APPLICATION TO AMEND THE SPECIFICATIONS FOR STEVIOL GLYCOSIDES, UNDER THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE* STANDARD 1.3.1 – FOOD ADDITIVES TO INCLUDE HIGH-PURITY REBAUDIOSIDE M

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Application to Amend the Specifications for Steviol Glycosides, Under the *Australia and New Zealand Food Standards Code* Standard 1.3.1 – Food Additives to Include High-purity Rebaudioside M

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Application to Amend the Specifications for Steviol Glycosides, Under the *Australia and New Zealand Food Standards Code* Standard 1.3.1 – Food Additives to Include High-purity Rebaudioside M

A. GENERAL REQUIREMENTS

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

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

Each point is addressed in the sections that follow.

A.1 Form of the Application

This application to amend the *Food Standards Australia New Zealand Food Code* ("the Code") is prepared pursuant to Guideline 3.1.1 – General Requirements and Guideline 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019), which require the following structured format to assess an application for a new food additive:

A. General information on the application;
B. Technical information on the food additive;
C. Information on the safety of the food additive; and
D. Information on dietary exposure to the food additive.

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

A.2 Applicant Details

Sichuan Ingia Biosynthetic Co., Ltd. is a manufacturer of non-caloric high-quality sweeteners for the food, flavour, and beverage industries. The contact details for Sichuan Ingia Biosynthetic Co., Ltd. are listed below.

In addition, Intertek Health Sciences Inc. is involved in the preparation, submission, and stewardship of this application. The contact details for Intertek Health Sciences Inc. are listed below.

A.3 Purpose of the Application

Sichuan Ingia Biosynthetic Co., Ltd. (hereinafter "Sichuan Ingia") is submitting this application to FSANZ concerning a high-purity rebaudioside M produced using enzymatic modification technology and is therefore seeking the amendment of the Code to permit Sichuan Ingia's high-purity rebaudioside M produced through enzymatic modification. This production process may also be referred to as "enzyme modification" or "bioconversion"; these processes refer to a steviol glycoside preparation obtained through enzymatic modification of a steviol glycoside extract to obtain higher quantities of a specified steviol glycoside (*e.g.,* rebaudioside M). Sichuan Ingia has developed a manufacturing process to produce high-purity rebaudioside M that utilises using enzymes sucrose synthase (SUS) and uridine diphosphate (UDP)-glucosyltransferase (91D2 and 76G1) derived from a genetically modified strain of *Escherichia coli* BL21 (DE3) that converts rebaudioside A extracted and purified from the leaves of *Stevia rebaudiana* Bertoni to rebaudioside M (referred to as "RM95"). The manufacturing process of Sichuan Ingia's RM95 is consistent with that of other enzymatic bioconversion processes used to produce steviol glycosides, specifically rebaudioside M and rebaudioside D, which are described in Annex 3 for enzyme-modified steviol glycosides by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2021) and already permitted for use in a range of conventional food and beverage products in Australia and New Zealand under Schedule 15. When manufactured as described, the final RM95 preparation meets or exceeds the ≥95% steviol glycoside purity criteria established by JECFA and the *Food Chemicals Codex* (FCC).

Currently, Schedule 3 of the *Australia New Zealand Food Standards Code* ("the Code") contains specifications for "*steviol glycosides produced by enzymatic conversion*" (S3—35), which includes rebaudioside M. This specification "*relates to a steviol glycosides preparation obtained from the leaves of the Stevia rebaudiana Bertoni plant*" and presents a range of permissible processes that exclude the use of Sichuan Ingia's ingredient. This application, therefore, aims to amend the Code to encompass the acceptability and permissibility of Sichuan Ingia's manufacturing methodology as another means to safely and effectively produce rebaudioside M. To that end, the following should be included in S3—35, to permit use of Sichuan Ingia's high-purity RM95 ingredient from enzymatic bioconversion:

> "(g) by enzymatic conversion of purified stevia leaf extract to produce rebaudioside M using the following protein engineered enzymes:
>
> (i) UDP-glucosyltransferases (EC 2.4.1.17) sourced from *Escherichia coli* BL21 (DE3) containing the UDP-glucosyltransferase genes from *Stevia rebaudiana*; and
>
> (ii) sucrose synthase (EC 2.4.1.13) sourced from *Escherichia coli* BL21 (DE3) containing the sucrose synthase gene from *Arabidopsis thaliana*;".

Similarly, Schedule 18 of the Code currently includes "Sucrose synthase (EC 2.4.1.13) sourced from *Escherichia coli* K-12 containing the gene for sucrose synthase from *Arabidopsis thaliana*" and "Uridine diphosphate (UDP) glucosyltransferase sourced from *Escherichia coli* K-12 containing the UDP glucosyltransferase gene from *Stevia rebaudiana*". This application is therefore aimed to amend these entries to include *Escherichia coli* BL21 (DE3) as a permissible source of these enzymes to encompass the permissibility of Sichuan's manufacturing process (either *via* the removal of the current K-12 strain identifier, or addition of BL21 (DE3) to the list of permitted sources).

This application does not intend to change the purity specification (≥95% steviol glycosides) or propose an extension for the use of rebaudioside M in additional food products nor does it propose to increase the permitted quantities of rebaudioside M in permitted food products.

A.4 Justification of the Application

A.4.1 Technological Function for the Food Additive

Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni and steviol glycosides obtained through enzymatic modification are already permitted for use as high-intensity sweeteners in Australia and New Zealand for the replacement of sucrose in reduced-calorie or no-sugar-added products. Sichuan Ingia's RM95 is comprised of at least 95% rebaudioside M and at least 95% total steviol glycosides, which would have more favourable sensory characteristics when compared to the major glycosides (*i.e.,* stevioside, rebaudioside A) and have taste profiles that are more reflective of sucrose.

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

The benefits to the consumer would mirror those for other steviol glycosides currently permitted for use in Australia and New Zealand. Sichuan Ingia's RM95 would be used similar to other steviol glycosides in foods and beverages to replace sugar, which will benefit consumers seeking products that have reduced caloric content. In addition, this would also include consumers with specific medical conditions that require reduced sugar intake, such as those with diabetes, as the consumption of steviol glycosides does not interfere with glucose homeostasis (EFSA, 2010). Amendment of the Code in a manner as described in Section A.3, to allow use of Sichuan Ingia's RM95, would provide food & beverage manufacturers with an alternative source of high purity rebaudioside M aside from those that are currently permitted, which would promote healthy market competition and ultimately benefit the Australian/New Zealand consumer.

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

A.5 Information to Support the Application

Technical information specific to Sichuan Ingia's manufacturing methodology for rebaudioside M is presented in detail in Section B, including information regarding the enzymes utilised and their use as processing aids. Since these enzymes are not approved processing aids in Australia and New Zealand, information regarding their manufacture and safety, including the source microorganism utilised to produce them, are presented pursuant to Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook*. FSANZ reviewed an application to expand the definition of steviol glycosides to include all steviol glycosides present in the *S. rebaudiana* Bertoni leaf in 2016, and as such reviewed the safety of steviol glycosides (FSANZ, 2017). More recently, FSANZ reviewed the safety of steviol glycosides, including rebaudioside M, within A1207 in May 2021 (FSANZ, 2021a). Since the safety of rebaudioside M and steviol glycosides in general have been previously reviewed and established by FSANZ, Section C provides a short summary of steviol glycoside safety and focuses on presenting: (a) new safety publications present in the scientific literature which have not previously been evaluated by FSANZ; and (b) recent opinions released by regulatory agencies and/or scientific bodies (*i.e.,* JECFA).

A.6 Assessment Procedure

Sichuan Ingia considers the most appropriate procedure to be adopted in assessing the application to be the General Procedure – Level 1. It is anticipated that this application will involve amending *Standard 1.3.1 – Food Additives* of the Code to modify the specifications outlined in Schedule 3 for

steviol glycosides produced by enzymatic conversion (section S3–35). Sichuan Ingia also requests that the evaluation be expedited.

A.7 Confidential Commercial Information (CCI)

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

A.8 Other Confidential Information

Sichuan Ingia requests that the identity of the companies that perform analysis testing (*i.e.,* stability, residue, *etc.*) remain confidential and that their identity not be disclosed to the general public. The identity and contact information for the companies and persons responsible for producing these data should be treated as confidential, as public disclosure of this information is not required for the safety assessment of this ingredient.

A.9 Exclusive Capturable Commercial Benefit (ECCB)

Sichuan Ingia is currently not the only manufacturer of rebaudioside M. Therefore, the application would not confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the *Food Standards Australia New Zealand Act 1991*, as there are other companies who would likely benefit from approval of this application.

A.10 International and Other National Standards

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

At its 82nd meeting in 2016, the JECFA Committee conducted a re-evaluation of the safety, dietary intake, and specifications for steviol glycosides. The safety of steviol glycosides and the acceptable daily intake (ADI) of 0 to 4 mg/kg body weight, expressed as steviol, were confirmed. The Committee reviewed a new manufacturing process for rebaudioside A that used a genetically modified strain of *Yarrowia lipolytica* to re-express the steviol glycoside biosynthetic pathway. This led to the issuance of a new specification monograph for "*Rebaudioside A from Multiple Gene Donors Expressed in Yarrowia lipolytica*" in 2016. The purity requirement for rebaudioside A from genetically modified *Y. lipolytica* is no less than 95% total steviol glycosides on a dried basis.

Additionally, the Committee considered data demonstrating the shared metabolism of all steviol glycosides and established new specifications for "*Steviol Glycosides from Stevia rebaudiana Bertoni*" in 2017. This expanded the definition of steviol glycosides to include a mixture of compounds with a steviol backbone conjugated to various sugar moieties. The purity requirement for steviol glycosides from *S. rebaudiana* Bertoni is no less than 95% total steviol glycosides on a dried basis.

More recently, JECFA adopted a framework to develop specifications for steviol glycosides produced through 4 methodologies, including enzymatic modification (also referred to as enzymatic conversion) (JECFA, 2021). The JECFA framework for steviol glycosides has been ratified by the *Codex Alimentarius* into the *General Standard for Food Additives* (GSFA), and thus adoption on a global scale is currently underway (Codex, 2023). The specifications for steviol glycosides produced by enzymatic conversion

share the same identity and purity (*i.e.,* ≥95% total steviol glycosides) requirements as steviol glycosides obtained from extraction of the leaves of *S. rebaudiana* Bertoni.

A.10.2 United States

In the United States (U.S.), steviol glycosides have Generally Recognised as Safe (GRAS) status for use as general purpose sweeteners in foods. Over 75 GRAS notices have been submitted to the U.S. Food and Drug Administration (FDA) covering purified individual steviol glycosides, mixtures of steviol glycosides, and glucosylated steviol glycosides, all with a total steviol glycoside content of no less than 95%. The U.S. FDA has raised no objections to the GRAS status of steviol glycoside products for use as general purpose sweeteners in foods, recognising the general safety of these substances. GRAS Notice (GRN) 799 was submitted by Sichuan Ingia for rebaudioside M produced by enzymatic bioconversion, which is the same product that is the subject of this application (U.S. FDA, 2018). The U.S. FDA responded with "no questions" to the GRAS status of Sichuan Ingia's RM95 produced *via* enzymatic bioconversion for use as a tabletop sweetener and as a general-purpose non-nutritive sweetener in foods (U.S. FDA, 2018).

A.10.3 Other Jurisdictions

Steviol glycosides are approved for use in a number of other jurisdictions, including the European Union (EU), Canada, Asia, Central/South America, Africa, and the Middle East. Further details of the regulatory approvals of steviol glycosides in Canada and the EU are presented in Sections C.4.3 and C.4.4, respectively. In several Asian countries, including Japan, China, Hong Kong, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Singapore, Taiwan, Thailand, and Vietnam, steviol glycosides are approved as food additives or sweetening agents. Japan, for example, has authorised various stevia extracts for use. The Food Safety and Standards Authority of India (FSSAI) has also approved the use of steviol glycosides as a non-nutritive sweetener.

In Central/South American countries and several Middle Eastern and African nations, steviol glycosides, stevioside, *S. rebaudiana* leaves, and highly refined stevia extracts are permitted for use as low-calorie sweeteners. Additionally, these glycosides are recognised as food additives in Switzerland and Russia.

A.11 Statutory Declaration

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

A.12 Checklists

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

B. TECHNICAL INFORMATION ON THE FOOD ADDITIVE

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

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

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

1. Technical information on the processing aid;

2. Information related to the safety of an enzyme processing aid;

3. Additional information related to the safety of an enzyme processing aid derived from a microorganism; and

4. Additional information related the safety of an enzyme processing aid derived from a genetically modified microorganism.

B.1 Nature and Technological Purpose of Rebaudioside M

B.1.1 Technological Purpose

Sichuan Ingia's RM95 is produced by the multi-step enzymatic bioconversion of rebaudioside A obtained from a leaf extract of *S. rebaudiana* Bertoni using SUS and two UDP-glucosyltransferase enzymes (91D2 and 76G1) derived from a genetically modified strain of *E. coli* BL21 (DE3). The final product is a highly purified preparation containing no less than 95% rebaudioside M. As per the technological purposes listed in Schedule 14 – *Technological purposes performed by substances used as food additives*, Sichuan Ingia's RM95 fulfils the function as an intense sweetener and a flavour enhancer, consistent with rebaudioside M and steviol glycoside preparations already approved for use in Australia and New Zealand. Sichuan Ingia does not intend for this application to extend the use of rebaudioside M or steviol glycosides in general to foods for which its use levels have not already been permitted; Sichuan Ingia intends to use their RM95 steviol glycoside preparation in the current food categories and at use levels currently permitted for steviol glycosides. Likewise, Sichuan Ingia does not intend to propose additional or different food matrices to which the addition of steviol glycosides has not already been approved.

B.1.2 Sweetness Potency

The sweetness equivalency to sucrose of Sichuan Ingia's RM95 produced *via* enzymatic bioconversion of rebaudioside A was evaluated by a sensory panel. Serial dilutions of sucrose (1.0, 2.5, and 5.0%) were prepared in bottled water at room temperature. The rebaudioside M solution was prepared in bottled water at room temperature. Participants (n=15) consumed the rebaudioside M solution and results were evaluated against the serially diluted sucrose samples starting with the lowest to the highest concentration. Results were averaged and converted to sweetness equivalency compared to sucrose. The results were consistent among all participants. Based on the results, rebaudioside M was determined to be 300 times sweeter than sucrose. The full study report is provided in Appendix D.

B.1.3 Stability

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

To confirm that these conclusions apply to Sichuan Ingia's RM95, a 6-month accelerated stability study was conducted on 5 representative non-consecutive batches of the final RM95 product (Lot Nos. 20220206,, 20220305, 20220105, 20220202, 20220102). The samples were stored at 40±2°C at a relative humidity of 75±5%. Rebaudioside M was observed to be stable over the course of the accelerated stability study, based on appearance, moisture content, and percent rebaudioside M

content measured by high-performance liquid chromatography (HPLC) compared to baseline (Table B.1.3-1).

Table B.1.3-1 Accelerated Stability of 5 Non-consecutive Lots of RM95

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
Lot No. 20220206			
0 months	White powder with sweet odour and taste	2.69	96.42
1 months		2.88	96.38
2 months		2.99	95.98
3 months		3.05	95.99
6 months		3.20	95.70
Lot No. 20220305			
0 months	White powder with sweet odour and taste	2.93	96.23
1 months		2.85	96.35
2 months		3.07	96.19
3 months		3.26	95.88
6 months		3.47	95.76
Lot No. 20220105			
0 months	White powder with sweet odour and taste	3.01	96.52
1 months		2.89	96.60
2 months		3.16	96.48
3 months		3.20	96.25
6 months		3.31	96.17
Lot No. 20220202			
0 months	White powder with sweet odour and taste	2.75	96.20
1 months		2.80	96.35
2 months		2.90	96.06
3 months		3.14	95.78
6 months		3.30	95.53
Lot No. 20220102			
0 months	White powder with sweet odour and taste	2.78	96.35
1 months		2.78	96.30
2 months		2.83	95.89
3 months		2.92	95.74
6 months		3.01	95.58

RM95 = rebaudioside M − rich (≥95% rebaudioside M) steviol glycoside preparation.

The long-term stability of RM95 (≥95% rebaudioside M; ≥95% total steviol glycosides) was investigated in 5 non-consecutive lots (Lot Nos. 20220206, 20220305, 20220105, 20220202, 20220102) at a temperature of 25±2°C and 60±10% relative humidity. Samples are to be maintained in commercial packaging for up to 36 months. The available results indicate that RM95 is stable for up to 24 months when maintained at room temperature (25±2°C) and a relative humidity of 60±10% (Table B.1.3-2).

Table B.1.3-2 Long-term Stability of 5 Non-consecutive Lots of RM95

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
Lot No. 20220206			
0 months	White powder with sweet odour and taste	2.69	96.42
3 months		2.70	95.87
6 months		2.83	95.75
12 months		2.88	95.65

Table B.1.3-2 Long-term Stability of 5 Non-consecutive Lots of RM95

Timepoint	Physical Characteristic	Moisture (%)	Rebaudioside M Content (%)
18 months		2.97	95.61
24 months		3.16	95.50
Lot No. 20220305			
0 months	White powder with sweet odour and taste	2.92	96.23
3 months		2.88	96.30
6 months		2.90	96.10
12 months		3.10	95.87
18 months		3.08	95.78
24 months		3.25	95.60
Lot No. 20220105			
0 months	White powder with sweet odour and taste	3.01	96/52
3 months		2.96	96.48
6 months		2.98	96.60
12 months		3.05	96.38
18 months		3.16	96.26
24 months		3.28	96.18
Lot No. 20220202			
0 months	White powder with sweet odour and taste	2.75	96.20
3 months		2.80	95.97
6 months		2.75	95.64
12 months		2.90	95.73
18 months		3.17	95.68
24 months		3.34	95.56
Lot No. 20220102			
0 months	White powder with sweet odour and taste	2.78	96.35
3 months		2.73	96.40
6 months		2.80	96.12
12 months		2.86	95.86
18 months		2.80	95.78
24 months		3.01	95.67

RM95 = rebaudioside M − rich (≥95% rebaudioside M) steviol glycoside preparation.

B.2 Information to Enable Identification of Rebaudioside M

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

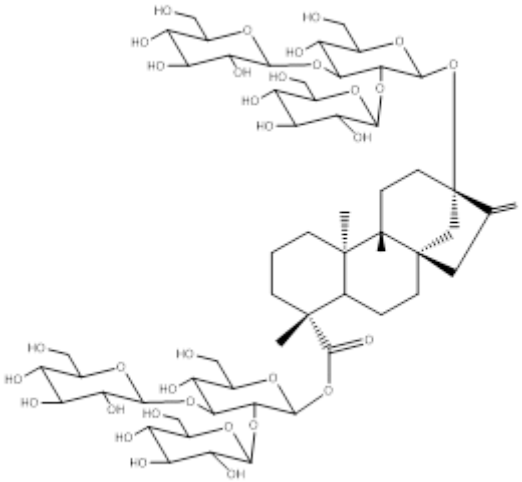
B.2.1 Identity of Substance

Sichuan Ingia's RM95 is produced by enzymatic bioconversion of rebaudioside A and the final product is a high-purity preparation containing no less than 95% rebaudioside M. Rebaudioside M is a minor naturally occurring steviol glycoside that is present in the leaves of *S. rebaudiana* Bertoni. Rebaudioside M is an *ent*-kaurene diterpenoid aglycone with a steviol backbone (Figure B.2.1-1). It is functionally related and structurally similar to rebaudioside A.

Chemical name: 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl

	3-O-β-D-glucopyranosyl β-D-glucopyranosyl ester
Common name:	Rebaudioside M
Synonyms:	Reb M, RM95
Chemical formula:	$C_{56}H_{90}O_{33}$
Molecular weight:	1291.29 Daltons
Chemical Abstracts Service (CAS) number:	1220616-44-3

Figure B.2.1-1 Chemical Structure of Rebaudioside M



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

Sichuan Ingia's RM95 is a white to off-white powder that is slightly soluble in water with a slight characteristic odour and sweet taste. Steviol glycosides are a group of compounds that share a similar molecular structure, where different sugar moieties are attached to the aglycone steviol (an *ent*-kaurene-type diterpenoid). Steviol glycosides include any compound containing a steviol backbone conjugated to any number or combination of the principal sugar moieties, including glucose, rhamnose, xylose, fructose, deoxyglucose, galactose, and arabinose (JECFA, 2021). Based on the similar chemical structure, all steviol glycosides including rebaudioside M share a common metabolic fate following consumption (Purkayastha *et al.,* 2016). Steviol glycosides are hydrolysed to steviol in the large intestine, which is subsequently absorbed and conjugated with glucuronic acid to form steviol glucuronide. The glucuronide metabolite is then excreted primarily *via* the urine in humans (Kraemer and Maurer, 1994; Koyama *et al*., 2003a,b; Geuns and Pietta, 2004 [unpublished]; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Wheeler *et al*., 2008; Roberts *et al.,* 2016).

B.4 Information on the Impurity Profile

Sichuan Ingia's RM95 produced *via* enzymatic bioconversion of rebaudioside A consists of ≥95% rebaudioside M and ≥95% total steviol glycosides. As described in Section B.6.1, Sichuan Ingia has established product specifications for rebaudioside M that are consistent with the specifications in Schedule 3 of the Code for "*steviol glycosides produced by enzymatic conversion*" (S3—35) and comply with the assay and impurity specifications in Annex 3 "*Enzyme-Modified Steviol Glycosides*" as described in the JECFA framework for steviol glycosides (JECFA, 2021). Any potential impurities contained within RM95 are discussed in greater detail within Section B.6.2, below.

B.5 Manufacturing Process

B.5.1 Overview

Sichuan Ingia's RM95 is produced *via* the enzymatic bioconversion of high-purity rebaudioside A using a strain of *E. coli* BL21 (DE3) that has been genetically modified to express the genes encoding for SUS and UDP-glucosyltransferases 91D2 and 76G1. The manufacturing process used to generate RM95 is consistent with that of other enzymatic bioconversion processes used to produce steviol glycosides, which are described in Annex 3 for enzyme-modified steviol glycosides in the JECFA framework for steviol glycosides (JECFA, 2021). The RM95 is obtained through enzymatic bioconversion of a high-purity rebaudioside A preparation (≥95% rebaudioside A; ≥95% total steviol glycosides) that is obtained through hot water extraction of the leaves of *S. rebaudiana* Bertoni. The formation of the activated sugar donor (UDP-glucose) is catalysed by SUS. The enzymes 91D2 and 76G1 then convert rebaudioside A into rebaudioside M through the enzymatic reaction shown in Figure B.5.4.2-1, and the crude rebaudioside M solution is purified and concentrated, yielding a final product that contains ≥95% rebaudioside M and ≥95% total steviol glycosides. Details of the method of manufacture, including raw materials and processing aids, the production strain and construction of the production strain, and the recombinant enzymes involved in the bioconversion process, are presented in Appendix A. Brief summaries are provided in the following sections.

B.5.2 Identity of Raw Materials and Processing Aids

All materials and processing aids utilised in the manufacture of Sichuan Ingia's' RM95 are food-grade and comply with relevant FCC or other internationally recognised standards. A list of all raw materials, processing aids, and filtration aids used in the manufacturing process to generate the enzymes is provided in Appendix A.

B.5.3 Details of the Manufacturing Process

In the first phase of manufacturing, a steviol glycoside primary extract containing ≥95% rebaudioside A (≥95% total steviol glycosides) is produced and purified according to the methodology outlined in the JECFA *Compendium of Food Additive Specifications* for steviol glycosides (JECFA, 2021).

The SUS and UDP-glucosyltransferase enzymes required for the enzymatic conversion process are generated by a strain of *E. coli* BL21 (DE3) that has been genetically modified to express the genes encoding for SUS and UDP-glucosyltransferases 91D2 and 76G1. The production strain is cultured for 5 to 6 hours and fermented with an induction agent (isopropyl β-D-1-thiogalactopyranoside [IPTG]) for 20 hours. The cells are then harvested through filtration and transferred to a reaction tank where purified rebaudioside A (≥95% rebaudioside A; ≥95% total steviol glycosides) is slowly added to the reaction tank. After the reaction period, the reaction mixture is filtered through a membrane to remove the precipitate and any remaining cells of the production strain. The crude solution containing the rebaudioside M is heated to deactivate any residual enzymes and to kill any remaining cells of the production strain.

The crude rebaudioside M solution is subjected to a series of purification and concentration steps that are consistent with the methodology described in Annex 3 of the JECFA framework for steviol glycosides (JECFA, 2021). The final high-purity rebaudioside M product (RM95; ≥95% rebaudioside M, ≥95% total steviol glycosides) is produced and the dried crystals are subsequently packaged. Further details of the production process are provided in Appendix A.

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

The enzymatic bioconversion reaction involves the use of enzymes that convert rebaudioside A to rebaudioside M. To begin, the formation of an activated sugar donor, UDP-glucose, is catalysed by SUS. The enzymatic bioconversion of rebaudioside A to rebaudioside M is then catalysed by the UDP-glucosyltransferase (91D2 and 76G1) enzymes. The gene encoding for the sucrose synthase enzyme was obtained from *Arabidopsis thaliana* and the genes encoding for the UDP-glucosyltransferase 91D2 and UDP-glucosyltransferase 76G1 enzymes are obtained from *S. rebaudiana* Bertoni. The source organisms for the genes that encode each of these enzymes have not been associated with any pathogenicity and/or toxigenicity; therefore, the introduction of the genes encoding for these enzymes is not expected to present any increased risk for pathogenicity and/or toxigenicity to the production organism, *E. coli* BL21 (DE3). The enzymes' function and species of origin are presented in Section B.5.4.1.

B.5.4.1 Information on the Identity of the Enzymes

Identification information on the SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes is provided below.

B.5.4.1.1 Sucrose Synthase

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

B.5.4.1.2 UDP-Glucosyltransferase 91D2

Source (strain):	*E. coli* containing DNA sequences encoding UGT and sucrose synthase enzymes
Common/Accepted Name:	Glucosyltransferase
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB):	2.4.1.17
Chemical/Systematic Name:	UDP-glucose β-D-glucosyltransferase

B.5.4.1.3 UDP-Glucosyltransferase 76G1

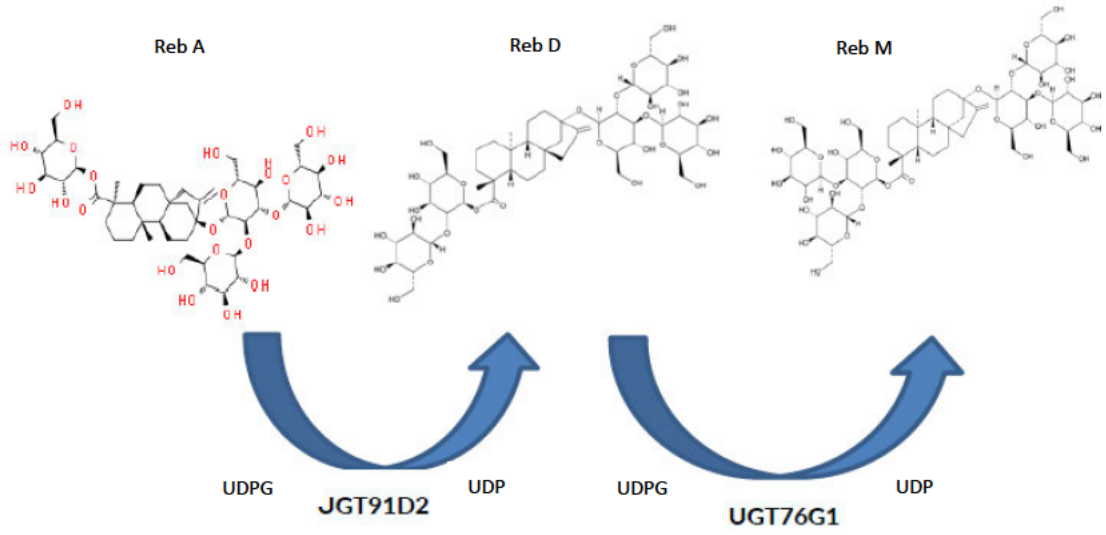
Source (strain):	*E. coli* containing DNA sequences encoding UGT and sucrose synthase enzymes
Common/Accepted Name:	Glucosyltransferase

Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB):	2.4.1.17
Chemical/Systematic Name:	UDP-glucose β-D-glucosyltransferase

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

The enzymatic conversion of rebaudioside A to rebaudioside M through the enzymatic activities of UDP-glucosyltransferase 91D2 and UDP-glucosyltransferase 76G1 is shown in Figure B.5.4.2-1 below. The formation of the activated sugar donor, UDP-glucose, is catalysed by SUS. Rebaudioside D is generated as an intermediate compound in the conversion of rebaudioside A to rebaudioside M.

Figure B.5.4.2-1 Enzymatic Bioconversion of Rebaudioside A to Rebaudioside M by UDP-Glucosyltransferase 91D2 and UDP-Glucosyltransferase 76G1



Reb = rebaudioside; UDP = uridine 5'-diphosphate; UGT = UDP-glucosyltransferase; UDPG = UDP-glucose.

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

The SUS and UDP-glucosyltransferase enzymes described in this application are only used as processing aids by Sichuan Ingia to produce RM95, similar to other glucosyltransferases used in the production of enzyme-modified steviol glycosides that are currently listed in Schedule 18 of the Code (differentiated from currently approved enzymes only by the strain of *E. coli*). In Canada, the enzymes are considered appropriate processing aids used in the production of enzyme-modified steviol glycosides. Sichuan Ingia's RM95, as described herein, is currently manufactured outside of Australia/New Zealand and is GRAS for use in the U.S. as a tabletop sweetener and as a general-purpose non-nutritive sweetener in foods (U.S. FDA, 2018).

B.5.4.4 Information on the Potential Toxicity of the Enzymes

The Basic Local Alignment Search Tool (BLAST) program maintained by the National Center for Biotechnology Information was used to conduct a sequence alignment query of the amino acid sequence of each enzyme against downloaded protein sequences obtained from a curated database of

8,522 venom proteins and toxins maintained by UniProt. Searches were conducted under the following conditions and criteria for significant sequence homology matches:

- Default search parameters: word size of 6, Expect threshold of 0.05, BLOSUM62 scoring matrix with default gap costs and composition adjustments.

- Structural homology/similarity criteria: identity >40%, E-value <0.001, bit-score >40.

No significant similarity to any toxins was identified from the sequence homology searches, indicating that the enzymes are not expected to pose any toxigenic concerns.

B.5.4.5 Information on the Potential Allergenicity of the Enzymes

The allergenicity potential of the SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes was evaluated using a bioinformatics approach. The searches were performed with AllergenOnline (Version 22, updated 25 May 2023). The searches were performed with the following criteria:

- Full-length sequence identity with cut-offs of greater than 50% identity and E-value smaller than $1x10^{-7}$;

- A "sliding window" of 80 amino acid sequences (*e.g.,* segments 1-80, 2-81, 3-82, *etc*.) derived from the full-length amino acid sequence of the protein; and

- 8 amino acid exact matches.

It should be noted that the searches were conducted following the guidelines described by FAO/WHO (2001) and Codex Alimentarius (2003, 2009). Matches greater than 35% over a window of 80 amino acids are suggestive of potential cross-reactivity with putative allergens; however, sequences sharing >35% identity over a window of 80 amino acids are common for many highly conserved proteins (Abdelmoteleb *et al.,* 2021). Although Abdelmoteleb *et al.* (2021) reported that all major and minor allergens were identified using an E-value threshold of $1x10^{-7}$, the degree of false positives obtained suggests that this threshold may not be sufficiently selective for use in risk assessment. Although the European Food Safety Authority (EFSA) Panel on Genetically Modified Organisms (EFSA, 2022) suggests that $1x10^{-7}$ may be a suitable E-value threshold, this has not yet garnered scientific consensus, and thus was not used as a threshold in the present assessment of allergenicity. Nonetheless, E-values were considered in the weight of evidence when assessing the relevance of sequences sharing >35% identity over at least 1 sliding window of 80 amino acids such that E-values $>1x10^{-7}$ over the full sequence are considered unlikely to pose a risk of allergenicity. It is further noted that the FAO/WHO (2001) and Codex Alimentarius (2003, 2009) guidelines recommend searches with the 80 amino acid sliding window and 8 amino acid exact matches, and do not include a recommendation to conduct searches with the full-length sequence. However, given that structural similarity between folded proteins may be evaluated using the full-length amino acid sequences, as noted by Aalberse (2000), Goodman *et al*. (2008), and Abdelmoteleb *et al*. (2021), any matches identified from the 80 amino acid sliding window or the 8 amino acid exact match searches were further evaluated for the degree of significance and identity over the full sequence and only considered further in the present allergenicity assessment if the match also had a percent identity >50% over the full sequence. The raw outputs are provided in Appendix E.

No significant identity matches were identified in the full-length sequence or 80-amino acid sliding window search that would be suggestive of an allergenic cross-reactive potential of these enzymes. In the 8 amino acid exact match with 76G1, 1 match to *alpha*-actinin (GI No. 1160577980) was identified. However, it should be noted that the utility of the exact match of 6 to 8 contiguous amino acids has been debated, and its usefulness in predicting potential allergenicity is unclear as these matches have

been known to produce “false positives” (Goodman *et al*., 2008; Ladics, 2019). The absence of exact matches of 6 to 8 amino acids between a query protein and a known allergen may suggest a lack of allergenicity, while an exact match of 6 to 8 amino acids may not necessarily suggest the protein to have allergenicity potential unless the query protein also shares >35% identity with a known allergen over an 80 amino acid window (Goodman *et al*., 2008). Therefore, it is not expected that this match would be suggestive of an allergenic concern of the enzyme. Furthermore, it should be highlighted that 76G1 is derived from *S. rebaudiana* Bertoni, which does not have a history of allergenic concern. Based on the available information, the allergenic risk of the SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes is considered to be low under the proposed conditions of use in the production of Sichuan Ingia’s RM95.

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

E. coli BL21 (DE3) was used as the parental microorganism to construct the production strain.*E. coli* belongs to the Enterobacterales family. The taxonomic identity of *E. coli* BL21 (DE3) is presented in Table B.5.4.6-1.

Table B.5.4.6-1 Taxonomic Identity of *Escherichia coli* BL21 (DE3)

Kingdom	Bacteria
Phylum	Pseudomonadota
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*
Strain	*Escherichia coli* BL21 (DE3)

ATCC = American Type Culture Collection.

E. coli are commensal residents of the gut microflora of humans and numerous other animal species. Strains of *E. coli* can be taxonomically classified into 5 different phylogroups (A, B1, B2, D, and E) based on the sequence similarity of housekeeping genes (Archer *et al.,* 2011). Human commensal strains are typically classified as Group A or B1 and non-related pathogenic strains of *E. coli* are classified under Groups B2, D, and E. Laboratory strains of *E. coli,* including strains K-12, B, C, and their derivatives are designated as Risk Group 1 organisms and are “not associated with disease in healthy adult humans” (Daegelen *et al.,* 2009; Archer *et al.,* 2019; National Institute of Health, 2019). *E. coli* BL21 (DE3) is widely utilised for the production of heterologous and homologous recombinant proteins and has an extensive history of use in universities, research organisms, and industry laboratories.

B.5.4.7 Pathogenicity/Toxigenicity of the Source Microorganism

The pathogenicity of *E. coli* B21 was evaluated by Chart *et al.* (2000). BALB/c mice (5/group) were administered 1×10^6 CFU of viable *E. col* B21 *via* the oral or peritoneal route. All animals were euthanized following a 7-day observation period. Animals administered *E. coli* BL21 displayed normal health throughout the observation period and viable colonies of *E. coli* BL21 could not be recovered from tissue samples. Furthermore, an oral toxicity conducted by Harper *et al.* (2011) demonstrated that administration of the *E. coli* BL21 (DE3) endotoxin to mice did not result in toxicity even at the highest dose administered (3.3 mg/kg body weight). O-antigen-positive strains of *E. coli* that can synthesize long-chain lipopolysaccharides are able to survive in normal and heat-activated serum. *E. coli* BL21 does not contain functional gene sequences encoding an O antigen polysaccharide (Jeong *et al.,* 2009). *Jeong et al.* (2009) thereby concluded that *E. coli* B21 “did not have the well-recognized pathogenic mechanisms required by strains of *E. coli* causing the majority of enteric infections”. *E. coli* BL21 (DE3) has been used as the production organism for a number of food ingredients that have been concluded

to be GRAS within the U.S. (*e.g.,* GRNs 876, 922, 923, 921, 925, 1015, and 1016). No safety concerns have been raised with its use in the production of food ingredients, particularly with respect to pathogenicity and/or toxigenicity. In addition, the DNA insert encodes only for the enzymes of interest and does not have any sequence similarity to other principal bacterially produced toxins as discussed in Section B.5.4.4.

B.5.4.8 Genetic Stability of the Source Microorganism

Polymerase chain reaction (PCR) analyses were conducted using the *E. coli* BL21 (DE3) production strain to evaluate the stability of the exogenous gene integration. 5 generations of the production strain were analysed *via* PCR for the presence of the gene encoding for the SUS enzyme. The results of the analyses indicate that the gene encoding for SUS was successfully integrated into the production strain and demonstrate the genetic stability of the production strain. Furthermore, 5 generations of the production strain were analysed *via* sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for expression of the recombinant SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes. The results of the analyses demonstrate the expression of the recombinant proteins by the production strain is stable across multiple generations. In addition, several steps are undertaken in the manufacturing process to inactivate and remove the enzymes from the final product, including heating and filtration steps. Furthermore, batch analyses demonstrate that the final product is of high purity (*i.e.,* contains ≥95% rebaudioside M) and is absent of residual DNA and protein that may be carried over from the production organism.

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

The genes encoding for the SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes from *Arabidopsis thaliana* and *S. rebaudiana* Bertoni, respectively, were synthesized *de novo*. The SUS and UPD-glucosyltransferase (91D2 and 76G1) genes were introduced into the expression vector using site-directed DNA integration to produce the recombinant plasmids. The SUS, 91D1 and 76G1 fragments and the expression vector were digested using restriction enzymes and the target fragments were ligated to produce the recombinant plasmids, which were then transformed into *E. coli* BL21 (DE3) competent cells using the calcium chloride transformation method. The cells were grown on an ampicillin-resistant medium. Colonies that were successfully transformed were obtained by ampicillin resistance screening. Stocks of the *E. coli* BL21 (DE3) production strains were stored in glycerol at -80°C. The genetic stability of the gene inserts and expression of the genes encoding for the enzymes were confirmed in multiple generations of the production strain. Further details of the construction of the production strain are provided in Appendix A.

B.6 Specification for Identity and Purity of RM95

B.6.1 Product Specifications for RM95

Sichuan Ingia has established food-grade specifications for RM95 produced *via* enzymatic conversion of rebaudioside A. As shown in Table B.6.1-1, the product specifications are consistent with the specifications in Schedule 3 of the Code for "*steviol glycosides produced by enzymatic conversion*" (S3—35) and comply with the purity requirements for enzyme-modified steviol glycosides as established by JECFA (2021). All methods of analysis are internationally recognised methods.

Table B.6.1-1 Product Specifications for RM95

Specification Parameters	Sichuan Ingia Rebaudioside M Specifications	JECFA (Enzyme-modified Steviol Glycosides) Specifications	Method of Analysis
Physical Parameters			
Appearance	White to light-yellow powder	White to light-yellow powder	Sensory evaluation

Table B.6.1-1 Product Specifications for RM95

Specification Parameters	Sichuan Ingia Rebaudioside M Specifications	JECFA (Enzyme-modified Steviol Glycosides) Specifications	Method of Analysis
Sweetness	200 to 350 times sweeter than sucrose (at 5% sucrose equivalency)	About 200 to 300 times sweeter than sucrose	Sensory evaluation
Odor	NS	Odourless or having a slight characteristic odour	Sensory evaluation
Particle Size	100% pass 80-mesh	NS	Ro Tap 25 g for 5 minutes
Solubility (%)	Freely soluble in a mixture of ethanol and water (50:50, v/v)	Very slightly soluble to freely soluble in water; slightly soluble to freely soluble in a mixture of ethanol and water (50:50 v/v)	JECFA Vol. 4
Chemical Parameters			
Rebaudioside M (%; wt/wt, on a dry basis)	≥95.0%	NS	HPLC (JECFA, 2017a)
Total Steviol Glycosides	≥95.0% (wt/wt, on a dry basis)	≥95% total steviol glycosides[a]	HPLC (JECFA, 2017a)
Loss on Drying (%; 105°C, 2 hours)	≤6.0	≤6	JECFA Vol. 4
pH	4.5 to 7 (1 in 100 solution)	4.5 to 7.0 (1 in 100 solution)	JECFA Vol. 4
Ash (%)	≤1.0	≤1	JECFA Vol. 4
Lead (mg/kg)	≤0.1	≤1	JECFA Vol. 4 – G-AAS
Arsenic (mg/kg)	≤0.1	≤1	JECFA Vol. 4 – HG-AAS
Mercury (mg/kg)	≤0.1	NS	JECFA Vol. 4 – CV-AAS
Cadmium (mg/kg)	≤0.1	NS	JECFA Vol. 4 – G-AAS
Residual Ethanol (mg/kg)	≤5,000	≤5,000	JECFA Vol. 4 – Method I
Residual Methanol (mg/kg)	≤200	≤200	JECFA Vol. 4 – Method I
Microbiological parameters			
Total Plate Count (CFU/g)	≤1,000	≤1,000	JECFA Vol. 4
Yeast and Mold (CFU/g)	≤100	≤200	JECFA Vol. 4
Escherichia coli (/g)	Negative	Negative	JECFA Vol. 4
Salmonella (/25 g)	Negative	Negative	JECFA Vol. 4

AAS = atomic absorption spectroscopy; CFU = colony-forming units; CV = cold vapor; G = graphite; HG = hydride generation; HPLC = high-performance liquid chromatography; JECFA = Joint FAO/WHO Expert Committee on Food Additives; NS = not specified; RM95 = rebaudioside M–rich (≥95% rebaudioside M) steviol glycoside preparation.

[a] Where steviol glycosides "*consists of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni including, glucose, rhamnose, xylose, fructose, deoxyglucose, galactose, and arabinose*" (JECFA, 2016, 2017a).

B.6.2 Product Analysis

B.6.2.1 Batch Analyses

5 non-consecutive batches of Sichuan Ingia's RM95 were analysed and the results, presented in Table B.6.2.1-1, demonstrate compliance with the defined product specifications. The Certificates of Analyses are provided in Appendix F.

Table B.6.2.1-1 Analytical Results for 5 Non-consecutive Batches of RM95

Specification Parameter	RM95 Specification	Manufacturing Lot Number				
		20240206	20240303	20240306	2024031	20240401
Physical Parameters						
Appearance	White to light-yellow powder	Complies	Complies	Complies	Complies	Complies
Sweetness	200 to 350 times sweeter than sucrose (at 5% sucrose equivalency)	Complies	Complies	Complies	Complies	Complies
Particle Size	100% pass 80-mesh	Complies	Complies	Complies	Complies	Complies
Solubility (%)	Freely soluble in a mixture of ethanol and water (50:50, *v/v*)	Complies	Complies	Complies	Complies	Complies
Chemical Parameters						
Rebaudioside M (%; wt/wt, on a dry basis)	≥95	97.52	97.47	97.48	97.43	97.63
Total Steviol Glycosides (%; wt/wt, on a dry basis)	≥95	99.52	99.33	99.52	99.33	99.46
Loss on Drying (%; 105°C, 2 hours)	≤6	3.10	3.23	2.86	2.93	3.05
pH	4.5 to 7 (1 in 100 solution)	5.5	5.4	5.3	5.5	5.4
Ash (%)	≤1.0	0.08	0.09	0.10	0.07	0.08
Lead (mg/kg)	≤0.1	0.04	0.06	0.06	0.03	0.06
Arsenic (mg/kg)	≤0.1	0.08	0.07	0.08	0.06	0.08
Mercury (mg/kg)	≤0.1	0.06	0.05	0.05	0.07	0.07
Cadmium (mg/kg)	≤0.1	0.03	0.07	0.07	0.056	0.05
Residual Ethanol (mg/kg)	≤5,000	756.8	987.3	648.28	915.7	655.3
Residual Methanol (mg/kg)	≤200	12.2	17.6	16.77	21.6	20.7
Microbiological Tests						
Total Plate Count (CFU/g)	≤1,000	<10	<10	<10	<10	<10
Yeast and Mould (CFU/g)	≤100	<10	<10	<10	<10	<10
Escherichia coli (/g)	Negative	Negative	Negative	Negative	Negative	Negative
Salmonella (/25 g)	Negative	Negative	Negative	Negative	Negative	Negative

CFU = colony-forming units; RM95 = rebaudioside M–rich (≥95% rebaudioside M) steviol glycoside preparation.

B.6.2.2 Residual DNA of the Production Strain

Five non-consecutive batches of Sichuan Ingia's RM95 were analysed for residual DNA from the genetically modified *E. coli* BL21 (DE3) production strain. Samples were analysed *via* quantitative PCR for the presence of the ampicillin resistance gene, the genes encoding for the SUS and UDP-glucosyltransferase (91D2 and 76G1) enzymes, and host *Escherichia coli* BL21 (DE3) genes. The results are presented in Table B.6.2.2-1 and demonstrate the levels of residual DNA from the production strain to be below each respective LOD in the final RM95 product. The study report is provided in Appendix G.

Table B.6.2.2-1 Results for Analysis of Residual Production Strain DNA in 5 Non-consecutive Batches of RM95

Specification Parameter	LOD	Manufacturing Lot Number				
		20240206	20240303	20240306	20240310	20240401
AmpR gene	3.3×10^{-4} ng/g	ND	ND	ND	ND	ND

Table B.6.2.2-1 Results for Analysis of Residual Production Strain DNA in 5 Non-consecutive Batches of RM95

Specification Parameter	LOD	Manufacturing Lot Number				
		20240206	20240303	20240306	20240310	20240401
SUS gene	3.3×10^{-4} ng/g	ND	ND	ND	ND	ND
UDP-glucosyltransferase 91D2 gene	3.3×10^{-4} ng/g	ND	ND	ND	ND	ND
UDP-glucosyltransferase 76G1 gene	3.3×10^{-4} ng/g	ND	ND	ND	ND	ND
Host *E. coli* BL21 (DE3) genes	3.3×10^{-2} ng/g	ND	ND	ND	ND	ND

*Amp*R = ampicillin resistance; LOD = limit of detection; ND = not detected; SUS = sucrose synthase; RM95 = rebaudioside M–rich (≥95% rebaudioside M) steviol glycoside preparation; UDP = uridine 5'-diphosphate

B.6.2.3 Residual Protein

Analysis of 5 non-consecutive batches of final RM95 product (Lot Nos. 20240206, 20240303, 20240306, 20240310, 20240401) was conducted using the bicinchoninic acid (BCA) assay, with a limit of detection of 2 ppm. Results from this analysis, presented in Table B.6.2.3-1, confirms the absence of residual protein in the final product. The Certificates of Analysis are provided in Appendix H.

Table B.6.2.3-1 Results for Analysis of Residual Protein in 5 Non-consecutive Batches of RM95

Specification Parameter	LOD	Manufacturing Lot Number				
		20240206	20240303	20240306	20240310	20240401
Residual Protein	2 ppm	ND	ND	ND	ND	ND

LOD = limit of detection; ND = not detected; RM95 = rebaudioside M–rich (≥95% rebaudioside M) steviol glycoside preparation

B.6.2.4 Residual Ampicillin and Isopropyl β-D-1-Thiogalactopyranoside

5 non-consecutive batches of Sichuan Ingia's RM95 were analysed for residual ampicillin and IPTG, due to their use in the seed inoculation and fermentation media. The results from these analyses are presented in Table B.6.2.4-1 and demonstrate the absence of residual ampicillin and IPTG in the final RM95 product. The Certificates of Analysis are provided in Appendix I.

Table B.6.2.4-1 Results for Analysis of Ampicillin and IPTG in 5 Non-consecutive Batches of RM95

Specification Parameter	LOD	Manufacturing Lot Number				
		20240206	20240303	20240306	20240310	20240401
Ampicillin	1 µg/kg	ND	ND	ND	ND	ND
IPTG	20 mg/kg	ND	ND	ND	ND	ND

IPTG = isopropyl β-D-1-thiogalactopyranoside; LOD = limit of detection; ND = not detected; RM95 = rebaudioside M–rich (≥95% rebaudioside M) steviol glycoside preparation; UDP = uridine 5'-diphosphate

B.7 Information for Food Labelling

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

B.8 Analytical Method for Detection

The analytical methods used to confirm that Sichuan Ingia's rebaudioside M meets the established chemical and microbial specifications (Section B.6.1) are internationally recognised (*e.g.*, Association of Analytical Communities [AOAC], *United States Pharmacopeia* [USP], JECFA). The rebaudioside M content in the final product is quantified according to the JECFA HPLC method for steviol glycosides described in Food and Agriculture Organization of the United Nations (FAO) JECFA Monograph 20 for "*Steviol Glycosides from Stevia rebaudiana Bertoni*" (JECFA, 2017a).

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

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

C. INFORMATION RELATED TO THE SAFETY OF THE FOOD ADDITIVE

In accordance with Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019) the safety information outlined must be provided to extend the use of a currently permitted food additive.

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

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

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

Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 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 that have been published since the last safety evaluation by FSANZ is provided in the following section.

C.1 Introduction

The safety of steviol glycosides is well established through numerous risk assessment and safety evaluations conducted by scientific and regulatory bodies, including the U.S. FDA, Health Canada, FSANZ, EFSA, and JECFA. Within these risk assessments and safety evaluations, it is generally recognised that steviol glycosides, including specified glycosides, share a common metabolic fate. These substances are hydrolysed to steviol in the large intestine, which is then absorbed and conjugated with glucuronic acid to form steviol glucuronide that is excreted in the urine. FSANZ recognises this shared metabolic fate and have expanded the definition of steviol glycosides to include all glycosides within the *S. rebaudiana* Bertoni leaf.

In 2016, FSANZ received an application to expand the definition of steviol glycosides to include all steviol glycosides present in the *S. rebaudiana* leaf. The safety of all steviol glycosides was reviewed by FSANZ at this time and an approval report was issued February 20, 2017 to expand the steviol glycoside definition (FSANZ, 2017). Similarly, FSANZ received an application on a steviol glycoside mixture produced by a genetically modified strain of *Y. lipolytica* expressing steviol glycoside biosynthesis genes. An approval report was issued on 27 September 2021 for this application (FSANZ, 2021b). Therefore, for this application, only safety studies conducted with steviol glycosides that were published since September 2021 were reviewed and summarised in the sections that follow. To identify scientific publications relevant to the safety of steviol glycosides and rebaudioside M, a comprehensive and detailed search of the published scientific literature was conducted up to January 2024. The search was limited to articles with full texts within peer-reviewed scientific journals and the following databases were accessed: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile®.

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

The metabolic fate of steviol glycosides is well established and discussed in the scientific literature, and is briefly discussed herein. It is generally recognised that steviol glycosides share a common metabolic fate (*i.e.,* absorption, distribution, metabolism, and excretion) due to the shared steviol backbone. Steviol glycosides are not hydrolysed in the upper gastrointestinal tract, owing to the presence of β-glycosidic bonds. Instead, steviol glycosides are digested by the gut microbiome in the colon, resulting in the release of steviol (Wingard *et al*., 1980; Hutapea *et al*., 1997; Gardana *et al*., 2003; Koyama *et al*., 2003a,b; Geuns *et al*., 2003, 2007; Renwick and Tarka, 2008; Nikiforov *et al.*, 2013; Purkayastha *et al.,* 2016). The rate of hydrolysis of a steviol glycoside is dependent on the complexity of its chemical structure (Wingard *et al*., 1980; Koyama *et al*., 2003b). However, despite the differences in chemical structure between different types of steviol glycosides, the rate of hydrolysis is generally similar as demonstrated by *in vitro* metabolic studies with human faecal homogenates (Purkayastha *et al.,* 2014, 2015, 2016). Following digestion by the gut microbiota, the released steviol is systemically absorbed into the portal vein and distributed to the liver, spleen, adrenal glands, fat, and blood (Nakayama *et al*., 1986; 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 and ultimately excreted alongside any unconjugated steviol or unhydrolysed fraction primarily in the urine and faeces (to a lesser extent) (Wingard *et al*., 1980; Nakayama *et al*., 1986; Kraemer and Maurer, 1994; Simonetti *et al*., 2004; Geuns *et al*., 2006, 2007; Roberts and Renwick, 2008; Wheeler *et al*., 2008). As previously discussed, the common metabolic fate of steviol glycosides is recognised by various scientific and regulatory bodies, who have concluded that the existing safety database for individual steviol glycosides, such as stevioside, rebaudioside A, and rebaudioside D, can be extrapolated to support the safety of another high-purity steviol glycoside.

C.3 Information on the Toxicity of Steviol Glycosides

C.3.1 Toxicological Studies

C.3.1.1 Repeat-dose Toxicity

The literature search identified 3 new studies that evaluated test article–related adverse effects of purified stevioside or rebaudioside A in obese or diabetic rats, or healthy rats with diabetic-related outcomes (Kurek *et al.,* 2021; Mubarak *et al.,* 2023). The results of these studies are summarised in Table C.3.1.1-1. These studies were not performed in accordance with standardised toxicological testing guidelines (*e.g.,* Organisation for Economic Co-operation and Development [OECD] Test Guidelines, U.S. FDA Redbook). These studies evaluated toxicologically relevant endpoints, such as haematology, clinical chemistry, and histopathology parameters, as well as glycaemia and oxidative stress, and therefore they were considered in the safety evaluation of Sichuan Ingia's rebaudioside M. In all studies, no test article–related adverse effects on any of the measured parameters were identified. In the study by Mubarak *et al.* (2023), obese Sprague-Dawley rats (30 males/group) were provided dried stevia leaves or purified rebaudioside A at a dose of 200 mg/kg body weight/day for 6 weeks. The study authors reported that the test article was administered orally, but did not specify the method (*e.g.,* diet or gavage or drinking water). The study authors only evaluated measures of oxidative stress and reported statistically significant differences in malondialdehyde and superoxide dismutase between the groups receiving each test article. It is noted that this study was not performed in accordance with standardised toxicological testing guidelines (*e.g.,* OECD Test Guidelines, U.S. FDA Redbook), and did not measure any toxicologically relevant endpoints. As a result, the utility of this study in the safety discussion of RM95 is limited. However, it is noted that a large body of repeated-dose toxicity studies on high-purity steviol glycosides exists, and the systemic toxicity of these compounds is well established. The results of these studies provide further supporting evidence on the established safety of steviol glycosides.

Table C.3.1.1-1 Summary of Newly Identified Repeated-dose Toxicity Studies of Steviol Glycosides

Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Dose in mg/kg bw/day	Parameters Evaluated	Significant Findings	Reference
Rat[a] (Wistar) 8 to 10 M/group	Oral (diet) 5 weeks	500 or 2,500 Pure stevioside or rebaudioside A	Blood biochemistry, histopathology	• No compound-related adverse effects on measured parameters.	Kurek *et al.* (2021)
Rat[b] (Sprague-Dawley) 30 M/group	Oral (diet) 6 weeks	200 Dried stevia leaves or purified rebaudioside A	Oxidative stress	• Statistically significant difference in MDA and SOD (oxidative stress).	Mubarak *et al.* (2023)

bw = body weight; M = male animals; MDA = malondialdehyde; SOD = superoxide dismutase.
[a] Animals were diabetic.
[b] Animals were obese.

C.3.1.2 Genotoxicity

The literature search identified 2 new studies that evaluated the potential genotoxicity of rebaudioside A (98.65% purity) in an *in vivo* chromosome aberration test (Yılmaz *et al.,* 2022). The results of this study are summarised in Table C.3.1.2-1. A weak positive effect was reported in the *in vivo* chromosome aberration test with a high-purity rebaudioside A (Yılmaz *et al.,* 2022). It should be noted that this study was not conducted in accordance with standardised toxicological testing guidelines (*e.g.,* OECD Test Guidelines), thus limiting their utility in the risk assessment of Sichuan Ingia's rebaudioside M. Furthermore, considering that the weak oxidative damage, cell cycle activity, and chromosomal aberration frequency effect reported by Yılmaz *et al.* (2022) were at test concentrations of up to 620 mg steviol/kg body weight/day, which is well above the JECFA ADI of 4 mg/kg body weight, the relevance of this finding is limited as it is not realistic to the estimated dietary exposure to steviol glycosides in the U.S. population. Given these discrepancies, the results of this study do not contradict the established lack of genotoxicity of high-purity steviol glycosides that is recognised globally. It is noted that long-term studies with stevioside have not suggested a carcinogenic effect of this compound (Toyoda *et al.,* 1997), and there is an extensive genetic toxicology database to support the lack of genotoxic and mutagenic effects of high-purity steviol glycosides (Brusick, 2008; Urban *et al.,* 2013).

Table C.3.1.2-1 Summary of Newly Identified Genotoxicity Studies of Steviol Glycosides

Test	Test System / Animal Species	Concentration/Dose	Results	Reference
***In Vivo* Studies**				
Chromosome aberration test	BALB/c mice 28 days (4/sex/group)	0, 470, 620, 940, or 1,880 mg/kg body weight/day steviol glycosides (rebaudioside A, 98.65% purity; equivalent to 155, 205, 310, or 620 mg steviol/kg body weight/day, respectively)	• Weak positive. • Dose-dependent increase in abnormal cells. • Slight increase in chromosome aberration and oxidative damage. • Increase in mitotic index in all groups.[a]	Yılmaz *et al.* (2022)

[a] This effect was described by the study authors to be insignificant in the test groups and control group, while the tabulated results suggest an increase in mitotic index. Given this discrepancy, it is difficult to interpret the overall findings.

C.3.1.3 Long-term Toxicity and Carcinogenicity

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

C.3.1.4 Reproductive and Developmental Toxicity

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

C.3.1.5 Immunotoxicity

Sánchez-Delgado *et al.* (2021) conducted a clinical study evaluating the effects of non-calorie sweeteners, including steviol glycosides, on nutrient and calorie intake, adipose mass, triglycerides, and serum proinflammatory cytokines as an immunotoxicity endpoint. The study was conducted over a 7-week period separated into 2 phases involving healthy individuals. In Phase I, a food frequency questionnaire was completed, and anthropometric and body composition measurements (weight, body mass index [BMI], total fat percentage, muscle mass, and waist circumference) were made at study initiation. A 1-week washout period was implemented to restrict food and drinks with added sugar and non-caloric sweeteners prior to administration initiation. In Phase II, blood samples were drawn from fasted participants to measure biochemical and immunological parameters (blood glucose, triglycerides, cholesterol, interleukin [IL]-1β, IL-6, IL-10, tumour necrosis factor [TNF]-α, and interferon [IFN]-γ) before and after the 6-week administration period. Subjects were randomly assigned 1 of 3 administration groups: Group 1 (n=12, eight 5-g packs of sucrose/day); Group 2 (n=13, four 1-g packs of sucralose/day, each pack containing 0.012 g of sucralose); and Group 3 (n=13, four 1-g packs of steviol glycoside/day, each packet containing 0.025 g of steviol glycosides). The composition of steviol glycosides was not specified. The assigned sweetener was added to drinks or food every day, and subjects were asked to restrict the use of added sugar or sweeteners in the rest of their diet during the administration phase. Intakes were monitored using 24-hour diet recalls and anthropometric and body composition parameters were measured weekly. Mean energy intake in the steviol glycoside group was reduced compared to baseline. Nutrient distribution showed a significant decrease in carbohydrate intake (p=0.002) and an increase in protein intake (p=0.0001) in the steviol glycoside group. No changes were observed in lipid intake, body weight, BMI, or muscle mass in the steviol glycoside group; however, body fat was significantly decreased (p=0.0287). Immunological parameters in the steviol glycoside group from baseline to Week 7 of the study showed a significant decrease in TNF-α concentrations (p=0.0029) and no significant change in IL-6 concentrations. Concentrations of IFN-γ and IL-10 were below the limit of detection. The authors concluded that, "*The data reported in the present study corroborates previously reported anti-inflammatory effects of steviol glycosides and support the notion that these compounds may have beneficial effects for human health* […]." The consumption of steviol glycosides did not lead to adverse effects or adversely affect the outcomes of immunotoxicity parameters.

C.3.2 Human Studies

In a 90-day open-label, single-arm pilot study, overweight participants with either normal blood sugar (healthy) (n=24) or prediabetes (n=21) replaced added sugar in their diets with a stevia-based test material in the form of a powder or pellet containing 2.19 or 20.51% w/w steviol glycosides, respectively (Raghavan *et al.*, 2023). The study authors evaluated a commercially available tabletop product that contains the sweetener with bulking agents, such as maltodextrin. Therefore, it would be difficult to determine the relevance of the reported findings to the sweetener itself. The primary outcomes measured were body weight and waist circumference and the secondary outcomes were blood glucose, body mass index, lipid levels, sugar consumption, glycated haemoglobin (HbA1c), and adverse events.

A statistically significant decrease in weight and waist circumference were reported in overweight participants with normal blood sugar and those who were prediabetic at study end compared to baseline. Postprandial blood glucose was statistically significantly different in healthy participants at 30 and 60 days compared to baseline, though not at 90 days. In prediabetic participants, no statistically significant differences were reported at any time point throughout the study. Statistically significant changes in body mass index were reported in both healthy and prediabetic participants at study end compared to baseline.

A slight but significant reduction in high-density lipoprotein was reported in healthy participants at study end compared to baseline. Slight changes in total cholesterol, low-density lipoprotein, very-low-density-lipoprotein, and triglycerides were reported; however, the changes were not statistically significant. In prediabetic participants, a slight but non-significant increase in high-density lipoprotein was reported at study end compared to baseline. Additionally, a non-significant reduction in total cholesterol and low-density lipoprotein was reported at Day 30 and 60 of the study, followed by an increase reported at Day 90 compared to baseline. Reductions in very-low-density lipoprotein and triglycerides were consistently reported, compared to baseline. In the prediabetic group, no significant changes to HbA1c were reported at study end compared to baseline. No adverse effects were reported in participants during the study. It was noted that the overall consumption of steviol glycosides by participants fell within the established ADI.

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

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

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

The safety of steviol glycosides has been extensively reviewed by JECFA at their 51st, 63rd, 68th, 69th, and 82nd meetings in 1998, 2004, 2007, 2008, and 2016 respectively (JECFA, 1999, 2006, 2007, 2009b, 2017b). Initially, the Committee established a temporary ADI for steviol glycosides of 0 to 2 mg/kg body weight, expressed as steviol, based on a no-observed-adverse-effect level of 970 mg/kg body weight/day (383 mg/kg body weight/day as steviol) from a 2-year study in rats (Toyoda *et al.*, 1997) and application of a safety factor of 200 (JECFA, 2006). In 2008, following review of additional animal and human studies evaluating the effects of steviol glycosides on blood pressure and blood glucose, the Committee concluded that the results from these studies were sufficient to remove the additional safety factor of 2, and established a full ADI of 0 to 4 mg/kg body weight (expressed as steviol) for steviol glycosides.

The JECFA Committee recently re-evaluated the safety, dietary intake, and specifications for steviol glycosides at its 82nd meeting in 2016. The safety of steviol glycosides as well as the ADI of 0 to 4 mg/kg body weight, expressed as steviol, were confirmed. Details of a new manufacturing process for rebaudioside A utilising a strain of *Y. lipolytica* that was genetically modified to overexpress the steviol glycoside biosynthetic pathway were submitted to and reviewed by the Committee. As a result, the Committee issued a new specification monograph for "*Rebaudioside A from Multiple Gene Donors Expressed in Yarrowia lipolytica*" (JECFA, 2016, 2017b). The Committee also reviewed data

demonstrating the shared metabolism of all steviol glycosides and issued new "tentative" specifications[1] for "*Steviol Glycosides from Stevia rebaudiana Bertoni*," which were subsequently published in a manner that superseded the tentative status (JECFA, 2017a), expanding the definition of steviol glycosides to "*a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties (glucose, rhamnose, xylose, fructose, arabinose, galactose and deoxyglucose) in any of the orientations occurring in the leaves of Stevia rebaudiana Bertoni*." The purity of steviol glycosides from *S. rebaudiana* Bertoni must be no less than 95% total steviol glycosides on the dried basis.

More recently, JECFA adopted a framework to develop specifications for steviol glycosides produced through 4 methodologies, including enzymatic modification (also referred to as enzymatic conversion) (JECFA, 2021). The JECFA framework for steviol glycosides has been ratified by *Codex Alimentarius* into the GSFA, and thus adoption on a global scale is currently underway (Codex, 2023). The specifications for enzyme-modified steviol glycosides (*i.e.,* steviol glycosides produced by enzymatic conversion) share the same identity and purity requirements as steviol glycosides obtained from extraction of the leaves of *S. rebaudiana* Bertoni: the final product must contain no less than 95% total steviol glycosides. As discussed in Section B.6.1, Sichuan Ingia's rebaudioside M meets or exceeds the identity and purity specification requirements for steviol glycosides produced by enzymatic conversion as established by JECFA (2021), and is manufactured in accordance with the processes described in Annex 3 of JECFA (2021); therefore, there are no anticipated safety concerns with respect to Sichuan Ingia's rebaudioside M.

C.4.2 United States Food and Drug Administration (FDA)

Steviol glycosides have a long history of regulatory evaluation by the U.S. FDA. In the 1970s and continuing into the 1990s, the FDA received numerous food additive petitions relating to the use of stevia leaves or steviosides as an alternative sweetener. Due to deficiencies in the technical and safety data at the time, these petitions were never filed by the FDA. In 1991, the FDA issued an alert banning the import of stevia leaves, steviosides, and foods containing stevia due to inadequate information to support the safety of these ingredients and their derivatives. The crude nature of these products, with a low steviol glycoside purity, was the primary safety concern. The FDA revised its import alert in 1995, following implementation of the *Dietary Supplement Health and Education Act of 1994*,[2] to allow stevia leaves, stevioside, or products containing stevioside to be imported if they were explicitly labelled as a dietary supplement or were used solely as a dietary ingredient in a dietary supplement product while maintaining that product labels must include a declaration on the part of the plant from which the stevia ingredient is derived (*e.g*., leaf), or whether the stevia ingredient is a crude stevia extract or a purified extract meeting established product specifications. The import alert was last revised in 2018 to highlight that steviol glycosides with ≥95% purity can be imported, provided the importer has documentation to demonstrate that they meet the minimum purity. Documentation to support the purity of the steviol glycosides may include an FDA "no questions" letter to a GRAS notice or Certificates of Analysis demonstrating that the steviol glycosides meet the established specifications (Perrier *et al.*, 2018). This revision underscores that any steviol glycoside imported into the U.S. or marketed within the U.S. must be of high purity (*i.e.*, ≥95% purity) and meet established specifications. As demonstrated by the large number of GRAS notices pertaining to high-purity steviol glycoside preparations within the U.S. FDA's GRAS Notice Inventory,[3] companies have sought the FDA "no questions" letter to support the GRAS status of their high-purity steviol glycosides (see Table C.4.2-1). These high-purity steviol glycosides include mixtures of different steviol glycosides, individual steviol glycosides such as stevioside, the group of major and minor rebaudiosides (*e.g.,* A, D, E, I, and M), or enzyme-modified steviol glycosides (also known as glucosylated steviol glycosides). Overall, the FDA have consistently raised "no questions" on

[1] The tentative status was removed at the 84th meeting and full specifications are to be published that include the additional sugar moieties arabinose and galactose.
[2] https://ods.od.nih.gov/About/DSHEA_Wording.aspx.
[3] U.S. FDA GRAS Notice Inventory is available at: https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices.

the GRAS status of these high-purity steviol glycosides when used as general-purpose sweeteners, suggesting that there is a general recognition on the safety of high-purity steviol glycosides within the U.S.

Table C.4.2-1 Summary of GRAS Notices Submitted to the United States Food and Drug Administration for Steviol Glycosides (Current as of December 2023)

Company	Substance	FDA Response	GRAS Notice No.
Steviol Glycosides by Leaf Extraction			
Whole Earth Sweetener Company LLC (subsidiary of Merisant)	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	No questions	252
Cargill, Inc.	Rebaudioside A purified from *Stevia rebaudiana* (Bertoni) Bertoni	No questions	253
McNeil Nutritionals	Purified steviol glycosides with rebaudioside A as the principal component	No questions	275
Blue California	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	278
Sweet Green Fields, LLC	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	282
Wisdom Natural Brands	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	287
Sunwin USA, LLC and Wild Flavors	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	303
Sunwin USA, LLC and Wild Flavors	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	304
Pyure Brands, LLC	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	318
PureCircle USA, Inc.	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	323
GLG Life Tech, Ltd.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	329
GLG Life Tech, Ltd.	Stevioside purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (stevioside)	No questions	348
GLG Life Tech, Ltd.	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	349
Guilin Layn Natural Ingredients, Corp.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	354
BrazTek International Inc.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	365
Sinochem Qingdao Co., Ltd.	Purified steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	367
Zhucheng Haotian Pharm Co., Ltd.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	369
GLG Life Tech Corporation	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	380
Chengdu Wagott Pharmaceutical	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	388
Chengdu Wagott Pharmaceutical	Steviol glycosides with stevioside as the principal component	No questions	389
Daepyung Co., Ltd.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	393

Table C.4.2-1 Summary of GRAS Notices Submitted to the United States Food and Drug Administration for Steviol Glycosides (Current as of December 2023)

Company	Substance	FDA Response	GRAS Notice No.
Daepyung Co., Ltd.	Steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	395
MiniStar International, Inc.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	418
PureCircle USA, Inc.	Rebaudioside D purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside D)	No questions	456
Almendra Limited	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside A)	No questions	461
Qufu Xiangzhou Stevia Products Co., Ltd.	Rebaudioside A purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni	No questions	467
PureCircle, Ltd.	Purified steviol glycosides with rebaudioside X as the principal component	No questions	473
GLG Life Tech Corporation	High-purity steviol glycosides (minimum purity 95%)	No questions	493
GLG Life Tech Corporation	High-purity Rebaudioside M	No questions	512
Almenda (Thailand) Limited	Steviol glycosides with rebaudioside A and stevioside as the principal components	No questions	516
GLG Life Tech Corporation	High-purity rebaudioside C	No questions	536
GLG Life Tech Corporation	High-purity rebaudioside D	No questions	548
Procuctora Alysa SpA	High-purity steviol glycosides (minimum purity 95%) consisting primarily of rebaudioside A	No questions	555
PureCircle, Ltd.	Purified steviol glycosides	No questions	619
Cargill, Inc.	Steviol glycosides produced in *Saccharomyces cerevisiae*	No questions	626
Hunan Huacheng Biotech Inc.	High-purity steviol glycosides (minimum purity 97%) consisting primarily of rebaudioside A	No questions	638
Xinghua GL Stevia Co., Ltd.	Purified steviol glycosides	No questions	702
Shangdong Shengxiangyuan Biotechnology	Purified steviol glycosides	No questions	733
GLG Life Tech Corporation	Steviol glycosides (minimum purity 95%)	No questions	790
Steviana Bioscience (Suzhou) Inc.	Purified steviol glycosides	No questions	795
Zhucheng Haotian Pharm Co., Ltd	Purified steviol glycosides from the leaves of *Stevia rebaudiana* (Bertoni)	No questions	983
Steviol Glycosides by Enzymatic Conversion			
Blue California	Rebaudioside M	No questions	667
Blue California	Rebaudioside D	No questions	715
PureCircle Limited	Steviol glycosides consisting primarily of rebaudioside M	No questions	745
Blue California	Rebaudioside E	No questions	823
Blue California	Rebaudioside I	No questions	911
Blue California	Rebaudioside B	No questions	968
Manus Bio, Inc.	Rebaudioside M obtained by enzymatic treatment of steviol glycosides purified from the leaves of *Stevia rebaudiana* (Bertoni)	No questions	1010
Sichuan Ingia Biosynthetic Co., Ltd.	Rebaudioside D	No questions	764
Tate & Lyle	Rebaudioside M	No questions	780
Sichuan Ingia Biosynthetic Co., Ltd.	Rebaudioside M	No questions	799

Table C.4.2-1 Summary of GRAS Notices Submitted to the United States Food and Drug Administration for Steviol Glycosides (Current as of December 2023)

Company	Substance	FDA Response	GRAS Notice No.
Manus Bio, Inc.	Rebaudioside I obtained by enzymatic treatment of steviol glycosides purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside I)	No questions	1106
Tate & Lyle	Enzyme-modified steviol glycosides	No questions	1140
Sichuan Ingia Biosynthetic Co., Ltd	Rebaudioside I obtained by enzymatic treatment of steviol glycosides purified from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni (rebaudioside I)	No questions	1178
Steviol Glycosides by Fermentation			
DSM Nutritional Products, LLC	Rebaudioside A from *Yarrowia lipolytica*	No questions	632
PureCircle Limited	Steviol glycosides consisting primarily of rebaudioside M	No questions	744
DSM Food Specialties/DSM Nutritional Products North America	Steviol glycosides consisting primarily of rebaudioside M produced in *Yarrowia lipolytica*	No questions	759
Amyris, Inc.	Rebaudioside M	No questions	812
Cargill, Inc.	Rebaudioside M	FDA ceased to evaluate at the notifier's request	867
Cargill, Inc.	Rebaudioside M	No questions	882
Glucosylated Steviol Glycosides			
NOW Foods	Enzyme-modified steviol glycoside preparation (EMSGP)	No questions	337
Toyo Sugar Refining Co., Ltd. and Nippon Paper Chemicals Co., Ltd.	Enzyme-modified steviol glycosides	No questions	375
Daepyung Co., Ltd.	Enzyme-modified steviol glycosides	No questions	448
Daepyung Co., Ltd.	Enzyme-modified steviol glycosides	No questions	452
PureCircle, Ltd.	Glucosylated steviol glycosides (minimum purity 80%)	No questions	607
GLG Life Tech Corporation	Enzyme-modified steviol glycosides	No questions	656
PureCircle USA	Glucosylated steviol glycosides (minimum purity 95%)	No questions	662
Haigen-BGG Natural Ingredients Limited	Glucosylated steviol glycosides	No questions	821
Jiang Su Svetia Biotechnology Co., Ltd.	Purified steviol glycosides	No questions	838
Sinochem Health Company Ltd.	Purified steviol glycosides	No questions	839
GLG Life Tech Corporation	Rebaudioside M	No questions	846
Qufu Shengren Pharmaceutical Co., Ltd	Glucosylated steviol glycosides	No questions	858
Daepyung Co., Ltd.	Glucosylated steviol glycosides	No questions	878
Shandong Shengxiangyuan Biotechnology Co., Ltd	Enzyme-modified steviol glycosides	No questions	970
Zhucheng Haotian Pharm Co., Ltd	Enzyme-modified steviol glycosides	No questions	999

FDA = Food and Drug Administration; GRAS = Generally Recognized as Safe.

C.4.3 Health Canada

Canada broadened the definition of steviol glycosides to include rebaudioside M, indicating a shared metabolic process among various steviol glycosides leading to hydrolysis into steviol, conjugation with glucuronic acid, and eventual elimination through urine in humans. Subsequently, in 2017, Health Canada extended the definition to encompass all steviol glycosides in the *S. rebaudiana* Bertoni plant. Safety assessments conducted by Health Canada in both instances concluded that the expanded definitions posed no safety concerns (Health Canada, 2016, 2017). This expansion supports the notion that safety data from 1 specific steviol glycoside can be applied to support the safety of others.

C.4.4 European Food Safety Authority (EFSA)

In a recent evaluation regarding a proposed amendment to the specifications of steviol glycosides, EFSA did not agree to expand the definition to include all individual steviol glycosides. The decision was based on uncertainties regarding the rate and extent of the metabolism of different steviol glycosides to steviol (EFSA, 2018a). Similarly, in an assessment of glucosylated steviol glycosides, EFSA concluded that the provided data was insufficient to evaluate their safety due to limited evidence on the complete hydrolysis of these compounds. As a result, metabolic fate data for steviol glycosides could not be applied in a read-across approach (EFSA, 2018b). EFSA has issued positive scientific opinions on various high-purity steviol glycoside preparations obtained through enzymatic conversion and/or microbial fermentation of a production organism expressing the biosynthesis pathway genes, indicating that the Agency does not expect these high-purity steviol glycoside mixtures to present any safety concerns (EFSA, 2019, 2021, 2023).

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

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

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

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

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

Each point is addressed in the following sections.

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

The currently approved food uses and use levels for steviol glycosides in Australia and New Zealand, as described under Schedule 15, are presented in Table D.1-1 below (FSANZ, 2021c). Sichuan Ingia intends to market RM95 for use as an intense sweetener under the same conditions of use as those presently authorised for steviol glycosides.

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

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

GMP = Good Manufacturing Practice.

D.2 Exposure Data

Rebaudioside M is proposed for use as a sweetener under the same conditions as currently authorised for steviol glycosides in Australia and New Zealand. Since rebaudioside M is intended to directly replace other steviol glycosides, the intake levels are expected to be the same as those already present in the market. Therefore, a separate intake assessment for rebaudioside M was not conducted for this application. It is important to note that use levels for steviol glycosides are expressed as steviol equivalents, not specified for any particular steviol glycoside. Instead, the levels are based on the total content of the aglycone, steviol, in the final food product resulting from the addition of any steviol glycoside meeting the appropriate specifications.

D.3 Use of the Food Additive in Other Countries

In the U.S., Sichuan Ingia's rebaudioside M produced *via* enzymatic bioconversion has GRAS status for use as a tabletop sweetener and a general purpose non-nutritive sweetener in foods (GRN 799 – U.S. FDA, 2018). GRN 799 was filed with the U.S. FDA on the same substance, rebaudioside M produced *via* enzymatic bioconversion, which is the subject of this application.

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