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

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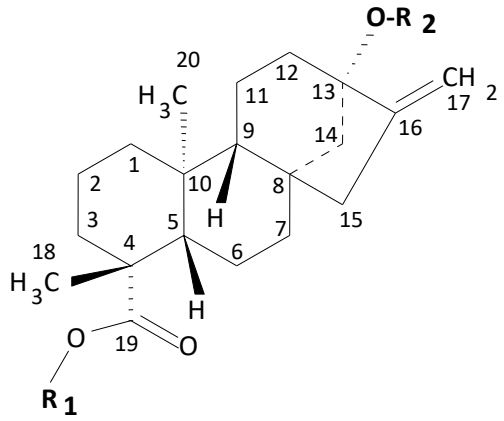
Application to Amend the Specifications for Steviol Glycosides, Under Australia and New Zealand Food Standards Code – Standard 1.3.1 – Food Additives, to Include a New Manufacturing Method for Selected Steviol Glycosides from Stevia Leaf Extracts with Highly Purified Stevioside and Rebaudioside

INTRODUCTION

Steviol glycosides are natural constituents of the *Stevia rebaudiana (S. rebaudiana)* Bertoni plant and more than 50 different steviol glycosides have been identified in the extracts obtained from the leaves of this plant. Although each steviol glycoside generally has its own unique taste profile and sweetness intensity (*i.e.,* some are up to 350 times sweeter than sugar), the molecular structures of all steviol glycosides are similar, consisting of a common steviol backbone linked to different sugar moieties (glucose, rhamnose, xylose, fructose, deoxyglucose, arabinose, galactose, and/or other sugar moieties) (Figure 1). Despite these differences in the type and number of sugar conjugates at R_1 and R_2, all steviol glycosides share a common metabolic pathway and are ultimately hydrolysed by the gut microflora in the intestine to steviol. Steviol is absorbed into the bloodstream and conjugated with glucuronic acid to form steviol glucuronide, so that it may be excreted from the body.

Figure 1 Backbone Structure for Steviol Glycosides



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

Steviol glycosides are currently approved for use as a food additive by Food Standards Australia New Zealand (FSANZ) under *Part 1.3 – Substances Added to or Present in Food* of the Australia New Zealand Food Standards Code (*The Code*). Under *The Code*, steviol glycosides are permitted for use as intense sweeteners and are considered safe for inclusion in food provided they are used at levels at or below that outlined in *Schedule 15 – Substances that may be used as Food Additives* (FSANZ, 2016[1]). Currently as listed in *Schedule 3 – Identity and Purity* of *The Code*, "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (S3-35) must be obtained from leaves of the *S. rebaudiana* Bertoni plant through (a) extraction with hot water; or (b) enzymatic conversion of purified stevia leaf extract to produce rebaudioside M (reb M) using uridine diphosphate (UDP)-glucosyltransferase and sucrose synthase sourced from strains of *Pichia pastoris*.

Due to recent advances in biotechnology, alternative manufacturing methods have been developed to yield higher amounts of 'minor' steviol glycosides that are present in the leaves of *S. rebaudiana* Bertoni (*i.e.,* reb M, D, and AM), which have been shown to have preferential taste characteristics. PureCircle Limited (PureCircle) has developed a novel enzymatic conversion process to produce high purity steviol glycoside preparations from stevia leaf extracts that are comprised primarily of 'minor' glycosides. Specifically, enzymes derived from genetically modified strains of *Escherichia coli* (*E. coli*) K-12[2], namely UDP-glucosyltransferases (EC 2.4.1.17) and sucrose synthase (EC 2.4.1.13), are utilised to convert highly purified steviol glycosides rebaudioside A (reb A) (>95%) and/or stevioside (>95%) extracted from the leaves of *S. rebaudiana* Bertoni to reb M and rebaudioside D (reb D) and/or to rebaudioside AM (reb AM; an isomer of reb D), respectively. The final purified products contain ≥95% steviol glycosides and meet the current purity requirements in Australia and New Zealand for steviol glycosides and the definition established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). PureCircle is therefore seeking to amend *Standard 1.3.1* and related Schedules for steviol glycosides (*i.e.,* S3-35) to allow for the inclusion of this enzymatic conversion manufacturing process as an alternate method to produce high purity steviol glycoside preparations. Consistent with the use of already permitted steviol glycoside preparations, selected steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are intended for use as natural, low-calorie, high-intensity sweeteners that offer numerous technological advantages and benefits to consumers and are suitable for use by individuals with diabetes, as well as others who follow a low-glycaemic diet. However, in comparison to existing steviol glycoside preparations containing only major steviol glycosides, steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract provide improved flavour and taste characteristics in various foods.

TECHNOLOGICAL INFORMATION

Steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are produced in accordance with current Good Manufacturing Practices (cGMP) and meet appropriate food-grade specifications. In stage 1 of the manufacturing process reb A and/or stevioside are extracted from the leaves of *S. rebaudiana* Bertoni and purified to no less than 95% purity, consistent with the production process already defined for recognised steviol glycosides. In the second stage, the *E. coli* K-12 production strains carrying the expression vectors for the corresponding enzymes are fermented to produce the required UDP-glucosyltransferases and sucrose synthase. The enzymes are purified and then utilized in stage 3 of the manufacturing process to convert the purified reb A and/or stevioside obtained in stage 1 to intermediate glycosides reb D and/or reb E, and the primary final glycosides reb M and/or reb AM, respectively. Depending on the length of the enzymatic reaction, preparations with different distributions of individual steviol glycosides can be obtained. In

[1] FSANZ (2016). *Food Standards Australia New Zealand Application Handbook. 1 March 2016*. Canberra, Australia / Wellington, NZ: Food Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/changes/pages/applicationshandbook.aspx.

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

the last stage of manufacturing, the steviol glycoside mixture is purified according to the methodologies for the manufacture of steviol glycosides as described in the Chemical and Technical Assessment (CTA) published by FAO/JECFA (FAO, 2016[3]). The final purified steviol glycoside products produced by enzymatic conversion contain ≥95% total steviol glycosides, determined primarily as the sum of reb A, D, and M and/or stevioside and reb AM, which is consistent with the purity criteria for steviol glycosides established by JECFA and the current purity requirements for "Steviol glycosides from *Stevia rebaudiana* Bertoni" (S3-35) as listed in *Schedule 3* of *The Code*.

Compositional data was provided by PureCircle showing the consistent distribution of individual steviol glycosides in 3 example commercial preparations of steviol glycosides produced by enzymatic conversion of highly purified reb A or stevioside from stevia leaf extract (3 non-consecutive lots of each commercial product). Likewise, analyses of these same batches demonstrate that the manufacturing process produces a consistent product that conforms to the established purity specifications for steviol glycosides in Australia and New Zealand. Furthermore, no residual protein, DNA, or *E. coli* was detected in the final product, supporting that steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are high purity preparations that do not contain impurities arising from the production process.

BIOLOGICAL AND TOXICOLOGICAL DATA

The safety of steviol glycosides has been thoroughly investigated by several advisory scientific bodies and regulatory agencies, including FSANZ, and it has been recognised that steviol glycosides as a group of substances share a similar backbone structure, and that these substances undergo a common metabolic pathway following ingestion (*i.e.*, removal of the sugar units resulting in the common metabolite steviol that is absorbed, conjugated with glucuronic acid, and excreted from the body). Where an acceptable daily intake for steviol glycosides has been established by a scientific or regulatory body, including FSANZ, it has been set at 0 to 4 mg/kg body weight.

New data was provided in the dossier that clearly demonstrates that steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are metabolised to steviol by human faecal homogenates (*i.e.,* gut microflora) under anaerobic conditions at similar rates as steviol glycosides extracted from *S. rebaudiana* Bertoni. Existing data on the *in vitro* hydrolysis of steviol glycosides were also re-assessed and summarised to allow for improved comparison of the rates of hydrolysis of individual steviol glycosides extracted from *S. rebaudiana* Bertoni (*e.g.,* reb A, B, C, D, E, F, and M, steviolbioside, and dulcoside A) (Purkayastha *et al.,* 2016[4]). Results of these studies provide confirmation that in the presence of human faecal homogenates, all steviol glycosides are metabolised to steviol at similar hydrolysis rates, irrespective of the identity of the individual glycoside or the manufacturing process employed. This indicates that the type and number of sugar units attached to the steviol backbone does not significantly impact the time it takes for the intestinal microflora to metabolise the glycosides to steviol. Thus, the safety database that has been established for individual steviol glycosides (*e.g.,* stevioside, reb A, and reb D) can be extrapolated to support the safe use of purified steviol glycosides in general, including steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract.

[3] FAO (2016). Steviol glycosides. In: *82nd JECFA - Chemical and Technical Assessment (CTA)* [82nd meeting held June 7-16, 2016]. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives Meeting (JECFA). Available at: http://www.fao.org/3/a-br566e.pdf.

[4] Purkayastha S, Markosyan A, Prakash I, Bhusari S, Pugh G Jr, Lynch B and Roberts A, 2016. Steviol glycosides in purified stevia leaf extract sharing the same metabolic fate. Regulatory Toxicology and Pharmacology: RTP, 77, 125-133.

In addition to the studies related to the metabolic fate of steviol glycosides, a few other genotoxicity, reproductive and developmental toxicity, and special investigative studies (*e.g.*, related to glucose/insulin homeostasis) have become available since FSANZ's most recent evaluation of steviol glycosides (A1157 – FSANZ, 2018[5]). The results of these new studies do not raise any additional questions regarding the established safety conclusions related to steviol glycosides.

DIETARY EXPOSURE

Steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract are intended for use as a high-intensity sweetener in foods and beverages under the same conditions of use as presently authorised for steviol glycosides, and either in place of existing steviol glycoside preparations already in the Australia/New Zealand marketplace or in combination with such preparations, up to but not greater than the maximum permitted use-levels (as steviol equivalents). As such, intakes of steviol glycosides produced by enzymatic conversion (as steviol equivalents) will be the same as for steviol glycosides already available in the Australia and New Zealand marketplace and a separate intake assessment was not performed for the purpose of this food additive amendment.

CONCLUSION

Overall, steviol glycosides as a group share the same structural backbone, steviol, and are differentiated only on the basis of the type and number of sugar units attached to the steviol backbone. Given the similar metabolic fate of steviol glycosides produced *via* enzymatic conversion with steviol glycosides extracted from *S. rebaudiana* Bertoni, the safety database that has been established for individual steviol glycosides from *S. rebaudiana* Bertoni (*e.g.,* stevioside, reb A, and reb D) which has been the basis for supporting steviol glycosides in general, can be extrapolated to support the safe use of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract. Consequently, the use of steviol glycosides produced by enzymatic conversion of highly purified reb A and/or stevioside from stevia leaf extract in foods and beverages for human consumption at the use-levels presently permitted in Australia and New Zealand for steviol glycosides would not present a significant risk to human health and is safe. Furthermore, the use of steviol glycosides produced by enzymatic conversion would provide technological benefits that cannot be achieved with the use of currently available steviol glycoside preparations. Therefore, a modification of the current specification for steviol glycosides (S3-35) in *The Code* is requested to modify the definition so that steviol glycosides produced *via* enzymatic conversion of purified stevia leaf extracts may be included under the same specification.

[5] FSANZ (2018). *Approval report – Application A1157: Enzymatic production of Rebaudioside M*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ), Australian Government. Available at: http://www.foodstandards.gov.au/code/applications/Documents/A1157%20Approval%20report.pdf.