for the Approval of Rebaudioside M from *Saccharomyces cerevisiae* expressing Steviol Glycoside Biosynthesis Pathway Genes

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# ADMINISTRATIVE INFORMATION

*(As per section 3.1.1 B of the Application Handbook as at 1 July 2019)*

## Applicant Details

*(As per section 3.1.1 B of the Application Handbook as at 1 July 2019)*

| | |
|---|---|
| Applicant | [redacted]<br>Head, Global Regulatory Affairs |
| Organisation | Amyris Inc.<br>https://amyris.com/ |
| Address: office & postal | [redacted] |
| Telephone | [redacted] |
| Email address | [redacted] |
| Primary contact | [redacted]<br>[redacted]<br>[redacted] |

## Nature of Business

*(As per section 3.1.1 B of the Application Handbook as at 1 July 2019)*

Amyris Inc. is a manufacturer of food ingredients and provides food and non-food applications worldwide.

## Details of other parties associated with the Application

*(As per section 3.1.1 B of the Application Handbook as at 1 July 2019)*

The following Scientific and Regulatory Consultants have been involved in the preparation, submission and stewardship of this application:

- [redacted], JK Food Reg (www.jkfoodreg.com.au)
- [redacted], FJ Fleming Food Consulting Pty Ltd

# 1. Application Information

## Status of Similar Applications

*(As per Section 3.1.1 D of the Application Handbook as at 1 July 2019)*

The Applicant has reviewed the Food Standards Development Work Plan (08 November 2019) and the FSANZ website and has identified the following similar current applications for approval of Steviol Glycosides:

| Application | Name | Applicant | Status |
|---|---|---|---|
| A1183 | Enzymatic production of Rebaudioside E | Blue California | Admin assessment report – 15/8/2019 |
| A1176 | Enzymatic production of Steviol Glycosides | PureCircle Limited | Call for submissions – ended 8/10/2019 |
| A1170 | Rebaudioside MD as a Steviol Glycoside from *Saccharomyces cerevisiae* | Cargill | Approval report published 27 June 2019 – gazetted |

## Assessment Procedure

*(As per section 3.1.1 F of the Application Handbook as at 1 July 2019)*

Amyris seeks to submit this application for consideration as a General Procedure, Level 2 (maximum of 380 variable hours) on the basis of the existing extensive safety database that exists for steviol glycosides and the shared metabolic fate with steviol glycosides which are currently approved for use in Australia and New Zealand.

The Application will be submitted as a **paid** Application.

## Confidential commercial information [CCI]

*(As per section 3.1.1 G of the Application Handbook as at 1 July 2019)*

This application **does contain** information that is confidential commercial information (CCI).

Amyris has provided information to support this application which it considers to be CCI.

This information is provided separately and clearly labelled as CCI.

## Exclusive capturable commercial benefit [ECCB]

*(As per section 3.1.1 I of the Application Handbook as at 1 July 2019)*

The Applicant understands that FSANZ consider this Application will confer an exclusive capturable commercial benefit for Amyris.

The Applicant notes they are not the only manufacturer of steviol glycosides - there are other manufacturers of steviol glycosides who would likely benefit from approval of this application.

# 2. Purpose of the Application

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

The purpose of this Application is to request an amendment to the *Australia New Zealand Food Standards Code* (hereafter the Code) to permit the use of Steviol glycosides Rebaudioside ("Reb") M that is produced by fermentation from *Saccharomyces cerevisiae* (*S. cerevisiae*), expressing steviol glycoside biosynthesis pathway genes, as a general-purpose sweetening agent.

The Applicant is seeking an amendment to steviol glycosides specifications within Schedule 3 (non-specific). The explanation is further expanded in section 3.1 below.

# 3. Justification for the Application

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

## 3.1 Need for the Proposed Change

*(As per section 3.1.1 D(a) of the Application Handbook as at 1 July 2019)*

Amyris' Rebaudioside M is like other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand and is intended to be used as a general-purpose sweetening agent.

Amyris Reb-M is produced by fermentation. Currently, under Schedule 3 – Purity and Identity of the Code, steviol glycosides from fermentation (S3—39) are required to conform to the following requirements:

(a) Is obtained from fermentation
(b) Is not obtained from the leaves of the *Stevia rebaudiana* Bertoni plant, and
(c) contains a prescribed steviol glycoside.

Amyris' steviol glycosides Reb-M contains not less than 95% total steviol glycosides.

The currently permitted prescribed steviol glycoside (Reb-MD) is derived from *Saccharomyces cerevisiae* strain CD15407 containing novel genes for the production of rebaudiosides.

Amyris' Rebaudioside M produced from *S. cerevisiae* meets all the above conditions but **does not** utilize the specific strain CD15407.

Thus, for Amyris to market this Reb-M product, an amendment to the Code is required.

## 3.2 Advantages of the Proposed Change

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

Rebaudioside M is most associated with improved sweetness quality (similar to sugar) when compared to major leaf derived steviol glycosides, such as rebaudioside A and stevioside.

Currently available steviol glycoside products available in the market have technological limitations with respect to sweetness, quality and taste.

The food additive industry is greatly interested in Rebaudioside M using alternative manufacturing processes such as yeast fermentation which is more efficient than the traditional leaf extraction processes, which yields only low levels of Rebaudioside. The Amyris manufacturing fermentation process for steviol glycoside mixtures that utilizes a genetically modified *S. cerevisiae* production organism can yield high concentrations of rebaudioside M.

Amyris' rebaudioside M provides an alternative to other steviol glycosides in the market with an improved sensory profile, and therefore, a better sweetness quality for consumers.

## 3.3 Disadvantage of the Proposed Change

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

The Applicant is not aware of any disadvantages of the proposed change.

Products containing Reb-M will be required to be labelled to indicate the presence of the food additive as a sweetener (960).

## 3.4 Public Health and safety issues

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

The Applicant has not identified any public health and safety issues in relation to the approval of Rebaudioside M produced from *S. cerevisiae* for use in the Australia/New Zealand food supply.

Refer **Sections 6.2 and 7.2** for information about the safety of the food additive and processing aid.

## 3.5 Consumer choice

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

No consumer choice issues related to the proposed change are foreseen.

Products containing Reb-M will be required to be labelled to indicate the presence of the food additive as a sweetener (960).

## 3.6 Support for the proposed change

*(As per section 3.1.1 D of the Application Handbook as at 1 July 2019)*

The Applicant has provided a letter from a potential customer. This is provided as Appendix CCI-6.

## 3.7 Regulatory Impact Information

*(As per section 3.1.1 D.1 of the Application Handbook as at 1 July 2019)*

Amyris Reb-M is intended to be marketed in the same approved food-uses and at the same use-levels as other steviol glycosides, which are already approved for many food applications not only within Australia and New Zealand (FSANZ, 2017a), and approved by the EU EFSA, Health Canada, the US FDA, Brazil ANVISA and other national food safety authorities.

### 3.7.1 Costs and Benefits of the Application

*(As per section 3.1.1 D.1.1 of the Application Handbook as at 1 July 2019)*

**Costs and Benefits – Consumer**

The consumer benefits for the use of Amyris Reb-M would be similar to those for steviol glycoside mixtures currently permitted for use in Australia and New Zealand.

The potential benefit to consumers includes:

- choice of additional products which become available due to the availability of another Reb-M for Australian and New Zealand food manufactures, and
- access to food products that are currently manufactured overseas with the use of this Reb-M.

Amyris Reb-M will be a replacement of sugar in foods, at a cost parity with sugar, for consumers who are seeking healthier foods and beverages with reduced calories from sugar.

The use of the Amyris Reb-M as a food additive would also benefit individuals with specific medical conditions that require reduced sugar intakes, such as diabetics, as determined by the European food safety authority EFSA (2010 report).

The proposed amendment places no additional economic cost on consumers.

**Costs and Benefits - Industry and Business**

Based on independent analysis, Amyris Reb-M provides improved sensory characteristics over major steviol glycosides (e.g. reb-A and stevioside) and has similar sugar stability, making it suitable for a wide variety of general sweetener applications.

This global safety approval would also provide Australia/New Zealand manufactured products an international commercial opportunity.

Use of the Amyris Reb-M will be at the discretion of business, therefore there are no direct costs imposed on industry.

**Costs and Benefits – Government**

The proposed amendment places no additional regulatory costs on government beyond the initial regulatory cost of approving this Reb-M.

### 3.7.2 Impact on International Trade

*(As per section 3.1.1 D.1.2 of the Application Handbook as at 1 July 2019)*

The Applicant notes that, in developing food standards, FSANZ must have regard to its WTO obligations; the promotion of consistency between domestic and international food standards; and the promotion of fair trading in food. These matters encompass consideration of international standards and trade issues.

Approval of the Amyris Reb-M would bring Australia and New Zealand into line with other countries where it is permitted for use (outlined under Section 5).

# 4. Information to Support the Application

*(As per section 3.1.1 E of the Application Handbook as at 1 July 2019)*

## 4.1 Data Requirements

The data and information provided to support the application fulfils the requirements for data as set out in the FSANZ Application Handbook:

- the source, author and year of evidence is identified
- it has been obtained using validated methods
- it represents Australian and New Zealand populations where possible, and
- it has been compiled from studies conducted under good laboratory practice (GLP).

Refer to **Sections 6 and 7** for information about the food additive and processing aid.

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## 4.2 FSANZ Act Objectives

Information is provided in this Application to address the objectives specified in Section 18 of the FSANZ Act 1991 as follows:

| High Order Policy Principles | Section of Application |
|---|---|
| 1(a) the protection of public health and safety | 6.2 & 7.2 |
| 1(b) the provision of adequate information relating to food to enable consumers to make informed choices | 6.1 |
| 1(c) the prevention of misleading or deceptive conduct | 6.1 |
| 2(a) the need for standards to be based on risk analysis using the best available scientific evidence | 6.2 & 7.2 |
| 2(b) the promotion of consistency between domestic and international food standards | 3.7 & 5.0 |
| 2(c) the desirability of an efficient and internationally competitive food industry | 3.7 & 5.0 |
| 2(d) the promotion of fair trading in food | 6.1 |
| 2(e) any written policy guidelines formulated by the Council for the purposes of this paragraph and notified to the Authority | N/A |

## 4.3 Policy Guidelines

*(As per section 3.3.2 of the Application Handbook as at 1 July 2019)*

Information is provided in this application to address the Policy Guideline - Addition to Food of Substances other than Vitamins and Minerals.

**Addition to Food of Substances other than Vitamins and Minerals**

The addition of substances other than vitamins and minerals to food where the purpose of the addition is to achieve a solely technological function should be permitted where:

| Specific Order Policy Principles – Technological Function | Section of Application |
|---|---|
| a) the purpose for adding the substance can be articulated clearly by the manufacturer (i.e. the 'stated purpose'); and | 3.0; 6.1 & 7.1 |
| b) the addition of the substance to food is safe for human consumption; and | 6.2 & 7.2 |
| c) the amounts added are consistent with achieving the technological function; and | 6.3 & 7.3 |
| d) the substance is added in a quantity and a form which is consistent with delivering the stated purpose; and | 6.3 & 7.3 |
| e) no nutrition, health or related claims are to be made in regard to the substance. | Not applicable |

# 5. International and Other National Standards

*(As per section 3.1.1 J of the Application Handbook as at 1 July 2019)*

## 5.1 International Standards

*(As per section 3.1.1 J.1 of the Application Handbook as at 1 July 2019)*

### 5.1.1 JECFA (Joint FAO/WHO Expert Committee on Food Additives)

Prepared at the 84th JECFA (2017), the Committee published JECFA Monograph 20, superseding tentative specifications prepared at the 82nd JECFA (2016) and published in FAO JECFA Monographs 19. The current JECFA specification for steviol glycosides refers only to those sourced from the leaves *of Stevia rebaudiana* Bertoni; it does not include steviol glycosides produced by fermentation[1]. A specification addressing other methods of manufacture of steviol glycosides (including fermentation) was prepared at the 87th JECFA meeting earlier in 2019; however, this specification has not yet been published.

An ADI of 0 - 4 mg/kg bw (expressed as steviol) was established at the 69th JECFA (2008). JECFA indicated, at its 87th meeting, that this ADI applies to steviol glycosides produced by the four methods discussed at the meeting. As noted above, this specification has not yet been published.

## 5.2 Other National Standards or Regulations

*(As per section 3.1.1 J.2 of the Application Handbook as at 1 July 2019)*

Steviol glycosides are approved for use in a number of jurisdictions, including the European Union, Canada, Asia, Central/South America, and Africa (Global Stevia Institute, 2017). A summary of Summary of Global Regulatory Approvals for Steviol Glycosides is provided in Table 2 below.

### 5.2.1 Australia/New Zealand

Schedule 15 of the Code provides permission for steviol glycosides to be added to a variety of processed foods in Australia and New Zealand (see the table to S15—5). This application does not seek amendment to these permissions.

Specifications for food additives, including steviol glycosides, must meet the requirements of Schedule 3 of the Code. Schedule 3 references sources of specifications that can be used to for compliance purposes (such as JECFA specifications) and lists specifications where other reference sources are not available. Noting the current lack of JECFA specifications for steviol glycosides produced by fermentation, FSANZ recently added a specification for steviol glycosides from fermentation (S3—39). However, this specification does not relate to the strain of yeast used by Amyris to produce Reb M.

[1] There is also a JECFA specification for Reb A from multiple gene donors expressed in *Yarrowia lipolytica* (FAO JECFA Monograph 19 (2016), which is not directly relevant to the Reb M that is the subject of this application

### 5.2.2 United States

In the U.S., Amyris' Rebaudioside M produced by *S. cerevisiae* has GRAS status for food and beverage uses (GRN 812 – U.S. FDA, 2018). The Food and Drug Administration (FDA) has provided no objections to the GRAS status of other steviol glycoside preparations, such as steviol glycosides (≥95% purity) extracted from the plant *S. rebaudiana*, enzyme-modified steviol glycosides, and steviol glycosides produced *via* microbial fermentation or enzymatic bioconversion for use as general purpose sweeteners in foods and beverages.

Effective GRAS notices pertaining to steviol glycosides with Reb-M are described in **Table 1** below.

**Table 1:** GRAS notices for steviol glycosides with Reb-M

| Year | Clearance |
|---|---|
| 2014 | GRN 512, High purity Rebaudioside M; FDA has no questions |
| 2016 | GRN 667, Rebaudioside M; FDA has no questions |
| 2018 | GRN 744, Steviol glycosides consisting primarily of rebaudioside M; FDA has no questions |
| 2018 | GRN 745, Steviol glycosides consisting primarily of rebaudioside M; FDA has no questions |
| 2018 | GRN 759, Steviol glycosides consisting primarily of rebaudioside M produced in *Yarrowia lipolytica*; FDA has no questions |

Of particular relevance, GRAS Nos. 744 and 745 received no questions from the FDA regarding the GRAS status of steviol glycosides consisting primarily of rebaudioside M for use as a general-purpose sweetener in foods, excluding meat and poultry products and infant formula, at levels in accordance with current good manufacturing practices. (U.S. FDA, 2018a, U.S. FDA, 2018b). Similar to Amyris's steviol glycosides Reb-M produced by fermentation, the final products in GRAS Nos. 744 and 745 contain ≥ 95% steviol glycosides, and consist of rebaudiosides A, B, C, D, E, F, M, stevioside, steviolbioside, rubusoside and dulcoside A in varying percentages.

### 5.3.3 Other Jurisdictions

In the European Union, commercially available steviol glycoside products must comply with the specifications for steviol glycosides (INS number 960) adopted by the European Commission in 2012 and recently updated in 2016 (EU, 2012, 2016).

**Table 2:** Summary of Global Regulatory Approvals for Steviol Glycosides[2]

| Jurisdiction | Safety Conclusions | Permitted Uses | Year Initially Approved | References for Evaluations |
|---|---|---|---|---|
| **Canada** | ADI to 4mg/kg bw | Sweetening agent | 2012 | Health Canada (2012a, b, 2016, 2017) |
| **Europe** | ADI to 4mg/kg bw | Sweetening agent | 2011 | EFSA (2010, 2015: EU 2011) |
| **ANZ** | ADI to 4mg/kg bw | Sweetening agent | 2008 | FSANZ (2008, 2015, 2017a) |
| **Japan** | N/A | General use as sweetener [1] | N/A | Marie (1991); Das et al (1992); Ferlow (2005); Japan Food Chemical Research Foundation (2014) |
| **Korea** | N/A | Sweetener in cookies, sugar products, beverages, seasonings, soy sauce, honey and so-ju | N/A | Kinghorn et al (1998); Chung et al (2005) |
| **India** | N/A | Food additive | N/A | FSSAI (2015); MOHFW (2016) |
| **Americas** | N/A | Food additive | N/A | PureCircle Stevia Institute (2018) |
| **South & North Asia and Asia Pacific** | N/A | Food additive | N/A | PureCircle Stevia Institute (2018) |
| **Middle East** | N/A | Food additive | N/A | PureCircle Stevia Institute (2018) |
| **Africa** | N/A | Food additive | N/A | PureCircle Stevia Institute (2018) |

[1] Three forms of purified stevioside

ADI = Acceptable daily intake; bw = body weight; EFSA = European Food Safety Authority; EU = European Union; FSSAI = Food Safety and Standards Authority of India; MOHFW = Ministry of Health & Family Welfare

[2] Reference is Application A1170

# 6. Substances added to Food – Food Additive

*(As per section 3.3.1 [Food Additives] of the Application Handbook as at 1 July 2019)*

## 6.1 Technical Information on the Food Additive

*(As per section 3.3.1 A of the Application Handbook as at 1 July 2019)*

### 6.1.1 Nature and Technological Purpose of Rebaudioside M

*(As per section 3.3.1 A.1 of the Application Handbook as at 1 July 2019)*

Amyris' Reb-M will perform the technological function listed in Schedule 14 - Technological purposes performed by substances used as food additives - Intense Sweetener which replaces the sweetness normally provided by sugars in foods without contributing significantly to their available energy.

Amyris's Rebaudioside M produced by fermentation is composed of ≥ 95% Reb-M and contains traces of other steviol glycosides, including those listed in Table 11. The final product contains ≥ 95% total steviol glycosides, which is consistent with the purity criteria for steviol glycosides as established JECFA (JECFA, 2017a). Amyris intends to market Rebaudioside M as a general-purpose sweetening agent in Australia and New Zealand. Amyris' Rebaudioside M is characterized by a sweetness intensity that is comparable to that of other high-intensity sweeteners (e.g., aspartame is approximately 200 times as sweet as sucrose, (DuBois *et al*., 1991) and provides an improved sensory profile as described further in the following section.

Evidence that the amounts proposed to be added are consistent with achieving the technological function is provided under Section 6.3.

The food additive is a powdered material and is incorporated homogenously and stably into the different food matrices to which it is proposed to be added in a similar way to other similar food additives.

**Taste Attributes**
Based on sensory testing performed on Amyris' Reb-M, it is found that it is 200-300 times sweeter than sucrose. This is consistent with literature findings comparing rebaudioside A (DuBois et al., 1991). Amyris' Reb-M was tested at a sweetness equivalence to a 5% sucrose solution in the sensory analysis. Sensory testing of Reb-M compared to Reb-A indicated that Reb-M was less astringent and bitter and left a less bitter lingering.

**Stability**
***General Stability of Steviol Glycosides***
The stability data of steviol glycosides have been reviewed by scientific advisory bodies involved in the evaluation of steviol glycosides safety (JECFA, the European Food Safety Authority (EFSA), and the Food Standards Australia/New Zealand (FSANZ)) and is also discussed in several published studies (Chang and Cook, 1983; Kroyer, 1999). Specifically, JECFA evaluated the stability of steviol glycosides under conditions mimicking their use in foods at their 68$^{th}$ meeting (JECFA, 2007). The Committee noted that steviol glycosides do not undergo browning or caramelization when heated and are reasonably stable under elevated temperatures used in food processing. Under acidic conditions (pH 2 to 4), steviol glycosides, are stable for at least 180 days when stored at temperatures up to 24°C. When exposed to

elevated temperatures (80°C, in water, 8 hours), however, 4 and 8% decomposition was reported in solutions of steviol glycosides at pH 4.0 and 3.0, respectively, indicating that the stability of steviol glycosides is pH and temperature dependent. When the temperature was increased to 100°C, expectedly higher rates of steviol glycoside decomposition (10 and 40% at pH 4.0 and 3.0, respectively) were reported. Based on the above, and in addition to 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 a recent publication, the structural and compositional stability of three commercial batches were evaluated to determine whether the manufacturing process adversely impacts steviol glycoside composition, with each batch containing a sample of untreated stevia leaves, the first water extract and high-purity end product (≥ 95% steviol glycosides) (Oehme et al., 2017). Changes in steviol glycoside composition were analyzed by HPLC-UV and HPLC-ESI-MS/MS. The authors reported that all nine JECFA-defined steviol glycosides were detected in all samples. The results also demonstrated that stevia extract processing does not chemically alter or modify the individual steviol glycoside content.

***Stability of Amyris' Rebaudioside M***

The general stability of steviol glycosides with a high reb M content (Lot 18RGT0511RM002) was assessed at pH 2, 5 and 8 for a total of 8 weeks at 4 different temperatures, 4, 22, 40, and 50°C. pH 2, pH 5 and pH 8 solutions were prepared using phosphoric acid and/or di-sodium hydrogen phosphate. Steviol glycosides with a high reb M content was suspended in 250 mL solution to obtain 1g/L concentration at each pH solution. Total steviol glycosides present in the stability samples were measured by HPLC at baseline as well as various time points over the study period, determined by the sum of the measured concentrations of the following specific steviol glycosides: rebaudiosides A, B, D, E, M, Steviol-19-O-B-D-glucoside, rubusoside, steviolbioside, Steviolmonoside, and stevioside. Steviol Glycosides with a high Reb-M content tested at pH level 2 was most stable when stored at 4°C and least stable at 50°C. However, at pH 2 when stored at 22°C, 40°C and 50°C, Reb-M degrades at a comparable level as reported in the reference GRN 744. Overall, no significant degradation is observed over 12 weeks for content tested at pH 2 stored at 4°C. Steviol Glycosides with a high Reb-M content tested at pH level 5 was stable when stored at 4°C, 22°C, 40 °C and 50°C for 12 weeks. No significant degradation is observed over 12 weeks for content tested at pH 5 stored at 4°C, 22°C, 40°C and 50°C. Steviol Glycosides with a high Reb-M content tested at pH level 8 was stable when stored at 4°C 22°C and 40 °C for 12 weeks No significant degradation is observed over 12 weeks for content tested at pH 8 stored at 4°C, 22°C and40°C. However, Reb-M shows slight degradation (0.74 g/Kg to 0.46 g/Kg) stored at 50°C for 12 weeks. **Table 3** summarizes the results of the stability for solutions of steviol glycosides with a high reb M content. Stability results are comparable to those reported in the reference GRN 744.

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**Table 3:** Stability data results (pH solution 2, 5, 8, and powder) Amyris's steviol glycosides reb M produced by fermentation

| | Time Point (wk) | Temperature (°C) | Steviol-19-O-B-D-glucoside (g/kg) | RebA (g/kg) | RebB (g/kg) | RebD (g/kg) | RebE (g/kg) | RebM (g/kg) | Rubus-oside (g/kg) | Steviol bioside (g/kg) | Steviol monoside (g/kg) | Stevioside (g/kg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH 2 | 0 | 4 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.99 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.01 | 0.03 | 0.00 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.99 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.98 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.98 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.98 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 22 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.02 | 0.02 | 0.00 | 0.84 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.68 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.66 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.55 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 40 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.05 | 0.01 | 0.00 | 0.24 | 0.00 | 0.07 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.12 | 0.00 | 0.13 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.20 | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.03 | 0.00 | 0.16 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 50 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.04 | 0.00 | 0.16 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.01 | 0.00 | 0.16 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.13 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.10 | 0.00 | 0.00 |
| | | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | **Time Point (wk)** | **Temperature (°C)** | **Steviol-19-O-B-D-glucoside (g/kg)** | **RebA (g/kg)** | **RebB (g/kg)** | **RebD (g/kg)** | **RebE (g/kg)** | **RebM (g/kg)** | **Rubuso side (g/kg)** | **Steviol bioside (g/kg)** | **Steviol monoside (g/kg)** | **Stevioside (g/kg)** |
| pH 5 | 0 | 4 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.81 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.92 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 22 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.81 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.03 | 0.02 | 0.00 | 0.78 | 0.00 | 0.01 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 40 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.81 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.87 | 0.00 | 0.00 | 0.00 | 0.00 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.90 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.89 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 50 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.81 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 | 0.88 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.01 | 0.02 | 0.00 | 0.88 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.82 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.79 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.02 | 0.02 | 0.00 | 0.72 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.68 | 0.00 | 0.00 | 0.00 | 0.00 |
| | **Time Point (wk)** | **Temperature (°C)** | **Steviol-19-O-B-D-glucoside (g/kg)** | **RebA (g/kg)** | **RebB (g/kg)** | **RebD (g/kg)** | **RebE (g/kg)** | **RebM (g/kg)** | **Rubusoside (g/kg)** | **Steviol bioside (g/kg)** | **Steviol monoside (g/kg)** | **Stevioside (g/kg)** |
| pH 8 | 0 | 4 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.74 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.84 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.86 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.84 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.86 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.86 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 22 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.74 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.83 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.86 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.84 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.85 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 40 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.74 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.82 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.01 | 0.03 | 0.00 | 0.82 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.76 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.75 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.69 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.00 | 0.04 | 0.00 | 0.65 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 0 | 50 | 0.00 | 0.00 | 0.00 | 0.02 | 0.00 | 0.74 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 2 | | 0.00 | 0.00 | 0.01 | 0.03 | 0.00 | 0.75 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 4 | | 0.00 | 0.00 | 0.01 | 0.04 | 0.00 | 0.68 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 6 | | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.59 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 8 | | 0.00 | 0.00 | 0.00 | 0.05 | 0.00 | 0.58 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 10 | | 0.00 | 0.00 | 0.02 | 0.06 | 0.00 | 0.51 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 12 | | 0.00 | 0.00 | 0.02 | 0.06 | 0.00 | 0.46 | 0.00 | 0.00 | 0.00 | 0.00 |

**Storage Stability**

The storage stability of steviol glycosides reb M produced by fermentation (Lot 18RGT0511RM002) was assessed. Powder samples were stored in aluminum food grade bags for up to 8 weeks at 1) 25°C, 60% relative humidity and 2) 40°C, 75% relative humidity. To assess storage stability, samples were tested by HPLC at baseline and at various time points, thereafter, based upon measured values of individual steviol glycosides as well as total steviol glycosides. Reb M to total steviol glycosides content stored at 25°C, 60% relative humidity and 40°C, 75% relative humidity storage conditions were stable, and no significant degradation was observed at 12 weeks. As reported in **Table 4**, steviol glycosides with reb M powder stored under both conditions for 12 weeks was stable in total steviol glycosides (TSG). Stability results are comparable to those reported in the reference GRN 744.

**Table 4:** Storage stability of Amyris's steviol glycosides reb M produced by fermentation (Lot 18RGT0511RM002), (g/kg)

| Time Point (wk) | Temperature (C°/%RH) | Steviol-19-O-B-D-glucoside (g/kg) | RebA (g/kg) | RebB (g/kg) | RebD (g/kg) | RebE (g/kg) | RebM (g/kg) | Rubus-oside (g/kg) | Steviol bioside (g/kg) | Steviol monoside (g/kg) | Stevioside (g/kg) | Reb-M/TSG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 25C/60% RH | 0.00 | 0.67 | 4.16 | 19.74 | 0.31 | 952.67 | 0.00 | 0.32 | 0.00 | 0.30 | 97.4% |
| 4 | | 0.00 | 0.68 | 5.49 | 19.16 | 0.25 | 861.21 | 0.00 | 0.24 | 0.00 | 0.00 | 97.1% |
| 8 | | 0.00 | 0.33 | 4.46 | 14.23 | 0.00 | 691.26 | 0.00 | 0.00 | 0.00 | 0.16 | 97.3% |
| 12 | | 0.00 | 0.00 | 5.38 | 15.88 | 0.00 | 803.00 | 0.00 | 0.00 | 0.00 | 0 | 97.4 % |
| 24 | | 0.00 | 0.46 | 5.26 | 19.33 | 0.31 | 798.39 | 0.00 | 0.00 | 0.00 | 0.24 | 96.9 % |
| 36 | | 0.00 | 0.00 | 5.22 | 19.58 | 0.00 | 848.78 | 0.00 | 0.00 | 0.00 | 0.00 | 97.2 % |
| | | | | | | | | | | | | |
| 0 | 40C/75% RH | 0.00 | 0.67 | 4.16 | 19.74 | 0.31 | 952.67 | 0.00 | 0.32 | 0.00 | 0.30 | 97.4% |
| 4 | | 0.00 | 0.79 | 6.62 | 21.06 | 0.28 | 962.67 | 0.00 | 0.39 | 0.00 | 0.37 | 97.0% |
| 8 | | 0.00 | 0.42 | 5.33 | 15.52 | 0.00 | 724.34 | 0.00 | 0.00 | 0.00 | 0.16 | 97.1% |
| 12 | | 0.00 | 0.00 | 5.61 | 16.38 | 0.00 | 811.96 | 0.00 | 0.00 | 0.00 | 0.00 | 97.4 % |
| 24 | | 0.00 | 0.47 | 5.75 | 19.20 | 0.37 | 794.51 | 0.00 | 0.00 | 0.00 | 0.23 | 96.8 % |
| 36 | | 0.00 | 0.00 | 5.49 | 19.09 | 0.00 | 831.15 | 0.00 | 0.00 | 0.00 | 0.00 | 97.1 % |

amyris

### 6.1.2 Information to Enable Identification of Rebaudioside M

*(As per section 3.3.1 A.2 of the Application Handbook as at 1 July 2019)*

Information to enable the identification of Amyris' Rebaudioside M, including the chemical name, structural formula, common name and synonyms and Chemical Abstract Service (CAS) registry number, is described below.

Amyris's Rebaudioside M produced by fermentation is manufactured using a strain of *S. cerevisiae* that has been modified through genetic engineering to express the steviol glycoside biosynthetic pathway and is composed of >95% Rebaudioside M and minor traces of other steviol glycosides (Table 5), which are identical to those that occur naturally in the stevia plant (*S. rebaudiana*). This is consistent with the steviol glycoside purity definition for steviol glycosides from *S. rebaudiana* established by JECFA (JECFA, 2017a).

**Table 5.** Steviol glycosides present in Amyris's steviol glycosides Reb-M produced by fermentation

| Common name | Mol. Wt. | CAS | Chemical Formula |
|---|---|---|---|
| Steviolmonoside | 481 | 60129-60-4 | C26H40O8 |
| Steviol-19-O-B-D-glucoside (i-Steviolmonoside) | 481 | N/A | C26H40O8 |
| Rubusoside | 643 | 64849- 39-4 | C32H50O13 |
| Steviolbioside | 643 | 41093- 60-1 | C32H50O13 |
| Stevioside | 805 | 57817- 89-7 | C38H60O18 |
| Rebaudioside B | 805 | 58543- 17-2 | C38H60O18 |
| Rebaudioside E | 967 | 63279- 14-1 | C44H70O23 |
| Rebaudioside A | 967 | 58543- 16-1 | C44H70O23 |
| Rebaudioside D | 1129 | 63279- 13-0 | C50H80O28 |
| Rebaudioside M | 1291 | 1220616- 44-3 | C56H90O33 |

### 6.1.3 Information on the Chemical and Physical Properties of RebM

*(As per section 3.3.1 A.3 of the Application Handbook as at 1 July 2019)*

JEFCA has published a monograph of identity published in FAO JECFA Monographs 20 (2017). This is provided as **Appendix 1.**

Amyris' Rebaudioside M is a white to off-white powder that has a clean taste with no abnormal or off odor and is freely soluble in water. All steviol glycosides are glycosylated derivatives of the aglycone steviol and therefore, all share the same backbone structure (Figure 1) and differ only with respect to the type and number of glycoside units at positions R1 and R2. Table 6 below provides a list of the other steviol glycosides that may be present in Amyris's steviol glycosides Reb-M produced by fermentation.

**Figure 1.** Backbone structure for steviol glycosides

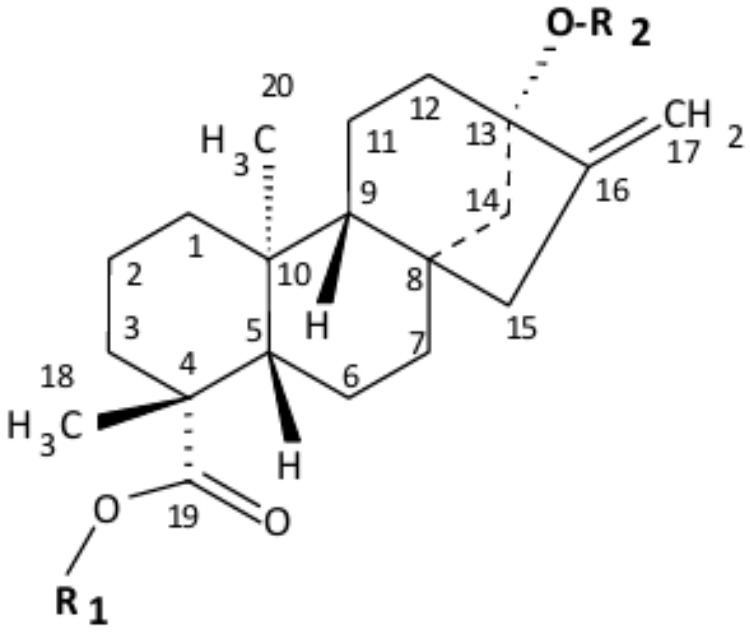


**Table 6:** Steviol glycosides present in Amyris's steviol glycosides Reb-M produced by fermentation

| Common name | Trivial formula | Mol. Wt. | $R_1$ | $R_2$ |
|---|---|---|---|---|
| Steviolmonoside | SvG1 | 481 | H | Glcβ1- [1] |
| Steviol-19-O-B-D-glucoside | SvG1 | 481 | Glcβ1- | H |
| Rubusoside | SvG2 | 643 | Glcβ1- | Glcβ1- |
| Steviolbioside | SvG2 | 643 | H | Glcβ(1-2)Glcβ1- |
| Stevioside | SvG3 | 805 | Glcβ1- | Glcβ(1-2)Glcβ1- |
| Rebaudioside B | SvG3 | 805 | H | Glcβ(1-2)[Glcβ(1-3)]Glcβ1- |
| Rebaudioside E | SvG4 | 967 | Glcβ(1-2)Glcβ1- | Glcβ(1-2)Glcβ1- |
| Rebaudioside A | SvG4 | 967 | Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1 |
| Rebaudioside D | SvG5 | 1129 | Glcβ(1-2)Glcβ1- | Glcβ(1-2)[Glcβ(1-3)]Glcβ1 |
| Rebaudioside M | SvG6 | 1291 | Glcβ(1-2)[Glcβ(1-3)]Glcβ1 | Glcβ(1-2)[Glcβ(1-3)]Glcβ1 |

[1] Glc - glucose

### 6.1.4 Information on the Impurity Profile

*(As per section 3.3.1 A.4 of the Application Handbook as at 1 July 2019)*

Amyris' Rebaudioside M contains no less than 95% and the specification limits and parameters are consistent as per JECFA guidelines (JECFA, 2017a). To ensure safety of the final product, heavy metals and microbiological parameters are tested. As Rebaudioside M is produced via yeast, absence of residual protein (via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)) and DNA (via polymerase chain reaction (PCR)) have been tested. Details are provided in section 6.1.6.

### 6.1.5 Manufacturing Process

*(As per section 3.3.1 A.5 of the Application Handbook as at 1 July 2019)*

**Overview**

Amyris Inc. intends to market steviol glycosides Reb-M produced by fermentation as a general-purpose sweetening agent, in accordance with permissions in the Code. Amyris Inc. steviol glycosides Reb-M will currently not be manufactured in in Australia or New Zealand and therefore the raw materials, production organism and fermentation nutrients will not enter Australia or New Zealand.

**Raw Materials, Processing Aids, and Equipment Specifications**

All raw materials, processing aids, and purification equipment used to manufacture Amyris's steviol glycosides Reb-M produced by fermentation are food-grade and have an appropriate regulatory status in the United States. **Table 7** below lists the raw materials, processing aids, equipment, and their respective technological function and regulatory status. The production process also utilizes food grade antifoaming agents that have an appropriate regulatory status for this use.

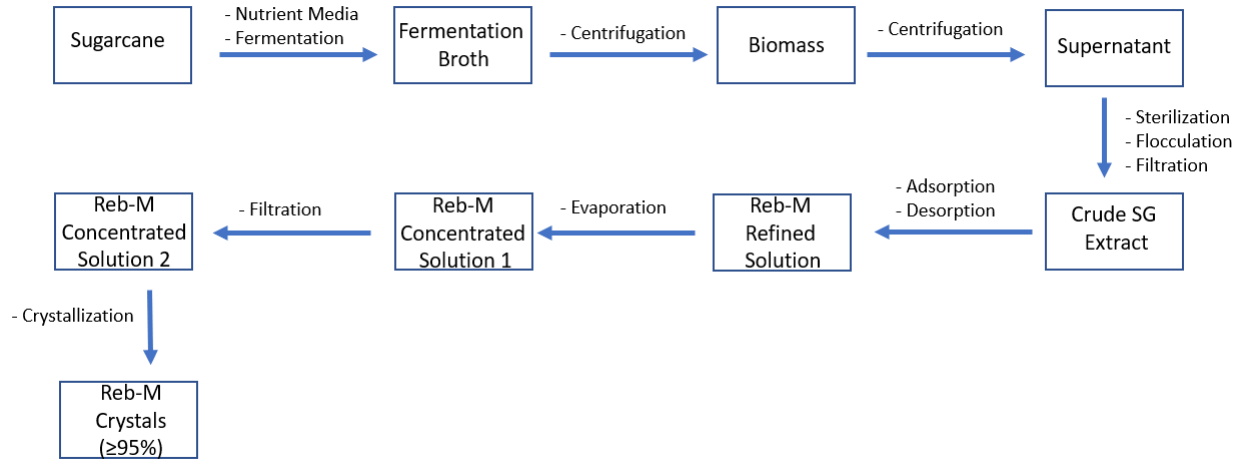
**Table 7:** Raw materials, processing aids, and equipment used in the manufacture of Amyris's steviol glycosides Reb-M produced by fermentation

| Raw Material/Processing Aid | Technological Function | Regulatory Status |
|---|---|---|
| *Indirect Additives - Fermentation Medium Ingredients* | | |
| Magnesium sulfate heptahydrate | Fermentation nutrient | No limitation other than cGMP as flavor enhancer, nutrient supplement, and processing aid, 21 CFR § 582.5443, 21 CFR § 184.1443 |
| Ammonium sulfate | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR § 582.1143, 21 CFR § 184.1143 |
| Baker's Yeast extract | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR § 184.1983 |
| Monopotassium phosphate ($KH_2PO_4$) | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §160.110 |
| Succinic acid | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §582.1091, 21 CFR §184.1091 |
| L-(+)-Lysine monohydrochloride | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §582.5411, 21 CFR §172.320 |
| Sodium hydroxide (NaOH) | Fermentation nutrient | pH control agent and processing aid with no limitation other than cGMP, 21 CFR §582.1763, 21 CFR §184.1763 |
| Ammonium Hydroxide ($NH_4OH$) | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR § 582.1139, 21 CFR § 184.1139 |
| Potassium hydroxide (KOH) | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §582.1631, 21 CFR §184.1631 |
| Ethylenediaminetetraacetic acid (EDTA) | Fermentation nutrient | Permitted in a number of foods as a food additive at specified levels, 21 CFR §172.135 |
| Zinc sulfate heptahydrate ($ZnSO_4 \bullet 7H_2O$) | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5997, 21 CFR §182.8997 |
| Copper sulfate ($CuSO_4$) anhydrous | Fermentation nutrient | Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1261 |
| Manganese (II) chloride tetrahydrate ($MnCl_2 \bullet 4H_2O$) | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5446, 21 CFR §184.1446 |
| Cobalt (II) chloride hexahydrate ($CoCl_2 \bullet 6H_2O$) | Fermentation nutrient | As an animal feed trace mineral (21 CFR §582.80) and agricultural chemical additive |
| Sodium molybdate dihydrate ($NaMoO_4 \bullet 2H_2O$) | Fermentation nutrient | As an agricultural chemical additive, chemical additive, processing aid; considered a plant nutrient under 40 CFR §180.920 and exempt from a tolerance in food |
| Iron (II) sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$) | Fermentation nutrient | Used as a nutrient supplement and processing aid with no limitation other than cGMP, 21 CFR §184.1315 |

| | | |
|---|---|---|
| Calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$) | Fermentation nutrient | Used as an anticaking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, processing aid, stabilizer and thickener, surface-active agent, synergist, texturizer in accordance with cGMP, 21 CFR §582.1193, 21 CFR §582.6193, 21 CFR §184.1193 |
| Biotin | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §582.5159, 21 CFR §182.8159 |
| para-amino-benzoic acid | Fermentation nutrient | EAFUS listed |
| Calcium pantothenate | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5212, 21 CFR §184.1212 |
| Nicotinic acid | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §184.1530 |
| Myo-inositol | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5370, 21 CFR §184.1370 |
| Thiamine.HCl | Fermentation nutrient | Used as a flavoring agent and nutrient supplement with no limitation other than cGMP, 21 CFR §582.5875, 21 CFR §184.1875 |
| Pyridoxine.HCl | Fermentation nutrient | Used as a nutrient supplement with no limitation other than cGMP, 21 CFR §582.5676, 21 CFR §184.1676 |
| Ammonium phosphate monobasic ($NH_4H_2PO_4$) | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR §184.1141a, 21 CFR §582.1141 |
| Sulfuric Acid ($H_2SO_4$) | Fermentation nutrient | GRAS when used in accordance with cGMP, 21 CFR § 184.1095 |
| Cane syrup / Brazilian maltose syrup | Raw material | GRAS |
| Ethanol, food-grade | Crystallization and desorption solvent | GRAS when used in accordance with cGMP, 21 CFR §184.1293 |
| Adsorption resin | Purification | Used in accordance with 21 CFR §177.2710 |

The flow chart for the manufacturing process is shown below in **Figure 2.**

**Figure 2.** Manufacturing process of Amyris's steviol glycosides Reb-M produced by fermentation




Amyris's steviol glycosides Reb-M produced by fermentation is manufactured using a strain of *S. cerevisiae* that has been modified through genetic engineering to express the steviol glycoside biosynthetic pathway. In the first stage of the manufacturing process food-grade sugarcane is mixed with the *S. cerevisiae* production strain and fermented to produce the Reb-M and other steviol glycosides. The fermentation broth goes through centrifugation to separate the biomass from the aqueous phase, followed again by centrifugation. The supernatant product is then sterilized, which then goes through flocculation and filtration to obtain the crude steviol glycosides extract. That extract enters an adsorption and desorption process to become the Reb-M refined solution, which is evaporated into a Reb-M concentrated solution. That solution is filtered and crystallized, which results in a final product that contains ≥ 95% Reb-M powder.

The purification processes used after fermentation are consistent with the methodologies for the manufacture of steviol glycosides as described in the Chemical and technical assessments of food additives (CTA) published by FAO/JECFA (FAO, 2016). Steviol glycosides Reb-M produced by fermentation is manufactured in a facility certified under Food Safety System Certification (FSSC) 22000:2010.

## 6.1.6 Specification for Identity and Purity of Rebaudioside M

*(As per section 3.3.1 A.6 of the Application Handbook as at 1 July 2019)*

JEFCA has published a monograph of identity published in FAO JECFA Monographs 20 (2017). This is provided as **Appendix 1.**

**Existing Specifications for Steviol Glycosides**

Four specifications are outlined in Schedule 3 of the Australia New Zealand Food Standards Code:

1. Rebaudioside M (S3-31),
2. Steviol glycoside mixtures containing rebaudioside M (S3-32)
3. Steviol glycosides from Stevia rebaudiana Bertoni (S3-35), and
4. Steviol glycosides from Reb MD from *Saccharomyces cerevisiae* strain CD15407 containing novel genes for the production of rebaudiosides (S3-39)

All specifications indicate that the total steviol glycoside content must be greater than or equal to 95% on a dry basis. This is consistent with what is outlined at JECFA (2017a) and the European Commission Regulation No 231/2012 (EU, 2012).

**Proposed Specifications for Rebaudioside M**
The product specifications for steviol glycosides Reb-M produced by fermentation are presented in Table 8 based on JECFA (2017a).

**Table 8:** Proposed product specifications for steviol glycosides Reb-M produced by fermentation

| Component | Limits | Unit of Measure |
|---|---|---|
| **Physical Analysis** | | |
| Appearance (powder) | White to off-white | N/A |
| Total steviol glycosides (anhydrous) | ≥ 95 | (wt/wt) % |
| Rebaudioside M Content (anhydrous) | ≥ 95 | (wt/wt) % |
| Ash | ≤ 1.0 | (wt/wt) % |
| Moisture (loss on drying) | ≤ 5.0 | (wt/wt) % |
| pH (measured at 1% dilution) | 4.5 – 7.0 | |
| Residual Ethanol | < 0.30 | % |
| Residual Methanol | < 0.02 | % |
| **Heavy Metals** | | |
| Lead (Pb) | < 1.0 | ppm |
| Arsenic (As) | < 1.0 | ppm |
| Cadmium (Cd) | < 1.0 | ppm |
| Mercury (Hg) | < 1.0 | ppm |
| Cobalt | < 0.03 | ppm |
| **Microbiological Analysis** | | |
| Total Plate Count (TPC) | < 1000 | CFU/g |
| Yeast | < 10 | CFU/g |
| Mold | < 10 | CFU/g |
| Total Coliforms | < 3 | MPN/g |
| E. coli | < 10 | CFU/g |
| Staphylococcus aureus | Non-detect | CFU/g |
| Salmonella | Negative / 25g | |
| Listeria | Negative / 25g | |
| Protein | Non-detect | ng / ml |
| DNA | Non-detect | pg / ul |

**Product Analysis**

***Physical and Chemical Analysis of Reb-M***

Data from the analysis of three non-consecutive lots of steviol glycosides Reb-M produced by fermentation, which demonstrate the consistency of manufacturing process and compliance with the physical and chemical specifications, are presented in **Table 9**. Note, the lot number is represented by the numbers at the front end of the code provided – i.e. 18RGT0506.

**Table 9:** Physical and chemical product analysis for 3 non-consecutive lots of Amyris's steviol glycosides Reb-M produced by fermentation

| Specification Parameter | Limit | Manufacturing Lot | | |
|---|---|---|---|---|
| | | **18RGT0506 RM001** | **18RGT0511 RM002** | **18RGT0606 RM003** |
| Appearance (powder) | White to off-white powder | White powder | White powder | White powder |
| Rebaudioside M content (anhydrous) by HPLC-UV | ≥ 95 wt% | 100 | 101 | 100 |
| Ash | ≤ 1.0 wt% | 0.01% | 0.02% | 0.02 % |
| Moisture (loss on drying) | ≤ 5.0 wt% | 1.02% | 1.31% | 0.1 % |
| pH (measured at 1% dilution) | 4.5 – 7.0 | 5.5 | 5.7 | 5.4 |
| Arsenic (As) | < 1.0 ppm | 0.001 ppm | 0.003 ppm | 0.003 ppm |
| Cadmium (Cd) | < 1.0 ppm | 0.003 ppm | 0.003 ppm | < 0.002 ppm |
| Lead (Pb) | < 1.0 ppm | 0.042 ppm | 0.025 ppm | 0.017 ppm |
| Mercury (Hg) | < 1.0 ppm | 0.001 ppm | 0.004 ppm | < 0.002 ppm |
| Cobalt | < 0.03 ppm | <0.03 ppm | < 0.03 ppm | < 0.03 ppm |
| Residual Ethanol | < 0.30% | 0.04% | < 0.02% | 0.16% |
| Residual Methanol | < 0.02% | < 0.01% | < 0.01% | < 0.01 % |

***Microbiological Analysis***

Data from the analysis of three non-consecutive lots of steviol glycosides Reb-M produced by fermentation, which demonstrate the consistency of manufacturing process and compliance with the microbiological specifications, are presented in **Table 10.**

**Table 10:** Microbiological product analysis for 3 non-consecutive lots of Amyris's steviol glycosides Reb-M produced by fermentation

| Specification Parameter | Limit | Manufacturing Lot | | |
|---|---|---|---|---|
| | | **18RGT0506RM001** | **18RGT0511RM002** | **18RGT0606RM003** |
| Total Plate Count (TPC)/Aerobic Plate Count (APC) | < 1000 CFU/g | 10 CFU/g | < 10 CFU/g | < 10 CFU/g |
| Yeast | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g |
| Mold | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g |
| Coliforms | < 3 MPN/g | < 3 MPN/g | < 3 MPN/g | < 3 MPN/g |
| Escherichia coli | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g |
| Staphylococcus aureus | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g | < 10 CFU/g |
| Salmonella | Negative / 25g | Not detected / 25g | Not detected / 25g | Not detected/25g |
| Listeria | Negative 25/g | Not detected / 25g | Not detected / 25g | Not detected/25g |
| Protein | Non-detect (ng/ml) | Not detected | Not detected | Not detected |
| DNA | Non-detect (pg/µl) | Not detected | Not detected | Not detected |

**Other Chemical Analysis**

***Steviol Glycoside Composition***

Data for three production lots of steviol glycosides Reb-M produced by fermentation in **Table 11** shows the difference in the distribution of steviol glycosides present in the mother liquor following the fermentation and in the final purified product following crystallization (measured by high performance liquid chromatography (HPLC)). Steviol glycosides Reb-M produced by fermentation produces a final product ≥95% Reb-M and other steviol glycosides such as those listed in **Table 11**. The manufacturing process purification steps are effective and produce a product with a consistent steviol glycoside distribution.

**Table 11:** Similarity of the stability of Amyris's steviol glycosides Reb-M produced by fermentation as compared to individual steviol glycosides as measured by HPLC

| | Lot 18RGT0506RM001 | | Lot 18RGT0506RM002 | | Lot 18RGT0606RM003 | |
|---|---|---|---|---|---|---|
| Steviol Glycoside (wt%) | Mother liquor | Crystal | Mother liquor | Crystal | Mother liquor | Crystal |
| Rebaudioside D | 0.082 | 1.90 | 0.086 | 2.09 | 0.596 | 1.66 |
| Rebaudioside M | 0.139 | 100.43 | 0.141 | 100.99 | 1.281 | 100.04 |
| Rebaudioside A | 0.005 | ND | 0.006 | ND | 0.045 | ND |
| Rebaudioside E | 0.025 | ND | 0.026 | ND | 0.230 | ND |
| Stevioside | 0.012 | ND | 0.014 | ND | 0.105 | ND |
| Rubusoside | 0.008 | ND | 0.009 | ND | 0.092 | ND |
| Rebaudioside B | 0.001 | 0.40 | 0.002 | 0.372 | 0.007 | 0.38 |
| Steviolbioside | 0.002 | ND | 0.002 | ND | 0.009 | ND |
| i-Steviolmonoside | 0.011 | ND | 0.012 | ND | 0.136 | ND |
| Steviolmonoside | 0.019 | ND | 0.018 | ND | 0.102 | ND |

***Protein Analysis***

To confirm the success of the purification and the absence of protein in steviol glycosides Reb-M produced by fermentation, the final product is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of steviol glycosides Reb-M produced by fermentation are dissolved to a concentration of 1,000 ppm, and about 10 µL from each dissolved sample is stained with 3X protein loading dye and loaded onto a precast polyacrylamide gel. Electrophoresis is conducted at 50 minutes at 200 V and the gel is stained with Coomassie Blue for 1 hour. Gels are de-stained by soaking in milli-q water. If protein is present in the sample, it will be visually detected on the gel (limit of detection = 0.1 µg protein). No visible protein bands have been detected in any batches of final product.

***Residual DNA Analysis***

To confirm the absence of residual DNA in steviol glycosides Reb-M produced by fermentation, a polymerase chain reaction (PCR) method was developed and primers were designed to amplify the gene of interest. Genomic DNA is extracted using a DNA extraction kit according to manufacturer's protocol. The thermal profile used is 2 minutes at 98°C followed by 35 cycles of 25 seconds at 98°C, 30 seconds at 55°C, and 60 seconds at 72°C (or longer than 60 seconds at 72°C if >1Kb) followed by 1 cycle for 2 minutes at 72°C. The genomic DNA is quantified by loading the sample with an agarose gel loading dye diluted to 1x and ran onto a 1% agarose gel for 20 to 30 minutes. The gel is visualized under UV light to image the DNA bands. Results of the PCR analysis have not detected any PCR products in any of the batches of final product (limit of detection for a single heterologous gene = 0.1 pg/µL DNA).

### 6.1.7 Information for Food Labelling

*(As per section 3.3.1 A.7 of the Application Handbook as at 1 July 2019)*

Reb-M is a steviol glycoside with the additive number 960.

Amyris Reb-M is a mixture of steviol glycosides and therefore will follow the same food labelling as previously established for steviol glycosides. All steviol glycosides, including Reb-M, are generally considered high-intensity sweeteners with modifying properties when added to a variety food product, and have been assigned the INS number 960. Therefore, Reb-M will be labelled under the functional class, sweetener, as “sweetener (960)” or “sweetener (steviol glycosides)”.

### 6.1.8 Analytical Method for Detection

*(As per section 3.3.1 A.8 of the Application Handbook as at 1 July 2019)*

Amyris’ Rebaudioside M is analyzed by reverse-phase high-performance liquid chromatography with DAD detection at 210nm (RP-HPLC-DAD). External standard calibration by steviol glycosides, Rebaudioside A, D, M and stevioside and relative response factors are used for the calculation of other Rebaudiosides by RP-HPLC-DAD detection at 210nm (RP-HPLC-DAD).

### 6.1.9 Potential Additional Purposes of the Food Additive when Added to Food

*(As per section 3.3.1 A.9 of the Application Handbook as at 1 July 2019)*

Amyris’ steviol glycosides Reb-M produced by fermentation was determined to be approximately 200-300 times sweeter than sucrose. Therefore, consumers looking to have a reduced calorie diet could look for Amyris’ steviol glycosides Reb-M products, as they will have reduced calories compared to sugar. Amyris’ steviol glycosides Reb-M could be used for people that require reduced sugar due to medical conditions. This is the same situation for other intense sweeteners already permitted in the Code.

## 6.2 Information Related to the Safety of the Food Additive

*(As per section 3.3.1 B of the Application Handbook as at 1 July 2019)*

Section 3.3.1 – Food Additives of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2019) 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.

**Introduction**

Over the last few decades, several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, the European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada, have reviewed the safety of steviol glycosides. Interest in the use of steviol glycosides as sweeteners initiated extensive testing of the compounds and, in turn, generated a large safety database. This database includes a thorough examination of the comparative metabolism and pharmacokinetics of steviol glycosides in experimental animals and humans, acute toxicity studies, short- 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 due to the predominance of stevioside in *S. rebaudiana* leaves (Toyoda et al., 1997), 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 on reb A (Curry and Roberts, 2008; Curry et al., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). Although the majority of toxicity studies have been conducted with either purified stevioside or reb A, the extensive information available on the common metabolic fate of steviol glycosides has permitted scientific bodies and regulatory agencies to extend their safety opinions to all steviol glycosides from the S. rebaudiana leaf, rather than just individual glycosides (JECFA, 2016a).

Given the metabolic fate of steviol glycosides, the safety of steviol glycosides Reb-M produced by fermentation can be established based on the conclusions of the steviol glycoside safety reviews, and on the publicly available scientific literature related to the safety of steviol glycosides. Furthermore, although the production strain is not present in the final product, information related to the safety of the *S. cerevisiae* parental and production strains was compiled, including assessment of the potential allergenicity of the heterologous gene sequences inserted in the production strain.

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

*(As per section 3.3.1 B.1 of the Application Handbook as at 1 July 2019)*

This application is not requesting an extension of use of steviol glycosides, which have already been assessed for safety by FSANZ and other agencies. FSANZ has very recently (April 2019) completed an assessment of a very similar application (A1170) for another steviol glycoside produced by fermentation of a similar strain of S. cerevisiae. JECFA has also recently considered specifications for steviol glycosides produced from four production methods, including fermentation (JECFA 87).

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

*(As per section 3.3.1 B.2 of the Application Handbook as at 1 July 2019)*

This application is not requesting an extension of use of steviol glycosides, which have already been assessed for safety by FSANZ and other agencies. FSANZ has very recently (April 2019) completed an assessment of a very similar application (A1170) for another steviol glycoside produced by fermentation of a similar strain of S. cerevisiae. JECFA has also recently considered specifications for steviol glycosides produced from four production methods, including fermentation (JECFA 87).

## 6.2.3 Safety assessment reports prepared by international agencies or other national government agencies, if available

*(As per section 3.3.1 B.3 of the Application Handbook as at 1 July 2019)*

JECFA established the current ADI at its 69th meeting (2008) and affirmed this at its 82nd meeting (2019). Both reports are referenced above in this application. JECFA also confirmed the ADI will apply to steviol glycosides derived from all four production methods considered at its 87th meeting (JECFA, 2019).

### 6.2.3.1 Metabolic Fate of Steviol Glycosides

***Microbial Degradation, Absorption, Distribution, Metabolism, and Elimination***

In vitro and ex vivo studies have demonstrated that steviol glycosides are not hydrolyzed by digestive enzymes of the upper gastrointestinal tract due to the presence of β-glycosidic bonds and are not absorbed through the upper portion of the gastrointestinal tract (Hutapea et al., 1997; 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 (Gardana et al., 2003; Renwick and Tarka, 2008). Several in vitro studies mimicking the anaerobic conditions of the colon, reviewed extensively by Renwick and Tarka (2008), have confirmed the ability of gut microflora from mice, rats, hamsters, and humans to hydrolyze steviol glycosides completely to steviol (Wingard et al., 1980; Hutapea et al., 1997; Gardana et al., 2003; Koyama et al., 2003a,b; Nikiforov et al., 2013; Purkayastha et al., 2016).

Steviol glycosides are hydrolyzed sequentially, removing one sugar moiety at a time, with differences in the degradation rates depending on the structural complexities of each steviol glycoside (Wingard et al., 1980; Koyama et al., 2003b). Stevioside, for example, is degraded to steviolbioside, steviolmonoside, and finally to steviol, with glucose released with each sequential hydrolysis, whereas rebaudioside A is first converted to either stevioside (major pathway) or rebaudioside B (minor pathway) prior to being ultimately degraded to steviol (Nakayama et al., 1986; Gardana et al., 2003; Koyama et al., 2003b). Despite these structural differences, several parallel in vitro comparisons between rebaudioside A and individual steviol glycosides have demonstrated a remarkable similarity with respect to the rate of hydrolysis of different steviol glycosides to steviol in the presence of human fecal homogenates, particularly during the first 24 hours of incubation (Purkayastha et al., 2014, 2015, 2016). For example, Reb-M and rebaudioside A (0.2 mg/mL) were incubated with human fecal homogenates samples at 37°C for up to 24 hours under anaerobic conditions, and by 16 hours both compounds were reported to be completely metabolized to steviol (Purkayastha et al., 2016). These experiments demonstrate that steviol glycosides are metabolized by human fecal homogenates to steviol at generally similar hydrolysis

rates, indicating that the number and location of sugar units attached to the steviol backbone does not significantly affect the rate of hydrolysis.

Steviol is absorbed systemically into the portal vein and distributed to a number of organs and tissues, including the liver, spleen, adrenal glands, fat, and blood (Nakayama et al., 1986; Sung, 2002 [unpublished]; Koyama et al., 2003b; Wang et al., 2004; Roberts and Renwick, 2008). In the liver, steviol is conjugated to glucuronic acid to form steviol glucuronide. In rats, free steviol (82 to 86% of chromatographed radioactivity), steviol glucuronide (10 to 12% of chromatographed radioactivity), and 2 unidentified metabolites (5 to 6% of chromatographed radioactivity) were identified in the plasma 8 hours after oral administration with either rebaudioside A or stevioside (Roberts and Renwick, 2008). Similarly, in humans steviol glucuronide was detected in the plasma following ingestion of stevioside or rebaudioside A, with maximal concentrations detected 8 and 12 hours after administration, respectively (Simonetti et al., 2004; Geuns et al., 2007; Wheeler et al., 2008). The toxicokinetic/ pharmacokinetic differences of steviol and steviol glucuronide were recently examined in rats and humans by Roberts et al. (2016) following administration of stevioside (40 mg/kg body weight). Peak plasma concentrations ($C_{max}$) of steviol were similar in both rats and humans but were slightly delayed in humans compared to rats. Similarly, $C_{max}$ values for steviol glucuronide were also delayed in humans but were approximately 25-fold higher in humans than rats. Systemic exposure to steviol and steviol glucuronide based on the area under the curve (AUC0-72h) was reported to be 2.8-fold and 57-fold greater in humans, when compared to rats, respectively. These data show that the extent of conjugation of steviol to glucuronic acid is higher in humans than in rats.

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

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

been established for individual steviol glycosides (e.g., stevioside, rebaudioside A, rebaudioside D) can be extrapolated to support the safe use of purified steviol glycosides in general, regardless of the steviol glycoside distribution of the preparation, including steviol glycosides Reb-M produced by fermentation.

***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 of *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes or extracted from the *S. rebaudiana* 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*, therefore, can be extended to include all steviol glycosides including those derived from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes.

**6.2.3.2 Recent Toxicological and Human Studies with Steviol Glycosides**

No safety concerns were identified in the literature research of steviol glycosides (2019).

***Repeated-Dose Studies in Animals***

Rebaudioside A (> 95% purity) produced by fermentation (by genetically modified yeast, Y. lipolytica) was administered to Sprague-Dawley rats as a dietary mixture at concentrations of 0, 500, 1,000, or 2,000 mg/kg body weight/day (N=20 per sex per group) for a total of 90 days (Rumelhard et al., 2016). No test article-related systemic or local toxicity was reported based on daily clinical observations and weekly physical examinations, and no deaths occurred in any group throughout the study. Males in the highest dose group experienced significantly lower changes in body weight, body weight gain, and cumulative body weight gain, resulting in mean body weights that were 5.9% lower than the control group at the end of the study. Females in the highest dose group also experienced some statistically significant decreases in body weight during the study, but at the end of the study, body weights between the synthesized rebaudioside A and control groups were equivalent. Consumption of rebaudioside A was not reported to influence food consumption. The study authors associated the changes in body weight with the decreased caloric value of the diet containing rebaudioside A and therefore did not consider these changes to be adverse. Neurological evaluations conducted during the final week of the study reported no differences between the control and test-article treated groups, and no ophthalmological findings were considered test-article related. Following 90 days of exposure, rebaudioside A was not reported to induce any changes in the hematology profile, serum chemistry, or urinalysis parameters, and had no effect upon gross pathological findings, organ weights, or histopathology. Based on these results, the authors concluded that the NOAEL for rebaudioside A (described as 'fermentative') was the highest dose tested (2,000 mg/kg body weight/day) and that the safety profile of rebaudioside A is similar to plant derived rebaudioside A (Rumelhard et al., 2016).

In another 90-day repeat-dose oral toxicity study, groups of male and female Sprague-Dawley rats (10/sex/group) were provided diets containing an ethanolic extract of *S. rebaudiana* Bertoni leaves at doses of 0, 1.04, 2.08, and 3.12% of the diet which correspond to targeted doses of 0, 830, 1670, and 2500 mg/kg bw/day. (Zhang et al., 2017). There were no mortalities and no treatment-related adverse clinical effects throughout the study. Clinical chemistry and hematological findings revealed no

consistent dose-dependent trends. Organ weights, macroscopic evaluations, and microscopic evaluations reported no treatment-related effects. It is noted that this study did not evaluate the complete set of organs recommended by the OECD (OECD, 1998b). The study also evaluated a test article that does not meet the purity specifications established by JECFA, which contained approximately 47.78% polyphenols (mostly isochlorogenic acids) with the remainder consisting of soluble fibers and glucose. Regardless of these limitations, the results of this study support the safety of stevia leaf-derived products.

***Genotoxicity***

The results of a bacterial reverse mutation assay, conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Test Guideline 471, was recently published in which the genotoxic potential of rebaudioside A (> 95% purity) produced by fermentation (by genetically modified yeast, Yarrowia lipolytica) was evaluated (Rumelhard et al., 2016). In the study, rebaudioside A was tested in Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, and Escherichia coli strain WP2 uvrA at concentrations of up to 5,000 µg/plate in the presence or absence of exogenous metabolic activation. The results indicate that the rebaudioside A produced by fermentation is not genotoxic. The same preparation was tested in an in vitro micronucleus assay in cultured peripheral human lymphocytes conducted in accordance with OECD Test Guideline 487 (Rumelhard et al., 2016). Consistent with the results of the preceding study, rebaudioside A was determined to lack genotoxic potential following incubation with lymphocytes in the presence and absence of exogenous metabolic activation at concentrations of up to 5,000 µg/mL. In studies using a crude ethanolic extract obtained from S. rebaudiana leaves, negative results were reported in a reverse mutation assay in S. typhimurium, an in vivo mouse micronucleus test, and an in vivo mouse sperm malformation assay; these findings support the safety of products derived from S. rebaudiana Bertoni leaves (Zhang et al., 2017). These findings corroborate the previous conclusions by JECFA (2010) that steviol glycosides and steviol are not genotoxic.

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

#### *6.2.3.3 Long-term Toxicity and Carcinogenicity*

As described in section C.3, the Long-term Toxicity and Carcinogenicity is summarized by scientific bodies and regulatory agencies. No new data was evaluated in relation to this endpoint.

#### *6.2.3.4 Reproductive and Developmental Toxicity*

As described in section C.3, the Reproductive and Developmental Toxicity is summarized by scientific bodies and regulatory agencies. No new data was evaluated in relation to this endpoint.

#### *6.2.3.5 Human Studies*

JECFA and EFSA (JECFA, 2009; EFSA, 2010) have previously demonstrated the safety of steviol glycosides in humans. No new data was evaluated in relation to this endpoint.

#### *6.2.3.6 Proposed Revision to the ADI for Steviol Glycosides*

The ADI for steviol glycosides of 4 mg/kg body weight/day (expressed as steviol) was calculated based on a NOAEL of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from the 2-year carcinogenicity study in rats conducted be Toyoda et al. (1997) and application of a safety factor of 100 (FSANZ, 2008; JECFA, 2009; EFSA, 2010; Health Canada, 2012a). As defined by the World Health Organization, the standard safety factor value of 100 to account for inter- and intra-species differences (a 10-fold factor for each) may be adjusted using chemical-specific adjustment factors (CSAFs). For example, using appropriate toxicokinetic/toxicodynamic data the safety factor of 10 that is applied to account for inter-species differences can be modified based on the chemical-specific data, and can be broken down into its 2 components that account for toxicokinetic (4-fold factor) and toxicodynamic (2.5-fold factor) differences.

Roberts et al. (2016) reported on the toxicokinetic differences of steviol and steviol glucuronide in rats and humans following a single oral dose of 40 mg stevioside/kg body weight. Blood samples were collected pre-dose and through 72 hours post-dose and were assayed for steviol and steviol glucuronide. Peak plasma concentrations (Cmax) of steviol were similar in both rats and humans (see below) but were slightly delayed in humans compared to rats. Cmax values for steviol glucuronide were also delayed in humans but were approximately 25-fold higher in humans than rats (approximately 4,440 ng/mL vs. 180 ng/mL). Systemic exposure to steviol and steviol glucuronide assessed using the area under the curve (AUC0-72h) was 2.8-fold (~1,650 ng·h/mL vs. ~590 ng·h/mL) and 57-fold (~136,000 ng·h/mL vs. ~2,400 ng·h/mL) greater in humans than rats, respectively. The AUC and Cmax data were used to calculate the CSAF as follows:

a) the AUC0-72 for free steviol in humans (1,631 ng·h/mL) was higher than the AUC in male and female rats (581 and 605 ng·h/mL, respectively), and therefore the ratio of AUC between humans and rats is 2.8

b) the Cmax values for free steviol in humans (77.21 ng/mL) were approximately equivalent to those in male and female rats (76.0 and 87.1 ng/mL, respectively), and therefore the ratio of Cmax values is approximately one, and

c) the standard safety factor of 4 for toxicokinetic interspecies differences can therefore be revised to range from 1 to 2.8.

Applying the CSAF of 1 to 2.8 for toxicokinetic differences between rats and humans when calculating the ADI for steviol glycosides revises the standard safety factor of 10 for interspecies differences to range from 2.5 [1(toxicokinetic) x 2.5(toxicodynamic)] to 7 [2.8(toxicokinetic) x 2.5(toxicodynamic)], and decreases the overall safety factor of 100 to range from 25 to 70. (human variability), providing an ADI between 6 and 16 mg/kg body weight, as steviol equivalents (Roberts et al., 2016). Currently, the ADI assigned by JECFA is 0 to 4 mg/kg body weight, as steviol equivalents for stevia leaf extracts.

**Summary of Steviol Glycoside Safety Opinions**

Over the last few decades, several scientific bodies and regulatory agencies, including the U.S. FDA, JECFA, the European Commission's Scientific Committee on Food (SCF), EFSA, FSANZ, and Health Canada, have reviewed the safety of steviol glycosides. Interest in the use of steviol glycosides as sweeteners initiated extensive testing of the compounds and, in turn, generated a large safety database. This database includes a thorough examination of the comparative metabolism and pharmacokinetics of steviol glycosides in experimental animals and humans, acute toxicity studies, short- 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 due to the predominance of stevioside in S. rebaudiana leaves (Toyoda et al., 1997), 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 on reb A (Curry and Roberts, 2008; Curry et al., 2008; Nikiforov and Eapen, 2008; Williams and Burdock, 2009). Although the majority of toxicity studies have been conducted with either purified stevioside or reb A, the extensive information available on the common metabolic fate of steviol glycosides has permitted scientific bodies and regulatory agencies to extend their safety opinions to all steviol glycosides from the S. rebaudiana leaf, rather than just individual glycosides (JECFA, 2016a).

Given the metabolic fate of steviol glycosides, the safety of steviol glycosides Reb-M produced by fermentation can be established based on the conclusions of the steviol glycoside safety reviews, and on the publicly available scientific literature related to the safety of steviol glycosides. Furthermore, although the production strain is not present in the final product, information related to the safety of the S. cerevisiae parental and production strains was compiled, including assessment of the potential allergenicity of the heterologous gene sequences inserted in the production strain.

## 6.3 Information Related to the Dietary Exposure to the Food Additive

*(As per section 3.3.1 C of the Application Handbook as at 1 July 2019)*

The use of Amyris Reb-M will be consistent with the current permissions for the use of steviol glycosides in Schedule 15 of the Code. This application does not seek additional permissions to those included in Schedule 15. Therefore, the dietary exposure will be consistent with previous dietary exposure assessments conducted by FSANZ for steviol glycosides.

### 6.3.1 A list of the food groups or foods proposed to contain the food additive, or changes to currently permitted foods

*(As per section 3.3.1 C.1 of the Application Handbook as at 1 July 2019)*

Amyris Reb-M is intended for use as an intense sweetener in accordance with the current permissions for steviol glycosides in Schedule 15 of the Code. This application does not seek additional permissions to those included in Schedule 15.

### 6.3.2 The maximum proposed level and/or the concentration range of the food additive for each food group or food, or the proposed changes to the currently permitted levels

*(As per section 3.3.1 C.2 of the Application Handbook as at 1 July 2019)*

See response in Section 6.3.1.

### 6.3.3 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

*(As per section 3.3.1 C.3 of the Application Handbook as at 1 July 2019)*

See response in section 6.3.1.

### 6.3.4 The percentage of the food group in which the food additive is proposed to be used or the percentage of the market likely to use the food additive

*(As per section 3.3.1 C.4 of the Application Handbook as at 1 July 2019)*

Amyris Reb-M 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. Amyris' Reb-M is intended to be a direct replacement for other steviol glycosides and therefore the expected intakes of Reb-M would be similar to the intakes for other steviol glycosides that are currently permitted in ANZ.

### 6.3.5 Information relating to the use of the food additive in other countries, if applicable

*(As per section 3.3.1 C.5 of the Application Handbook as at 1 July 2019)*

Amyris Reb-M is intended for use as an intense sweetener in jurisdictions where it is approved for use as set out in Section 5.

### 6.3.6 For foods where consumption has changed in recent years, information on likely current food consumption

*(As per section 3.3.1 C.6 of the Application Handbook as at 1 July 2019)*

There are no food groups for which consumption has changed in recent years.

# 7. Substances added to Food – Processing Aid

In this section information on the *S. cerevisiae* production organism is presented. The use of the production organism in this case is akin to the use of processing aids in the production of foods and food ingredients. To ensure all relevant information on the production organism is included in this application, the relevant requirements of Section 3.3.2 – Processing Aids of the FSANZ Application Handbook have been used as a guide; including subsections C, D and E (as indicated under the headings below).

## 7.1 Technical Information on the Processing Aid

*(As per section 3.3.2 A of the Application Handbook as at 1 July 2019)*

The material described in this section is representative of the commercial product.

### 7.1.1 Information on the type of processing aid

*(As per section 3.3.2 A.1 of the Application Handbook as at 1 July 2019)*

*Saccharomyces cerevisiae* (*S. cerevisiae*) expressing steviol glycoside biosynthetic genes is used as a processing aid to manufacture steviol glycosides, primarily composed of Rebaudioside M. *S. cerevisiae* is removed shortly after fermentation and is not present in the final preparation of Amyris' highly pure Rebaudioside M, as confirmed by residual protein and DNA analysis (Refer to sections 6.1.6).

### 7.1.2 Information on the Identity of the Processing Aid

*(As per section 3.3.2 A.2 of the Application Handbook as at 1 July 2019)*

The processing aid *S. cerevisiae* expressing steviol glycoside biosynthetic genes is the same yeast species (*Saccharomyces cerevisiae)* also known as brewer's yeast or baker's yeast; it has an extensive history of safe use in the food industry.

**Table 12:** Taxonomic Identity of *Saccharomyces cerevisiae*

| | |
|---|---|
| **Kingdom** | Fungi |
| **Phylum** | Ascomycota |
| **Class** | Saccharomycetes |
| **Order** | Saccharomycetales |
| **Family** | Saccharomycetaceae |
| **Genus** | Saccharomyces |
| **Species** | Saccharomyces cerevisiae |

### 7.1.3 Information on the Chemical and Physical Properties of the Processing Aid

*(As per section 3.3.2 A.3 of the Application Handbook as at 1 July 2019)*

The parental strain *S. cerevisiae* CEN.PK113-7D was genetically engineered to increase flux through the endogenous yeast mevalonate pathway to increase carbon flux to the farnesyl pyrophosphate (FPP) precursor as described by Westfall *et al.* (2012) and Meadows *et al.* (2016). The genetically engineered parental strain with high flux to FPP was converted to produce steviol glycosides, similar to the biosynthetic process that occurs naturally in the plant *Stevia rebaudiana*. **Table 13** provides a summary of the representative enzymes necessary to convert FPP to Rebaudioside M and their technological functions. Finally, because this processing aid is not present in the final product, as evidenced by no DNA or protein detected in the final product, there are no possible interactions of the processing aid with food.

**Table 13:** Summary of enzymes and their respective functions in Amyris's production strain

| **Enzyme** | **Function** |
|---|---|
| Geranylgeranyl pyrophosphate (GGPP) synthase | Converts FPP to GGPP |
| Copalyl diphosphate (CDP) synthase | Converts GGPP to CDP |
| Kaurene synthase (KS) | Converts CDP to kaurene |
| Kaurene oxidase (KO) | Converts kaurene to kaurenoic acid |
| Kaurenoic acid hydroxylase (KAH) | Converts kaurenoic acid to steviol |
| Cytochrome P450 reductase (CPR) | Works in conjunction with the P450 enzymes in pathway (KO and KAH) |
| UDP-glucosyl transferases (UGTs) | Adds a glucose to steviol or steviol glycosides |

### 7.1.4 Manufacturing Process

*(As per section 3.3.2 A.4 of the Application Handbook as at 1 July 2019)*

The parental strain, *S. cerevisiae* CEN.PK113-7D, is auxotrophic for histidine, leucine, tryptophan, uracil, and adenine through base-pair deletions or changes of HIS3, LEU2, TRP1, URA3, and ADE1, respectively. A steviol-glycoside producing yeast strain, herein referred to as the *S. cerevisiae* production strain, was generated by starting with the a high-flux FPP CEN.PK113-7D strain, as described by Westfall *et al.* (2012) and Meadows *et al.* (2016), and adding the genes necessary to convert FPP to steviol glycosides, primarily rebaudioside M. All DNA constructs used to create the production strain were integrated in site-specific, stable, and non-essential regions of the yeast genome via homologous recombination. These regions include, but are not limited to, genes such as *PDC6, NDT80, DIT1, GAS2, GAS4,* and *HO* as well as non-coding regions in the genome. The genes used to generate the production strain encode for enzymes that either are required for steviol glycoside synthesis and or improve the overall production efficiency of steviol glycosides. All promoters and terminators used to express the genes are native to S. cerevisiae or *Ashbya gossypii*, a yeast-like fungus.

All heterologous genes introduced into the production strain are derived synthetically from biosafety level 1 organisms and codon optimized for expression in *S. cerevisiae*. Table 14 lists the source organism for all heterologous genes present in production strain. The heterologous genes are not associated with any known allergens or toxins. In addition, the production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens.

The antibiotic resistance markers kanMX (confers resistance to G418), hphA (confers resistance to hygromycin B), and natA (confers resistance to nourseothricin) were used at various points during strain construction. The final production strain has all antibiotic resistance genes removed; the strain has been shown to be completely sensitive to those three antibiotics; the strain cannot grow on media containing any one of the three antibiotics. During the course of strain construction, the production strain is also restored to full prototrophy by insertion of copies of HIS3, LEU2, TRP1, URA3, and ADE1 from wild-type *S. cerevisiae* or *Ashbya gossypii*.

DNA constructs are inserted into the yeast genome via standard methods as described in Rothstein (1991). A single DNA construct contains genomic DNA homologous to the upstream and downstream DNA sequence of the desired locus for precise integration at the target site. The integrated DNA may contain one to four expression cassettes, each of which consists of a yeast promotor, the gene of interest, and a yeast terminator. DNA constructs with more than one expression cassette may contain spacer DNA obtained from amplified genomic DNA of *E. coli* K-12 to prevent interference during transcription. Spacer DNA is used as structural DNA elements inside of the integrated DNA constructs and they do not have sequence homology to yeast chromosomes. Spacer DNA does not express heterologous proteins as they do not encode functional protein sequences and do not include yeast promotors sequences. All DNA constructs are sequence verified before being integrated into a yeast strain. Correct integration is verified by PCR after transformation. Before being transferred for production, the final strain is whole genome sequenced to verify the correct integrations and sequences of all engineering and also to confirm that no unintended genome rearrangements or insertions occurred.

The parental strain is a stable haploid yeast and therefore does not undergo mating-type switching or mating events upon cell division (Jensen *et al.,* 1985). The production strain is rendered haploid negative ($HO^-$) by deletion of the *HO* gene; *HO* is replaced with a DNA construct containing a kaurene synthase gene and a copalyl-diphosphate synthase gene. Replacement of *HO* with a DNA construct ensures that the production strain remains haploid negative and will not undergo mating events/unwanted genetic rearrangement. The growth rate of the production strain is significantly slower than wild type yeast, due to the genetic engineering that has been down to route carbon to steviol glycosides instead of biomass. **Appendix CC-4** - Whole genome sequencing report Nov 2019 contains additional (confidential) information on the nature of the deleted genes in the Reb-M production strain.

As the DNA constructs are inserted by homologous recombination, the introduced genetic elements are stable, and the production strain does not contain any plasmid or other exogenous mobile genetic elements. The cell line stability is demonstrated by using primary and secondary cell banks and comparing productivities. Extended seed trains are routinely tested to ensure retention of phenotype over generations of the production strain. Furthermore, the production strain is consistently tested for contaminating bacteria and strain performance according to internal standard operation procedures.

Additional detail, including confidential nucleotide sequences of constructs are included in **Appendix CC-1** - RebM production strain genetic engineering report.

### 7.1.5 Specification for Identity and Purity

*(As per section 3.3.2 A.5 of the Application Handbook as at 1 July 2019)*

Amyris practices several quality control measures in accordance with current Good Manufacturing Practices (GMP)s and has established identity and purity criteria that must be confirmed before a seed culture stock of *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes are released for commercial production. Included in the release criteria are assessments of viability and purity parameters and morphology and growth characteristics

### 7.1.6 Analytical Method for Detection

*(As per section 3.3.2 A.6 of the Application Handbook as at 1 July 2019)*

Verification of the absence of *S. cerevisiae* expressing steviol glycoside biosynthesis material in final product is determined with production material by DNA analysis through Polymerase Chain Reaction (PCR) and Protein through SDS-PAGE analysis. The DNA limit of detection for a single heterologous gene is 0.1 pg/µL DNA and protein limit of detection by SDS-PAGE is 0.1 µg protein. The details of these methods and the results of these analyses are provided in pages 31 and 32.

## 7.2 Information Related to the Safety of the GM Processing Aid

*(As per section 3.3.2 C of the Application Handbook as at 1 July 2019)*

### 7.2.1 General Information on use of the Food Processing Aid in Other Countries

*(As per section 3.3.2 C.1 of the Application Handbook as at 1 July 2019)*

*Saccharomyces cerevisiae,* also known as brewer's yeast or baker's yeast, has an extensive history of safe-use in the food industry. In the U.S., according to 21 CFR §172.896 dried yeast, including S. cerevisiae, is permitted for use in food so long as the total folic acid content is no greater than 0.04 mg/g of yeast (U.S. FDA, 2017a). Protein isolated from S. cerevisiae (baker's yeast protein) and the dried cell walls of S. cerevisiae (baker's yeast glycan) are food additives permitted for the direct addition to food for human consumption (21 CFR §172.325 and 172.898, respectively) (U.S. FDA, 2017a). Baker's yeast extract, the concentrated or dried soluble component of mechanically ruptured cells of S. cerevisiae, is GRAS for use as a flavoring agent and adjuvant at a level not to exceed 5% in food (21 CFR §184.1983 - U.S. FDA, 2017a). Vitamin D2 baker's yeast, which is generated by exposing S. cerevisiae to UV light, resulting in the conversion of endogenous ergosterol to vitamin D2, is also a food additive permitted for direct addition to food for human consumption (21 CFR §172.381 - U.S. FDA, 2017a). Food enzymes produced by S. cerevisiae (e.g., invertase, GRN No. 88) (U.S. FDA, 2002) as well as several S. cerevisiae strains genetically-modified to alter the expression of specific endogenous enzymes or pathways (GRN No. 120, 175, 350, 422, 604) (U.S. FDA, 2002, 2003, 2006, 2011b, 2012, 2016c) have GRAS status with no objection from the U.S. FDA.

S. cerevisiae has been granted Qualified Presumption of Safety (QPS) status in the European Union by EFSA and therefore is considered safe for the derivation of genetically modified strain lineages intended for use in the production of food additives and enzymes, as long as the following qualification is met in the safety assessment: "Absence of resistance to antimycotics used for medical treatment of yeast infections in cases where viable cells are added to the food or feed chain S. cerevisiae this qualification applies for yeast strains able to grow above 37°C" (EFSA, 2017).

Despite the extensive history of safe use of *S. cerevisiae* in the food industry, rare reports of *S. cerevisiae* infections in humans indicate that *S. cerevisiae* is also regarded as an opportunistic pathogen. A comprehensive review conducted by Enache-Angoulvant and Hennequin (2007) reported 92 cases of *Saccharomyces* invasive infection, with the most common predisposing factors being antibiotic therapy and intravascular catheter. *S. cerevisiae* strain YJM789, for example, was isolated from the lung of an AIDS patient with polymicrobial pneumonia (Tawfik et al., 1989; Wei et al., 2007) and de Llanos et al. (2006) reported 4 clinical cases of *S. cerevisiae* detection in the blood. Amyris's steviol glycosides Reb-M produced by fermentation does not contain any viable production organisms, as evidenced by the absence of protein and residual DNA in the final product, and therefore the reports are of no safety concern.

### 7.2.2 Information on the Potential Toxicity of the Processing Aid

*(As per section 3.3.2 C.2 of the Application Handbook as at 1 July 2019)*

The production strain contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. The genes used to create the production strain are naturally occurring or from biosafety level 1 organisms, listed in **Table 14**. The fermentation broth is subjected to a heat treatment step to kill the yeast cells prior to the purification/concentration steps wherein the production strain is removed. As evidenced by the absence of protein and residual DNA in the final product and the high purity content of the steviol glycosides Reb-M produced by fermentation, the inserted DNA from these source organisms is of no safety concern.

**Table 14:** Source organisms for genes inserted in Amyris's production strain

| **Organism from which gene was derived** | **Description** |
|---|---|
| *E. coli K-12* | A non-pathogenic / non-toxic strain of E. coli |
| *Dickeya zeae* | Bacterium; harmless to humans |
| *Saccharomyces kluyveri* | Yeast similar to *S. cerevisiae*; laboratory model organism; harmless to humans |
| *Zymomonas mobilis* | Bacterium; makes ethanol; originally isolated from alcoholic beverages like African palm wine |
| *Blakeslea trispora* | Fungus that infects soy; used commercially to produce beta-carotene |
| *Arabidopsis thaliana* | Mouse-ear cress; a weed in the brassicaceae family (*i.e.,* broccoli and cauliflower) commonly used for molecular plant research |
| *Pisum sativum* | Garden pea |
| *Oryza sativa* | Rice |
| *Picea glauca* | White spruce |
| *Stevia rebaudiana* | Leaf extracts from this plantine are consumed and are classified as GRAS (Generally Recognized as Safe) |
| *Setaria italic* | Foxtail millet; a variety of cultivated millet |

All heterologous genes introduced into the production strain are derived synthetically from biosafety level 1 organisms and codon optimized for expression in *S. cerevisiae*. Table 14 lists the source organism for all heterologous genes present in production strain. The heterologous genes are not associated with any known allergens or toxins. In addition, the production strain is not toxigenic or pathogenic, and does not contain or produce any known pathogenicity-related proteins, toxins, allergens, or pyrogens. Additional detail to support the above information is provided in **Appendix CC-2** - Pathogenicity and toxicity report.

Furthermore, in the manufacturing process for rebaudioside M, the fermentation broth is subjected to heat treatment and undergoes several separation and purification steps to ensure that the production strain is removed from the final steviol glycoside product. Neither protein nor DNA is present in the final product of steviol glycosides Reb-M produced by fermentation, as defined in the product specifications, and the heterologous gene sequences inserted in the production strain does not present a health concern.

### 7.2.3 Information on the Potential Allergenicity of the Processing Aid

*(As per section 3.3.2 C.3 of the Application Handbook as at 1 July 2019)*

As demonstrated in 3 non-consecutive batches of steviol glycosides Reb-M produced by fermentation, the final product does not contain residual protein and DNA as per the defined product specifications. The potential for cross-reactivity among the inserted heterologous gene sequences in the production strain was investigated in accordance with the FAO/WHO protocol for bioinformatic allergenicity assessment (FAO/WHO, 2001). In the assessment, potential linear IgE epitopes were identified by searching for any match of 6 consecutive amino acids from each inserted gene sequence to an allergen database. Potential conformational IgE epitopes were identified by searching for greater than 35% sequence identity over a sliding 80-mer amino acid window. Amyris's steviol glycoside Reb-M produced by fermentation contains a total of 16 genes including two different copies of the KAH gene; therefore16 gene sequences were searched against the AllergenOnline Database Version 18 (available at http://www.allergenonline.org; updated March 23, 2018) maintained by the Food Allergy Research and Resource Program of the University of Nebraska (FARRP, 2017). The database contains a comprehensive list of putative allergenic proteins developed via a peer reviewed process for the purpose of evaluating food safety.

Part one of the bioinformatics assessment searched for 6-mer matches between the engineered Reb-M constructs, and the AllergenOnline database. This search returned 324 hits. Part two of the bioinformatics assessment, requiring >35% sequence similarity of any 80-mer amino acid window, returned 429 hits. In addition, total protein sequences queried for >35% similarity against the entire allergen database returned zero hits.

Based on the search of 6 consecutive amino acids, all inserted gene sequences had 100% identity to known allergens, however, it should be noted that the use of a 6-mer amino acid identity search can generate false positives (Goodman, 2006; EFSA, 2010). The FARRP indicates that a single identity match of 6 to 8 contiguous amino acids does not imply similar IgE binding in the absence of more extensive identity alignments (Goodman et al., 2008). Evaluation of sequence identity over a sliding 80-mer amino acid window indicated that several gene sequences had greater than 35% similarity to known allergen sequences. However, none of the sequences shared greater than 35% identity with any identified allergens over their full sequence length, indicating the unlikely potential for cross-reactivity to any known allergens. Therefore, based on the assessment conducted, the inserted heterologous gene

sequences in the production strain to produce steviol glycosides Reb-M produced by fermentation have low potential for allergenicity. Neither protein nor DNA is present in the final product of steviol glycosides Reb-M produced by fermentation, as defined in the product specifications, and the potential allergenicity of the heterologous gene sequences inserted in the production strain does not present a health concern.

The full risk assessment report is confidential and is provided as CC-5.

### 7.2.4 Safety Assessment Reports prepared by International Agencies or Other National Government Agencies, if available

*(As per section 3.3.2 C.4 of the Application Handbook as at 1 July 2019)*

Amyris' Reb-M produced by *S. cerevisiae* expressing steviol glycoside biosynthesis pathway processing aid has undergone a GRAS evaluation. The U.S FDA has responded with no questions regarding Amyris' Reb-M as a general purpose sweetener (U.S. FDA, 2018) and has also been concluded GRAS by a panel of qualified Experts.

### 7.2.5 Additional Information Related to the Safety of a Processing Aid Derived from a GM Microorganism

*(As per section 3.3.2 D of the Application Handbook as at 1 July 2019)*

#### Information on the Source Microorganism

*(As per section 3.3.2 D.1 of the Application Handbook as at 1 July 2019)*

A wild-type *S. cerevisiae* is used as the parental microorganism, herein referred to as the parental strain, to construct the processing aid *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes. The strain is derived from the well characterized and sequenced *S. cerevisiae* strain CEN.PK113-7D. (van Dijken 2000, Nijkamp, J. F. *et al., 2012*) The parental strain was converted into a steviol glycoside producing yeast, herein referred to as the production strain, by a series of site-specific genomic integrations of DNA constructs. The 18s rRNA sequence of the production strain is provided in Appendix CC-1.

#### Information on the Pathogenicity and Toxicity of the Source Microorganism

*(As per section 3.3.2 D.2 of the Application Handbook as at 1 July 2019)*

The *S. cerevisiae* species has a long history of safe use in the production of food and food ingredients. More specifically, as noted in Section 7.2.2 above, the *S. cerevisiae* production strain producing steviol glycosides, primarily Reb-M contains no known pathogenicity-related proteins, toxins, allergens, or pyrogens. All heterologous genes used to produce steviol glycosides are synthesized and derived from BSL1 organisms.

#### Information on the Genetic Stability of the Source Organism

*(As per section 3.3.2 D.3 of the Application Handbook as at 1 July 2019)*

The identity of the production strain is confirmed through whole genome sequencing. Additionally, as homologous recombination is used for the genetic transformation of the yeast, the genetic elements introduced are stably integrated into the genome. The lack of antibiotic or auxotrophic marker selection pressure in the production host contribute to the strain's stability. 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.

The cell line stability is demonstrated by using primary and secondary cell banks and comparing yields and productivities of the product during fermentation. Extended seed trains are routinely tested to ensure retention of phenotype over generations of the production strain. Production strain performance has been shown to be consistent over a number of fermentations.

The stability of the DNA integrations in the production strain has also been determined by colony PCR (cPCR) for every integration containing a heterologous gene before and after a 7-day fermentation, compared to a positive control. **Appendix CC-3** - Genome stability report contains confidential details of these cPCR results.

### 7.2.6 Additional Information Related to the Safety of a Processing Aid Derived from a Genetically-modified Microorganism

*(As per section 3.3.2 E of the Application Handbook as at 1 July 2019)*

**Information on the Methods used in the Genetic Modification of the Source Organism**
*(As per section 3.3.2 E.1 of the Application Handbook as at 1 July 2019)*

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

A description of the gene constructs is provided in Section 7.1.4 above and Appendix CC-1 - RebM production strain genetic engineering.

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

All genes used to create the production strain are synthesized and are based on deposited sequences. Modifications have been made to some of the inserted synthesized gene sequences. This is discussed in Section 7.1.4 above and in Appendix CC-1 - RebM production strain genetic engineering report.

*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 organization of all inserted genetic material*

A description of the methods used to construct the production strain is provided in Section 7.1.4 above and in Appendix CC-1 - RebM production strain genetic engineering report.

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

Information on the stability of the inserted genes is provided in Section 7.2.5 above and in Appendix CC-3 - Genome stability report.

## 7.3 Information Related to the Dietary exposure to the processing aid

*(As per section 3.3.2 F of the Application Handbook as at 1 July 2019)*

The processing aid is not present in the final steviol glycoside Reb-M product and is therefore not consumed in products containing the intense sweetener.

# 8. Foods Produced using Gene Technology

*(As per section 3.5 of the Application Handbook as at 1 July 2019)*

In this section information on the *S. cerevisiae* production organism is presented.

## 8.1 The Nature of the Genetic Modification

*(As per section 3.5 A.3 of the Application Handbook as at 1 July 2019)*

### 8.1.1 Description of the method used to transform the host organism

*(As per section 3.5 A.3(a) of the Application Handbook as at 1 July 2019)*

The method used to transform the host organism is described in detail in section 7.1.4 above.

### 8.1.2 Description of the construct and the transformation vectors used, including:

*(As per section 3.5 A.3(b) of the Application Handbook as at 1 July 2019)*

(i) the size, source and function of all the genetic components including marker genes, regulatory and other elements

Appendix CC-1 (confidential) lists all DNA (genes, markers, regulatory elements) inserted into the host organism (CC-1, sections 5 and 6). Nucleotide sequence detail is also included in CC-1 (section 6) for the DNA constructs inserted into the production strain. CC-1 (section 3) also lists the source and function of all heterologous DNA and engineering conducted on native yeast genes.

(ii) a detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.

Appendix CC-1 (sections 5 and 6) also contain physical orientation of the genetic components inserted into the chromosomes in the production strain. Listing restriction sites is only relevant for plasmid DNA which is not included in this yeast strain.

### 8.1.3 A full molecular characterisation of the genetic modification in the new organism

*(As per section 3.5 A.3(c) of the Application Handbook as at 1 July 2019)*

(i) identification of all transferred genetic material and whether it has undergone any rearrangements

CC-1 (confidential) includes detailed description of all transferred genetic material in the production strain. CC-4 (confidential) demonstrates that no rearrangements were detected via whole-genome sequencing. Whole-genome sequence data confirms that all intended genetic engineering steps in the RebM production strain were made correctly and that no unexpected genetic events had occurred during engineering.

(ii) a determination of the number of insertion sites, and the number of copies at each insertion site

As noted above, CC-1 includes detailed description of all transferred genetic material, including the number of copies at each insertion site. CC-3 – Genome stability report demonstrates that the intended integrations were inserted. Additionally, CC-4 highlights that whole-genome sequencing confirms no unintended integrations occurred; therefore, the expected copy number of each integration is correct.

(iii) full DNA sequence of each insertion site, including junction regions with the host DNA

CC-1 (section 6) provides DNA sequences for each insertion site, including all upstream and downstream homology regions in the inserted DNA. These homology regions target the inserted DNA to the genome and specify exactly where the DNA will integrate. The new DNA is inserted between the homology regions. The (native) DNA sequences in the homology regions are retained in the final strain.

(iv) a map depicting the organisation of the inserted genetic material at each insertion site

As noted in the above point, CC-1 (section 6) provides DNA sequence information on the precise location of each integration into the genome.

(v) details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).

As noted above, section 6 of CC-1 lists all DNA sequence information for inserted DNA (including for all ORFs and their junctions. As noted in section 7.1.4 above, all DNA integrations are sequence verified prior to insertion. The whole-genome sequencing information in CC-4 also confirms the ORFs and junctions.

### 8.1.4 A description of how the line or strain from which food is derived was obtained from the original transformant.

*(As per section 3.5 A.3(d) of the Application Handbook as at 1 July 2019)*

We note that this guideline appears to be aimed at food derived from a genetically modified plant or animal. However, the method used to transform the host organism is described in section 7.1.4. Detailed information on every transformation and modification of the original yeast strain is included in sections 5 and 6 of CC-1. Together, this information describes how the RebM production strain was derived from the wild type yeast.

## 8.2 New Proteins

*(As per section 3.5 B.2 of the Application Handbook as at 1 July 2019)*

### 8.2.1 Information on the potential allergenicity of any new proteins

*(As per section 3.5 B.2(b) of the Application Handbook as at 1 July 2019)*

Section 7.2.3 and CC-5 provide detail on potential allergenicity of new proteins produced in the production strain. CC-5 describes a bioinformatic prediction of allergenicity; specifically, that the engineered gene constructs for steviol glycosides Reb-M produced by fermentation product have low risk of potential allergenicity. In addition, as noted in section 7.2.3, no residual protein is present in the final steviol glycosides Reb-M produced by fermentation product. Section 4 of CC-1 provides detail on the new amino acid sequences for each of the altered proteins in the production organism. These amino acid sequences were used in the allergenicity bioinformatics study described in CC-5.

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