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

EXECUTIVE SUMMARY

PREPARED BY:
Sichuan Ingia Biosynthetic Co., Ltd.
2-111, No. 368 Tianfu 2nd Street, Hi-Tech Zone
Chengdu, Sichuan, China
610041

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EXECUTIVE SUMMARY

Sichuan Ingia Biosynthetic Co., Ltd. (hereinafter "Sichuan Ingia") is submitting an application to Food Standards Australia New Zealand (FSANZ) for an amendment to the *Food Standards Australia New Zealand Food Code* ("the Code"), to include a high-purity rebaudioside M ingredient produced using enzymatic modification technology. This production process may also be referred to as "enzyme modification" or "bioconversion", referring to the process of obtaining a steviol glycoside preparation 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 process that produces high-purity rebaudioside M utilising sucrose synthase and 2 uridine-5'-diphosphate (UDP) glucosyltransferase enzymes 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"). This manufacturing process is consisten with that of other enzymatic bioconversion processes used to produce steviol glycosides, which are described in Annex 3 for enzyme-modified steviol glycosides by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and already permitted for use in a range of conventional food and beverage products in Australia and New Zealand under Schedule 15. Sichuan Ingia's RM95 product, when manufactured as described, meets or exceeds the ≥95% steviol glycoside purity criteria established by JECFA and the *Food Chemicals Codex* (FCC); however, to permit use of Sichuan Ingia's ingredient, Schedule 3 of the *Australia New Zealand Food Standards Code* ("the Code") for "*steviol glycosides produced by enzymatic conversion*" (S3—35), should be modified to include rebaudioside M produced by enzymatic conversion using the protein engineered enzymes utilised by Sichuan Ingia.

Therefore, the applicant 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. Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni and steviol glycosides obtained *via* enzymatic modification are already permitted for use in Australia and New Zealand for the replacement of sucrose in reduced-calorie or no-sugar-added products. In comparison to already permissible steviol glycoside preparations (such as steviosides and rebaudioside A), RM95 has more favourable sensory characteristics, and has a taste profile that is more reflective of sucrose. The availability and use of RM95 as an alternative steviol glycoside ingredient on the Australia and New Zealand marketplace is expected to promote healthy market competition, which will ultimately benefit Australian and New Zealand consumers. Notably, this application is not intended to change the purity specification (≥95% steviol glycosides) as it is currently defined in the Code, nor is it to propose an extension for the use of rebaudioside M in additional food products or increase the permitted quantities of rebaudioside M in specified food products.

The RM95 ingredient 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 sucrose synthase and 2 UDP-glucosyltransferase enzymes. The production strain is cultured for 5 to 6 hours and fermented with an induction agent for 20 hours. Cells are then harvested through filtration and transferred to a reaction tank where purified rebaudioside A is added slowly. After the reaction period, the crude solution containing the rebaudioside M is heated to deactivate any residual enzymes and to kill any remaining cells of the production strain and is subjected to a series of purification and concentration steps. The final rebaudioside M product is a high-purity ingredient that contains no less than 95% rebaudioside M and no less than 95% total steviol glycosides.

Product specifications for RM95 produced *via* enzymatic conversion of rebaudioside A have been established to align with the specifications included in Schedule 3 – *Identity and purity* of the Code for "*steviol glycosides produced by enzymatic conversion*" (S3—35) and to comply with the most recent purity requirements for enzyme-modified steviol glycosides established by JECFA. Results from batch analysis of 5 non-consecutive lots of RM95 demonstrate that the applicant's manufacturing process produces a consistent product that meets the defined specifications. In addition to chemical and microbiological analyses, non-consecutive lots of the final RM95 have been analysed and demonstrated to be absent of residual DNA from the production

strain (including DNA inserts encoding for the enzymes utilised in the bioconversion, ampicillin resistance genes and *E. coli* host DNA), residual protein, and residual ampicillin and isopropyl β-D-1-thiogalactopyranoside from the seed inoculation and fermentation media used during the production of RM95.

The safety of steviol glycosides is well-established through numerous risk assessment and safety evaluations conducted by scientific bodies and regulatory agencies, including the United States (U.S.) Food and Drug Administration (FDA), Health Canada, FSANZ, the European Food Safety Authority (EFSA), and JECFA. Within these risk assessments and safety evaluations, it is generally recognised that steviol glycosides, including individually specified glycosides, share a common metabolic fate. In brief, these substances are hydrolysed to steviol in the large intestine by the gut microbiome, where it is then absorbed and conjugated with glucuronic acid to form steviol glucuronide for excretion in the urine. In 2016, FSANZ reviewed the safety of steviol glycosides following an application to expand the definition of steviol glycosides to include all steviol glycosides present in the *S. rebaudiana* Bertoni leaf. More recently, FSANZ received multiple applications on a rebaudioside M ingredient from *Saccharomyces cerevisiae* and a steviol glycoside mixture produced by a genetically modified strain of *Yarrowia lipolytica* expressing steviol glycoside biosynthesis genes. Within each of these reviews, the safety of rebaudioside M, and steviol glycosides in general, were established by FSANZ. Therefore, for this application, a comprehensive and detailed search of the published scientific literature was conducted from September 2021 to September 2024 to identify any recent scientific publications relevant to the safety of steviol glycosides and rebaudioside M. Results from the studies identified in the literature search did not change the overall conclusion of safety that has been established for rebaudioside M and steviol glycoside preparations.

Since RM95 is produced *via* enzymatic bioconversion of high-purity rebaudioside A using a strain of *E. coli* BL21 (DE3) that has been genetically modified, the safety of the *E. coli* BL21 (DE3) was also considered in this application. *E. coli* BL21 (DE3) is a non-pathogenic and non-toxigenic microorganism that is widely used for the heterologous and homologous recombinant proteins and has an extensive history of use in universities, research organisms, and industry laboratories. No safety concerns have been raised with its use in the production of food ingredients with respect to pathogenicity and 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. Furthermore, a series of bioinformatic searches were conducted to evaluate the potential allergenicity of sucrose synthase and two UDP-glucosyltransferases; the proteins encoded by genes required for RM95 production. No evidence of allergenicity was identified in any of the sequence alignment searches.

Sichuan Ingia's RM95 is proposed for use as a sweetener under the same conditions as currently authorised for steviol glycosides in Australia and New Zealand. As such, the use of RM95 produced *via* enzymatic conversion of rebaudioside A is expected to be fully substitutional to the other steviol glycosides currently marketed in Australia and New Zealand, and the anticipated intakes of this ingredient are unlikely to change from the current levels in the result of a successful application. A separate intake assessment for RM95 produced *via* enzymatic conversion of rebaudioside A therefore was not performed for this application.

The totality of evidence provided in this application supports the safe use of RM95 produced *via* enzymatic conversion of rebaudioside A as a high-intensity sweetener in Australia and New Zealand for the replacement of sucrose in reduced-calorie or no-sugar-added products. RM95 is a high-purity product containing no less than 95% rebaudioside M and no less than 95% total steviol glycosides. It is generally recognised that steviol glycosides, including individually specified glycosides, share a common metabolic fate. Therefore, the extensive safety database that exists for steviol glycosides can be applied to establish the safety of RM95 produced *via* enzymatic conversion of rebaudioside A. The weight of the scientific evidence presented in the accompanying application indicates that consumption of RM95 produced *via* enzymatic conversion of rebaudioside A, through its use as a high-intensity sweetener for the replacement

of sucrose in reduced-calorie or no-sugar-added products, does not present a significant risk to human health and is safe for the intended use.