FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

13 November 2018

[63-18]

Supporting document 1

Risk and technical assessment – Application A1157

Enzymatic production of Rebaudioside M

Executive summary

Blue California's application seeks permission in the Australia New Zealand Food Standards Code (the Code) for a Rebaudioside M (Reb M) from a novel production method. The traditional method of production uses hot water extraction of the *Stevia rebaudiana* Bertoni leaf, followed by purification and recrystallization using methanol or ethanol. Conversely, Blue California uses an enzymatic process to manufacture Reb M using enzymes sourced from genetically modified strains of *Pichia pastoris*.

Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni, including Reb M, are already permitted for use as a food additive in the Code, with maximum permitted levels (MPLs) in a variety of food categories and at GMP levels in tabletop sweeteners in Schedule 15. Reb M exhibits preferential sensory characteristics when compared to the major glycosides, being more reflective of sugar. Hence it is a useful food additive in formulations for reduced-calorie or no-sugar-added products, where it replaces sugar.

An acceptable daily intake (ADI) of 0-4 mg/kg bodyweight for steviol glycosides, expressed as steviol, was established by FSANZ in 2008 and JECFA in 2009. This ADI is appropriate for Reb M produced using enzymes from genetically modified *Picha pastoris* as it is chemically the same as Reb M extracted traditionally from *Stevia rebaudiana* Bertoni and would therefore follow the same metabolic pathway in humans. Toxicological and other relevant data published subsequent to FSANZ's previous assessments of steviol glycosides raised no concerns regarding the safety of steviol glycosides and did not indicate a need to amend the ADI.

The Blue California Reb M complies with purity specifications of JECFA. The *P. pastoris* source organism for the enzymes used to produce Reb M has a long history of industrial use, is commonly used for recombinant gene expression and is not toxigenic. No major allergens are used to culture the yeast or at any other stage of the production process and sufficient information was provided concerning potential homology between the novel enzymes and known allergens for FSANZ to conclude there is no concern.

In conclusion, FSANZ's hazard assessment has not identified any safety concerns associated with Blue California's Reb M produced using enzymes from genetically modified *P. pastoris*.

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1 Introduction and description of substance

Blue California's application seeks permission in the Australia New Zealand Food Standards Code (the Code) for a Rebaudioside M (Reb M) from a non-traditional production method. The traditional method of extraction uses hot water extraction of the *Stevia rebaudiana* Bertoni leaf, followed by purification and recrystallisation using methanol or ethanol. Conversely, Blue California uses a novel multi-step enzymatic pathway process to manufacture Reb M using the genetically modified enzymes uridine diphosphate - glucosyltransferase (UDP-glucosyltransferase) and sucrose synthase. This process facilitates the transfer of glucose to purified stevia leaf extract via glycosidic bonds. The enzymes utilised are sourced from strains of *Pichia pastoris*.

Schedule 3 of the Code contains specifications for Reb M and for steviol glycoside mixtures containing Reb M in S3—31 and S3—32, respectively, for which the Blue California Reb M complies. Both specifications refer to primary source specifications for steviol glycosides contained within section S3—2, being either S3—2(1)(b) [the FAO JECFA Monograph], S3-2(1)(c) [the Food Chemicals Codex] or S3—2(1)(d) [European Commission Regulation No 231/2012 (EU, 2012) laying down specifications for food additives]. Specifications for steviol glycosides from these primary sources, including Reb M, indicate that the ingredient is extracted from the leaves of *Stevia rebaudiana* Bertoni. S3—35 contains a specification for steviol glycosides extracted using the traditional hot water extraction method, but not for any other method. As such, the Reb M for which this application is being made by Blue California does not comply with specifications in S3—35.

Blue California are not requesting a change to the purity specification (≥95% steviol glycosides) or proposing an extension for the use of Reb M in additional food products nor do they propose to increase the permitted quantities of Reb M in permitted food products.

2 Food technology assessment

2.1 Identity and chemical properties

Blue California's Reb M is produced by enzymatic conversion of purified stevia leaf extract. The final product is a high purity preparation containing no less than 95% Reb M, which is a minor, naturally occurring, steviol glycoside that is present in the leaves of *S. rebaudiana* Bertoni. Blue California has established product specifications for Reb M that are consistent with the specifications in schedule 3 of The Code for "Reb M" in S3—31 and S3—32 if included in a steviol glycoside mixture.

Chemical name: 13-[(O-β-D-Glucopyranosyl-(1-2)-O-[β-D-glucosylpyranosyl-(1-3)]-β-Dglucosylpyranosyl) oxy]-kaur-16-en-18-oic acid (4-)-O-β-D-glucosylpyranosyl-(1-2)-O-[β-Dglucosylpyranosyl-(1-3)]-β-D-glycosylpyranosyl ester.

Common name: Reb M

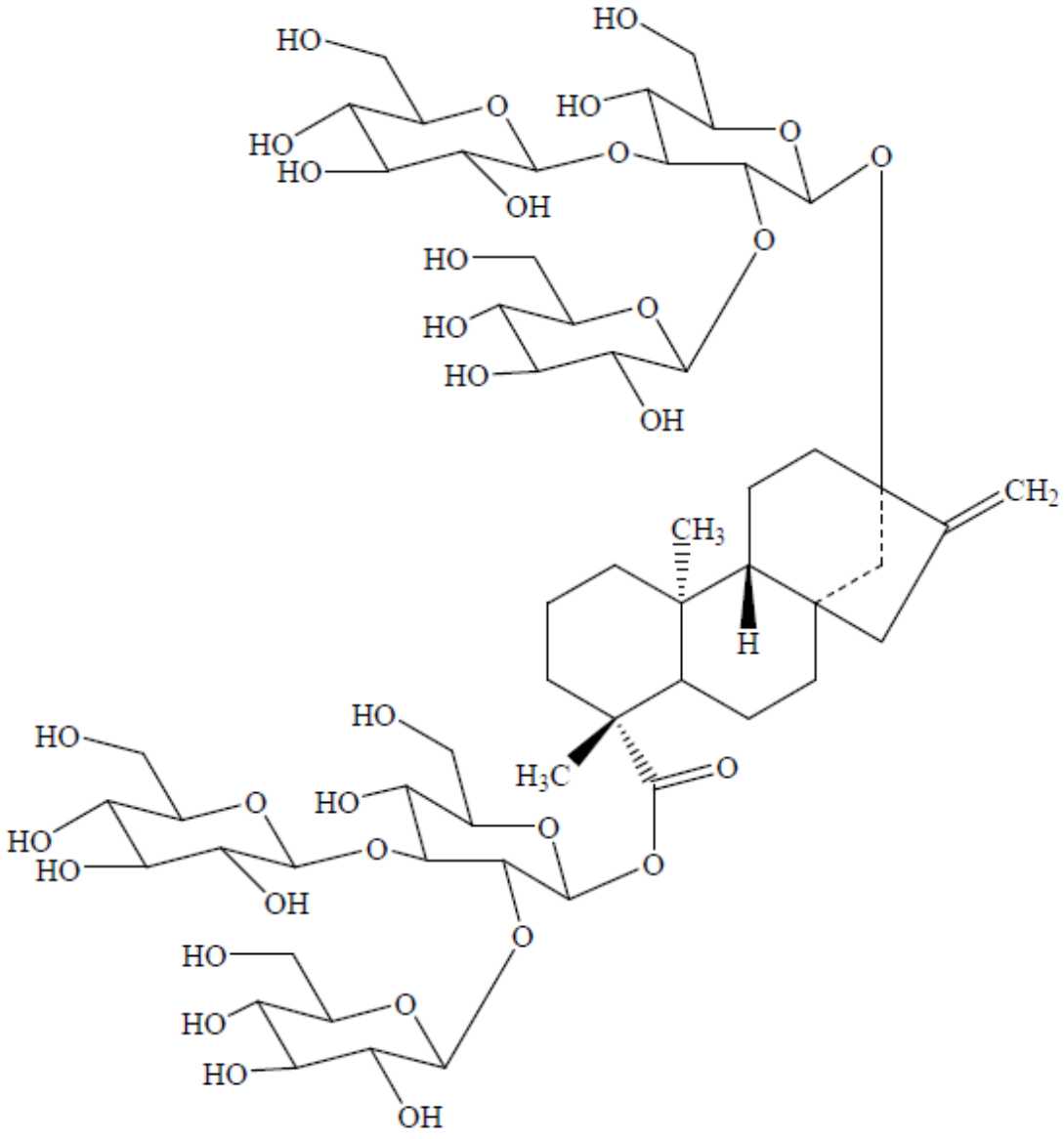
Synonyms: Reb M, Rebaudioside X, Reb X

Chemical formula: C56H90O33

Molecular weight: 1291.29 Daltons

CAS Number: 1220616-44-3

Figure 1 Chemical Structure of Reb M



2.2 Information on the Physical and Chemical Properties of Reb M

Blue California's Reb M is a white powder that is freely soluble in water with a slight characteristic odour, consistent with Reb M extracted from the leaves of *S. rebaudiana* Bertoni. 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 diterpene1. 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, 2016).

2.3 Information on the Identity of the Enzymes

Three enzymes are used as processing aids in the conversion of the stevia leaf extract to Reb M. These enzymes have been protein engineered to contain a UDP-glucosyltransferase and a sucrose synthase component. The three enzymes are produced from two strains *of P. pastoris,* Yeast A and Yeast B. Yeast A expresses the enzyme UGT-A and Yeast B expresses the two enzymes, UGT-B1 and UGT-B2.

UDP-glucosyltransferase

Source (strain):	*Pichia pastoris* containing DNA sequences encoding UDP-glucosyltransferase and sucrose synthase enzymes
Common:	Glucosyltransferase
EC Number:	2.4.1.17
Systematic Name:	UDP-glucose β-D-glucosyltransferase
CAS Number:	9030-08-4

Sucrose Synthase

Source (strain):	*Pichia pastoris* containing DNA sequences encoding UDP-glucosyltransferase and sucrose synthase enzymes
Common:	Sucrose synthase
EC Number:	2.4.1.13
Systematic Name:	NDP-glucose:D-fructose 2-α-Dglucosyltransferase
CAS Number:	9030-05-1

2.4 Specifics of the enzyme reaction

UDP-glycosyltransferases are involved in the transfer of a sugar residue from an activated donor molecule (*e.g.,* UDP-glucose) to an acceptor molecule (Richman *et al.,* 2005). Steviol glycoside synthesis in the *S. rebaudiana* Bertoni plant involves successive glucosylation steps starting with steviol to form steviolmonoside, followed by steviobioside, and then stevioside. Specifically, UDP-glucosyltransferase UGT76G1 catalyses the reaction of stevioside to form Reb A by glucosylation at the C-4 carboxyl group (Richman *et al.,* 2005; Humphrey *et al.,* 2006). In this reaction, the activated sugar donor (UDP-glucose) and

[1] biosynthetic intermediates used in the production of specific plant hormones.

fructose are formed from UDP and sucrose, the reaction of which is catalysed by sucrose synthase (Humphrey *et al.,* 2006). The reaction mechanism of UDP-glucosyltransferase and sucrose synthase to form Reb A from stevioside is shown in Figure 2 below, taken from the Application. These coupled activities of UDP-glucosyltransferase and sucrose synthase were adapted by Blue California to the efficiently produce Reb M from stevia extract (Mao *et al*., 2016a, b).

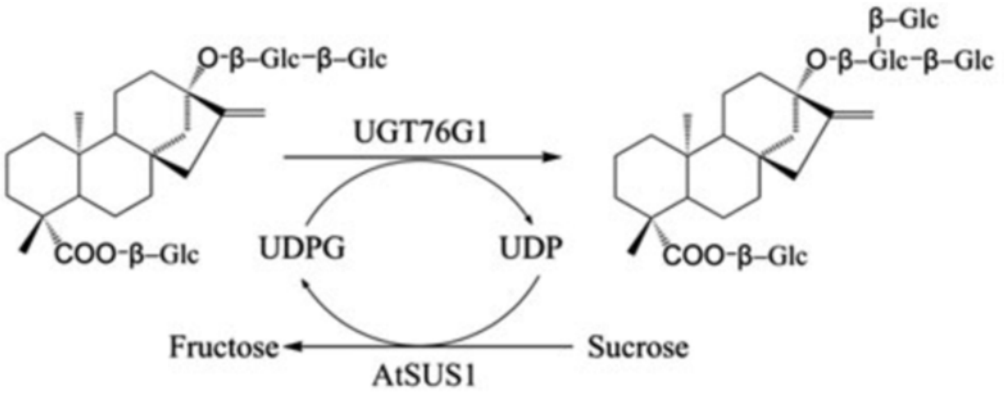


Figure 2 Mechanism of Formation of Reb A from Stevioside with UDP-Glucosyltransferase and Sucrose Synthase

2.5 Technological purpose

Steviol glycosides extracted from the leaves of *S. rebaudiana* Bertoni, including Reb M, are already permitted for use as food additives in the Code, with the International Numbering System assignation 960. The technological purpose of steviol glycosides as a food additive is that of an intense sweetener which replaces the sweetness normally provided by sugars in food, without contributing significantly to their available energy. Hence it is valuable for use in foods such as reduced-energy or no-added sugar products. Steviol glycosides are permitted at MPL's in a variety of food categories and at GMP level for tabletop sweeteners in Schedule 15. The technological purpose of this particular Reb M from Blue California does not differ from currently permitted steviol glycosides, rather it is the method of manufacture that differs.

2.6 Technological justification

The primary reason for developing alternative methods to the traditional extraction methods for steviol glycosides is that not all glycosides are naturally produced to the same degree in the leaves of *S. rebaudiana* Bertoni. For example, stevioside is a major glycoside present in the leaves of the plant, constituting about 5 to 10% in dry leaves (JECFA, 1999), whereas Reb M is a minor glycoside that is present at much lower levels. Some of the minor glycosides, such as Reb M, have more favourable sensory characteristics when compared to the major glycosides (e.g. stevioside, Reb A) and have taste profiles that are more reflective of sucrose. Hence the development of the new technology to produce a glycoside with preferential sensory characteristics for product development.

The sweetness equivalency to sucrose of Blue California's Reb M produced via enzymatic conversion of purified stevia leaf extract was determined to be 200 times sweeter than sucrose upon evaluation by a sensory panel. The full study report was provided with the application.

2.7 Manufacturing process

Blue California uses a novel multi-step enzymatic pathway process to manufacture its high purity Reb M (≥95%). This uses enzymes to facilitate the transfer of glucose to purified stevia leaf extract via glycosidic bonds.

The manufacturing process is divided into 2 stages, see figure 3:

Stage 1. Enzyme production
Two strains of *P. pastoris* undergo fermentation to generate three enzymes, UGT-A, UGT-B1 and UGT-B2. Following the fermentation step, the enzymes are isolated from the source microorganisms.

Stage 2. Reb M production
The enzymes are mixed with stevia extract (≥95% steviol glycosides), extracted from the leaves of *S. rebaudiana* Bertoni to generate Reb M.

The resulting Reb M undergoes a series of purification and isolation steps to generate the final high-purity Reb M (≥95%),

Blue California's Reb M is currently manufactured in China and will not be manufactured in Australia or New Zealand. All materials and processing aids utilised in its manufacture are food-grade and comply with relevant Food Chemical Codex or other internationally-recognised standards. The enzymes are produced by strains of *P. pastoris*. Blue California's Reb M is manufactured in compliance with Good Manufacturing Practice (GMP).

Further details on the manufacturing process and additional information regarding the source microorganisms, enzymes and other processing aids and raw materials can be found on pages 17 – 19 of the application.

2.8 Manufacturing flow chart

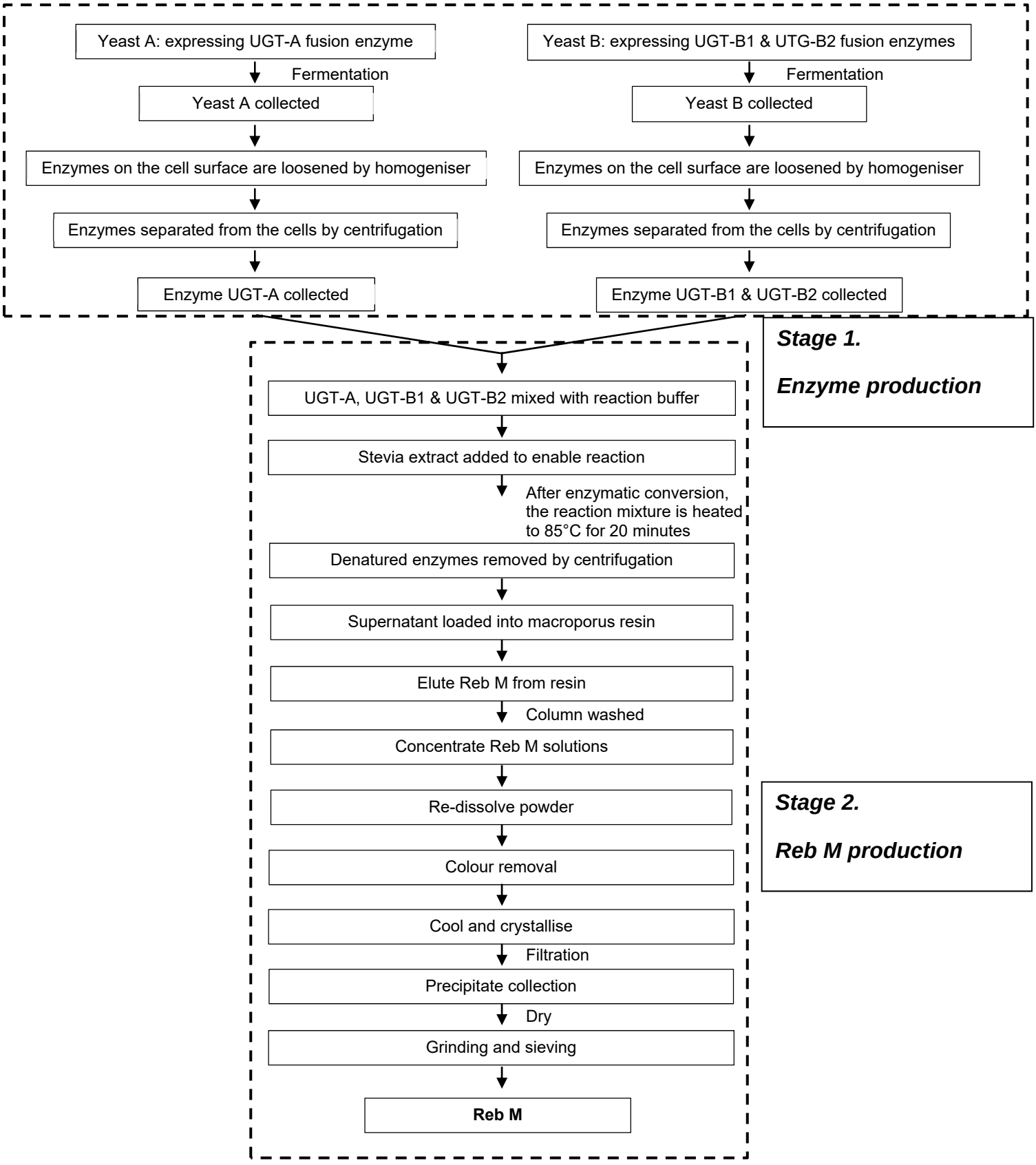


Figure 3 Schematic of the production process of Blue California's Reb M

2.9 Product specification

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (JECFA 2016) and in the Food Chemicals Codex (Food Chemicals Codex 2014). These primary sources of specifications are listed in the table to section S3—2 in schedule 3, Identity and Purity. Enzyme preparations need to meet these enzyme specifications. Schedule 3 also includes specifications for heavy metals (section S3—4) if they are not specified within specifications in sections S3—2 and S3—3.

Blue California's application contains a comprehensive product specification in Table B.6.1-1 on pages 23 – 24 for Reb M produced via enzymatic conversion of purified stevia leaf extract. The product specifications are consistent with the specifications in schedule 3 of the Code for "Reb M" in sections S3—31, S3—32 and S3—35. They also comply with the assay and impurity specifications in the FAO JECFA Monograph 19 for "steviol glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2016b

Table 1 Comparative Reb M Specifications

Analysis	***Specifications***		
	Blue California	**JECFA**	**the Code (S3—35)**
Appearance/Description	White powder	White to light yellow powder	White to light yellow powder
Purity (%)	≥ 95	≥ 95	≥ 95
Solubility	Soluble in water	Freely soluble in water	Freely soluble in water
pH (1% solution)	5.0-7.0	4.5-7.0	4.5-7.0
Total ash (%)	<1	≤1	<1
Loss on drying	≤6	≤6	≤6
Residual ethanol (mg/kg)	<1000	<5000	<5000
Residual methanol (mg/kg)	<200	≤200	≤200
Arsenic (mg/kg)	<0.5	≤1.0	≤1.0
Lead (mg/kg)	<0.5	≤1.0	≤1.0

Certificates of analyses for five non-consecutive batches of Blue California's Reb M were provided with the application to demonstrate compliance with the defined product specifications

2.10 Analytical method for detection

The analytical methods used to confirm that Blue California's Reb M meets the established chemical and microbial specifications and are listed in Table B.6.1-1 on page 23 of the application. The methods are internationally recognised, such as the Association of Official Analytical Chemists [AOAC], U.S. Pharmacopeia [USP], and JECFA. The Reb M content in the final product is quantified according to the JECFA HPLC method for steviol glycosides described in FAO JECFA Monograph 19 for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (JECFA, 2016b). Details of the HPLC method and chromatographic data were provided with the application. These showed that the Blue California Reb M met and exceeded the acceptance criteria for Reb M content, purity and moisture.

2.11 Product Stability

Blue California provided results of a 6-month accelerated stability study conducted on 5 representative batches of their Reb M product, when stored at 40±2°C at a relative humidity of 75±5%. Reb M was observed to be stable over the course of the accelerated stability study, based on appearance, moisture content, and percent Reb M content. The Blue California Reb M has a shelf life of 2 years.

JECFA have concluded that “steviol glycosides, including steviol glycosides extract preparations containing higher levels of new glycosides, are thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage” (JECFA, 2007).

2.12 Food technology conclusion

The food technology assessment concludes that Blue California’s Reb M produced by a novel multi-step enzymatic pathway process meets the specifications currently listed in the Code. Blue California demonstrated that its particular method of production of Reb M produces a consistent product that conforms to these specifications. The Blue California Reb M is of high purity and contains no impurities resulting from its unique manufacturing process. Its technological purpose matches that of currently permitted Reb M preparations produced by the traditional method of hot water extraction.

3.0 Safety assessment

The enzymatic process used to convert leaf-extracted stevia to Reb M involves three enzymes from a genetically modified source. The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of these enzymes, specifically by considering the:

- history of use of the host and gene donor organisms (source microorganisms),
- characterisation of the genetic modification(s), and
- stability of the production strain
- safety of the novel proteins.

3.1 History of use

3.1.1 Host organism

Pichia pastoris

P. pastoris is non-toxigenic (Jiang *et al*. 2012) and has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) for production of enzymes, specifically phytase and fumonisin esterase (EFSA 2017). The yeast has been in commercial use for over 40 years, and has been used successfully for the production of many industrial enzymes and proteins of pharmaceutical relevance (Ahmad *et al*. 2014). In 2010 it was noted that *P. pastoris* is the second most-used host for recombinant gene expression, *Escherichia coli* being the most commonly used (Sørensen 2010).

3.1.2 Gene donor organism(s)

Genes for three unique enzymes were introduced into the *Pichia* production strains. The enzymes included two types of UDP-glucosyltransferase and a sucrose synthase. The donor organisms for the genetic material used to generate the enzymes cannot be identified in this report as it is confidential commercial information (CCI). FSANZ’s assessment confirmed a history of safe use exists for all donor organisms, with no safety concerns being identified.

3.2 Characterisation of the genetic modification

Full details of the genetic modification to the production organism were provided to FSANZ for assessment but cannot be disclosed as they are CCI. A summary of FSANZ's assessment of this information appears below.

3.2.1 Description of the introduced DNA

The applicant has developed three protein engineered enzymes: UGT-A, UGT-B1 and UGT-B2; to be used as processing aids, for the production of RebM. These enzymes were expressed in two different production strains. One production strain expresses UGT-A and the second expresses both UGT-B1 and UGT-B2.

Expression cassettes for transformation of the host strains were constructed using standard laboratory techniques and transformations were performed using standard methods.

3.2.2 Characterisation of the introduced DNA

The genomic DNA from both production strains was characterised with no safety concerns being identified.

3.2.3 Stability of the production organisms and inheritance of the introduced DNA

The production strains and introduced DNA were found to be stable.

3.3 Safety of the novel proteins

A relevant factor to consider in relation to the safety of the novel proteins is whether they will be present in the Reb M product (Figure 3). Reb M is purified after enzymatic conversion and no protein detectable.

3.3.1 History of safe use

The genes that encode the enzymes come from organisms with a long history of safe use.

3.3.2 Bioinformatics analyses of UGT-A, UGT-B1 and UGT-B2

The applicant provided results from *in silico* analyses comparing the amino acid sequences of UGT-A, UGT-B1 and UGT-B2 to known protein toxins and virulence factors identified in the NCBI Protein databases. A BLASTP search (v2.6.1) found homology to enzymes from toxigenic bacteria but the enzymes themselves are not toxins. The sequences for UGT-B1 and UGT-B2 did show limited similarity to an animal venom; however, this was not considered to be biologically significant.

3.4 Safety assessment conclusion

Two yeast production strains have been generated expressing three enzymes. Molecular analyses indicated no safety concerns.

4.0 Hazard Assessment

4.1 Previous FSANZ Assessments

FSANZ assessed steviol glycosides in 2008 (Application A0540) and established an ADI of 0-4 mg/kg bw/day steviol. At that time, ten steviol glycosides were known; stevioside, dulcoside, steviolbioside, rubudioside, and rebaudiosides A, B, C, D, E and F.

FSANZ updated the hazard assessment of steviol glycosides in 2011 (Application A1037) but did not find reason to change the ADI established in 2008.

Reb M was assessed and approved in 2015 (Application A1108). FSANZ expanded the definition of steviol glycosides to include all steviol glycosides found in the leaves of *S. rebaudiana* Bertoni, in February 2017 (Application A1132). The ADI of 0-4 mg/kg bw/day steviol was maintained.

4.2 Objectives of the Assessment

Information on the safety of steviol glycosides and Reb M previously assessed by FSANZ is summarised in this report, with detailed review generally limited to relevant information that postdates the approval dates of A1108 and/or A1132 as applicable. The assessed data on Reb M include information on toxicokinetics and metabolism, genotoxicity, short term toxicity in laboratory animals, and human tolerance studies. The submitted data, together with previous FSANZ assessments and assessment by JECFA (FAO/WHO 2016a) are considered suitable to assess the hazard of Reb M produced using UDP-glucosyltransferase and sucrose synthase from *Pichia pastoris*.

4.4 Characteristics of Reb M manufactured using enzymes from GM *P. pastoris*

Information on the chemical and physical properties of Reb M is presented in the Food Technology Assessment. The applicant's product is ≥95% Reb M, and the product specifications are consistent with the existing specifications in Schedule 3 of the Code for "Reb M" and "steviol glycoside mixtures containing Reb M". They also comply with the assay and purity specifications in FAO JECFA Monograph 19 for "Steviol Glycosides from *Stevia rebaudiana* Bertoni" (FAO/WHO 2016b). The application includes results of pesticide residue analysis of five representative batches of the final Reb M product, none of which shows any pesticide residues.

4.5 Toxicological data

4.5.1 Toxicokinetics and metabolism

The information on toxicokinetics and metabolism provided by the applicant was previously evaluated by FSANZ in A1132. Briefly, all known steviol glycosides are hydrolysed to steviol at similar rates. The number of sugar moieties in the glycosides, and the sugars present in those moieties, do not have any marked effect on the rate of hydrolysis. It is concluded that the steviol glycosides share a common metabolic pathway.

In rats dosed with 40 mg/kg bw stevioside, values for peak plasma concentration (C_{max}), time of peak concentration (T_{max}) and total plasma exposure over time (AUC) for steviol were similar in male and female rats. However in rats dosed with 1000 mg/kg bw, C_{max}, and AUC were significantly higher, and T_{max} later, in females compared to males. Likewise, values for C_{max}, T_{max} and AUC for steviol glucuronide were similar between the sexes when rats were dosed with 40 mg/kg bw stevioside, but higher in females than males in rats dosed with 1000 mg/kg bw.

Toxicokinetics in human subjects are limited to data from males. C_{max} for plasma steviol was similar in rats and humans however, T_{max} occurred later in humans than in rats. In both species, steviol was metabolised by conjugation to steviol glucuronide, but maximum concentrations of steviol glucuronide were approximately 25-fold greater in human subjects than in rats.

4.5.2 Genotoxicity

FSANZ has previously concluded (A1132) that the weight of evidence from experimental studies indicates that steviol glycosides are unlikely to be genotoxic. More recent studies of the genotoxicity of steviol glycosides evaluated as a part of this assessment provide further support that conclusion.

Bacterial reverse mutation assay of rebaudioside A (Rumelhard et al. 2016) Regulatory status: GLP according to OECD Guideline 471

The test article was Reb A, produced under Good Manufacturing Practice (GMP) from a genetically modified strain of the yeast *Yarrowia lipolytica*, and purified to >95%.

The bacterial test strains used were *Salmonella typhimurium* strains TA1535, TA 1537, TA98 and TA100, and *Escherichia coli* strain WP_2 *uvr*A. Experiments were conducted in triplicate, both with and without metabolic activation by S9 mixture, by the direct plate incorporation method. The test article solvent, and negative control article, was dimethyl sulfoxide (DMSO). For experiments conducted without S9, the positive control articles were as follows: sodium azide for TA1535; 2-nitrofluorene for TA98; methyl-methanesulfonate for TA100; acridine mutagen ICR-191 for TA1537 and 4-nitroquinolone N-oxide for WP_2 *uvr*A. For experiments conducted with S9, the positive control was 2-aminoanthracene. With the exception of sodium azide, which was dissolved in water, all positive control articles were dissolved in DMSO.

Test article concentrations were 0, 52, 164, 512, 1600 and 5000 µg/plate in the first experiment and 492, 878, 1568, 2800 and 5000 µg/plate in the second experiment. The concentration of S9 was 5% v/v in the first experiment and 10% v/v in the second experiment. Incubation conditions were not specified. The OECD Guideline recommends incubation at 37°C for 48 to 72 h.

No cytotoxic effects, and no biologically significant increase in the number of revertant colonies were observed at any concentration of Reb A in either the first or second experiment. The positive control articles induced significant increases in numbers of revertant colonies, confirming the validity of the experiments.

It was concluded from these experiments that the test article was not cytotoxic or mutagenic under the conditions of the bacterial reverse mutation assay.

In vitro micronucleus assay of Reb A with human peripheral lymphocytes (Rumelhard et al. 2016). Regulatory status: GLP, according to OECD Guideline 487

This assay was conducted using the same Reb A produced from genetically modified *Yarrowia lipolytica* and dissolved in DMSO, as described above.

Cultured human peripheral lymphocytes, obtained from a healthy male donor, were exposed to the test article for 3 h with S9 mix (1.8% v/v) for metabolic activation, or 3 for 24 h without metabolic activation. Concentrations of test article were 0, 512, 1600 and 5000 µg/mL. All experiments were conducted in duplicate. Incubations at 37°C were conducted in a controlled environment with 80-100% humidity, and 5% CO_2 in air. Mitomycin C was used as a clastogen, at final concentrations of 0.25 µg/mL and 0.15 µg/mL in the 3 and 24 h incubations respectively, while colchicine was used as an aneugen at final concentrations of 0.1 µg/mL and 0.05 µg/mL in the 3 and 24 h incubations respectively, as positive control articles in cultures without S9. In cultures with S9, cyclophosphamide monohydrate was used as the positive control article at a final concentration of 15 µg/mL Cells that were exposed to the test article for 3 h were washed and re-suspended in the presence of cytochalasin B (5 µg/mL) and incubated for a further 24 h, so that cells that had completed once cell division

would be binucleate and readily identified.

At the end of incubation, cells from all incubations were processed and mounted on slides, and the number of micronuclei/1000 cells recorded.

No cytotoxic effects were observed as a results of exposure to Reb A for 3 h, with or without S9 mixture. Exposure to Reb A for 24 h without S9 was associated with a slight (17%) decrease in relative cell growth at the highest concentration of 5000 µg/mL. When compared to negative control cultures, exposure to Reb A under all three experimental conditions was not associated with an increase in micronuclei. In contrast, the positive control articles all induced significant increases in micronuclei, confirming the validity of the assays. It was concluded that Reb A produced by genetically modified *Yarrowia lipolytica* was not clastogenic or aneugenic in the *in vitro* micronucleus assay.

MTT assay and alkaline comet assay of stevioside using human HCT 116 and CCD18Co cell lines (Sharif et al. 2017) Regulatory status: Non-GLP

Stevioside is one of a number of plant-derived chemicals being investigated for prevention and/or treatment of cancer. Ideally, an anticancer agent will have little or no effect on nontarget cells. The purpose of these assays was to assess the cytotoxicity and genotoxicity of stevioside on a model nontarget cell, CCD18Co, a cell line derived from normal human colon myofibroblasts. The cytotoxicity of stevioside to HCT 116, derived from human colorectal cancer, was also investigated. No regulatory guidelines were mentioned. The methods used in this paper are not part of the standard genotoxicity screen used in toxicology, but the paper is considered to be valid supplementary information.

For the MTT assay, both cell lines were cultured in monolayer, seeded in a 96 well plate, and treated with stevioside at concentrations of 0, 12.5, 25, 50, 100 and 200 µM for 24 h. Following incubation, 20 µL of 3-[4,5-dimetilthiazole-2-il]-2,5-difeniltetrazolium bromide (MTT salt) (0.5% w/v) was added to each well and the plate was incubated for a further 4 h. At the end of the incubation the medium was discarded, formazin crystal was dissolved by addition of DMSO, the plate was shaken for 5 minutes to achieve uniformity and absorbance was read at 570 nm.

For the alkaline comet assay cells were seeded in a 6-well plate for 24 h before being treated with 0 or 200 µM stevioside for 24 h. The alkaline comet assay was performed according to the method of Tice et al. (2000) and tail length (% DNA tail) and tail moment (tail length x DNA tail) were quantified using appropriate software.

Stevioside had a statistically significant cytotoxic effect in the MTT assay only at the highest concentration, 200 µM. At this concentration there was 20% and 30% cell death in HCT 110 and CCD18Co cells respectively. The difference between the two cell lines was not statistically significant, and the cytotoxicity at 200 µM stevioside was not considered to be severe.

CCD18Co cells exposed to stevioside did not show any significant changes in tail moment compared to untreated cells. HCT 116 cells exposed to stevioside showed a significant increase in tail moment compared to untreated cells. Rebaudioside had no effect on tail DNA in either type of cell. The authors concluded that stevioside did not show cytotoxic or genotoxic effects in normal cells in this assay.

Chromosomal aberration assay of Stevia in human lymphocytes (Uçar et al. 2018) Regulatory status: Non-GLP

The test article for this non-GLP study was a commercial Stevia extract containing steviol glycosides at a purity of 99%. The test system consisted of lymphocytes harvested from

peripheral venous blood of two healthy adult women and two healthy adult men. All blood donors' non-smokers aged between 25 and 26 years, who had not taken any medications for at least 3 weeks and had not been radiographed for at least 3 months.

Human lymphocytes, cultured at 37°C, were exposed to stevia concentrations of 0, 1, 2, 4, 8 or 16 µg/mL at 24 and 48 hours from culture initiation. A positive control culture was exposed to mitomycin C at 0.20 µg/mL. Cell survival was measured using Trypan Blue dye exclusion, and the viability of the negative control cultures was 98%. After 70 hours of incubation in the presence of the test or positive control article, colchicine was added to all cultures at 0.06 µg/mL. Two hours after addition of colchicine, cells were harvested, fixed, mounted on slides and stained for examination. One hundred metaphases per blood donor, equal to a total of 400 metaphases, were examined for each concentration. The entire experiment was conducted twice.

No significant differences in the frequency of chromosomal aberrations were observed between the cells exposed to the Stevia extract and the negative control cells. A significant increase in the frequency of chromosomal aberrations in the cells exposed to mitomycin C confirmed the validity of the assay.

Micronucleus assay of Stevia in human lymphocytes (Uçar et al. 2018) Regulatory status: Non-GLP

This experiment was conducted with the same test article and human lymphocytes from the same human donors as described above for the chromosomal aberration assay by the same authors. Human lymphocytes were incubated at 37°C for 44 or 72 hours from initiation. Cytokinesis was arrested with cytochalasin B at a final concentration of 5.2 µg/mL. The lymphocytes were exposed to the Stevia extract at concentrations of 0, 1, 2, 4, 8 or 16 µg/mL at for 24 and 48 hours. A positive control culture was exposed to mitomycin C at 0.20 µg/mL. After 72 hours of incubation, cells were harvested, processed, mounted on slides and stained for examination. Micronuclei were scored from 1000 binucleate cells per donor, equal to 4000 binucleate cells per concentration. The entire experiment was conducted twice.

Treatment with Stevia extract did not have a significant effect on the frequency of micronuclei, when compared to the negative control cultures. The positive control article, mitomycin C, induced the expected significant increase in the frequency of micronuclei.

4.5.3 Short term studies in animals

Ninety day dietary study of Reb A in Sprague-Dawley rats (Rumelhard et al. 2016) Regulatory status: GLP, in compliance with US FDA and OECD 408 guidelines.

This study was conducted using the Reb A, produced from a genetically modified strain *Yarrowia lipolytica* and purified to >95%, used for the genotoxicity assays. Stability of the test article in the diet for up to 10 days was confirmed pre-study, and fresh diet was prepared weekly. Homogeneity and dose analysis of the test article in the diet were confirmed during the study.

Rats were aged 5-6 weeks at time of receipt. They were housed 4/cage by sex under standard laboratory conditions of temperature, humidity, air changes and light/dark cycle. Water and food were provided *ad libitum*, except during scheduled fasts such as prior to blood collection, and activities such as neurotoxicity assessments. After acclimation for 2 weeks, rats were assigned using a computerized randomization procedure to one of four groups, 20/sex/group, and fed a commercial diet supplemented with 0, 500, 1000 or 2000 mg/kg bw/day Reb A.

Endpoints during the in-life phase were mortality, clinical findings, bodyweight changes, food consumption, and, in the final week of the study, ophthalmological findings, open-field motor activity and performance on a Functional Observational Battery (FOB). Blood for haematology and serum chemistry evaluations was collected from 10 rats/sex/group in Study Weeks 1 and 6. In Week 12, 10 rats/sex/group were subject to overnight urine collection in metabolism cages. The same rats were anaesthetized prior to necropsy and blood was collected from the vena cava for haematology, clinical chemistry and coagulation assays. All rats were subject to complete gross necropsy and collection of a comprehensive list of organs. Fresh organ weights were recorded for adrenals, brain, epididymides, heart, kidneys, liver, ovaries with oviducts, pituitary, prostate, spleen, testes, thymus and uterus, and fixed weights of thyroids with parathyroids were also recorded. Histopathological examination was performed on all tissues from control and high-dose rats, as well as any abnormal tissues from rats in other groups.

All rats survived to scheduled termination and there were no test article-related effects on clinical observations, food consumption, neurological performance as measured by motor activity and FOB, ophthalmological findings, haematology, clinical chemistry, coagulation parameters, gross necropsy findings, organ weights, organ weight ratios or histopathological findings. Group mean values for body weight gain and cumulative body weight gain were significantly lower in 2000 mg/kg bw/day males than in male controls. The group mean terminal body weight of the 2000 mg/kg bw/day males was 5.9% lower than that of control males. In females in the same group, significantly lower group mean bodyweights, relative to female controls, were found in some weeks but there was no effect on terminal group mean bodyweights.

Calculated group mean test article consumptions were 0, 516, 1026 and 2057 mg/kg bw/day in males and 0, 509, 1016 and 2021 mg/kg bw/day in females. The slightly lower bodyweight in the 2000 mg/kg bw/day males was not considered to be an adverse effect. A No Observed Adverse Effect Level of 2057 mg/kg bw/day in male and 2021 mg/kg bw/day in female Sprague-Dawley rats.

4.5.4 Chronic and carcinogenicity studies in animals

No chronic or carcinogenicity studies of steviol glycosides postdating Application A1132 were submitted or located by literature search. Such studies are not considered necessary for the hazard assessment of rebaudioside M, because there is no evidence that steviol glycosides are genotoxic and there is also no evidence of preneoplastic changes in short term studies in animals.

4.5.5 Developmental and reproductive studies in animals

No developmental or reproductive studies of steviol glycosides postdating Application A1132 were submitted or located by literature search.

4.5.6 Special studies in animals

Twenty-eight day dietary study of minor steviol glycosides in rats (Aranda-González et al. 2016) Regulatory status: Non-GLP

The purpose of this study was to investigate the effects on blood sugar of rebaudiosides B, C and D, dulcoside A and steviolbioside. The test subjects were male Wistar rats individually housed under standard controlled laboratory conditions, weighing between 200 and 2509 g at the start of the experiment, and provided with food and water *ad libitum*. Diabetes was induced in 35 fasted rats by intraperitoneal injection of 120 mg/kg bw nicotinamide, followed 15 minutes later by injection of 65 mg/kg bw streptozotocin. Rats were tested for hyperglycaemia two weeks later.

Rats were randomly assigned to groups within two cohorts; normoglycaemic rats and hyperglycaemic rats. The day prior to study start, all rats were given an acute intraperitoneal glucose tolerance test (IPGTT). Control normoglycaemic rats were given 1 g/kg glucose and distilled water. Treated normoglycaemic rats were given 1 g/kg glucose and 5 mg/kg glibenclamide, or 20 mg/kg bw Reb B, Reb C, Reb D, dulcoside A or steviolbioside. There were 6 rats/group in the normoglycaemic cohort, with the exception of glibenclamide group in which there were 12 rats. In the hyperglycaemic cohort, there were 4 rats/group and the treatment groups were the same, but with an additional group dosed with 180 mg/kg bw metformin. Blood samples were collected at 0, 15, 30, 60 and 120 min after IP injection. For the following 28 days, all rats were fed the same doses of the same treatments in incorporated into pulverized rodent pellets. Regular food was withdrawn each day and replaced with the treated food until it was consumed, when regular food was restored. Body weight was measured weekly. After 28 days, another IPGTT was performed on all rats, with only glucose administered to all groups.

In the acute IPGTT, none of the groups treated with steviol glycosides exhibited a significant effect on blood glucose when compared to the control group, whereas in the rats treated with glibenclamide, there was a significant decrease in the AUC_{net} for blood glucose. This response was expected because glibenclamide stimulates pancreatic beta cells to secrete insulin. Twenty-eight days of feeding with steviol glycosides or glibenclamide did alter the response to IPGGT. The hyperglycaemic rats treated with metformin, a drug which reduced hepatic glucose production, showed the expected decrease in AUC_{net} for blood glucose in the IPGGT. It was concluded that 20 mg/kg bw/day of the tested minor steviol glycosides did not have an antihyperglycaemic effect in normoglycaemic or induced-diabetic Wistar rats.

This study was not intended to be a toxicology study. However it is noted that there were no adverse effects of dietary exposure to 20 mg/kg bw/day of the tested steviol glycosides for 28 days in male Wistar rats.

Ten-day repeat-dose oral gavage study of stevioside in NMRI-Haan mice (Ilic et al 2017) Regulatory status: non-GLP

The purpose of this study was to evaluate the anti-diabetic effect of stevioside in mice. The test article was stevioside hydrate, 98% purity. The test system comprised male NMRI-Haan mice, weighing 23-29 g at study start. They were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water, but other husbandry conditions are not specified. Mice were assigned to eight groups of 6 mice/group. The first two groups were gavaged with saline or 20 mg/kg bw stevioside in saline for 10 days, followed by a glucose tolerance test (GTT) on Day 10. The second pair of groups were gavaged with saline or 20 mg/kg bw stevioside in saline for 10 days, followed by an adrenalin test on Day 10. The third pair of groups were gavaged with saline or 20 mg/kg bw stevioside in saline for 10 days, followed by intraperitoneal injection of 150 mg/kg bw alloxan, while the final pair of groups were treated with intraperitoneal alloxan before being gavaged with saline or 20 mg/kg bw stevioside in saline for 10 days. At the end of the experiment all mice were killed and the pancreas collected from each mouse. After fixation, sections of pancreas were stained for insulin and glucagon using immunohistochemistry.

Treatment with stevioside prevented a significant rise in blood glucose in the GTT but not in the adrenalin test. Pre-treatment with stevioside resulted in a significantly lower alloxan-induced hyperglycaemia, compared to control mice, and this was associated with less loss of β cells from the endocrine pancreas. Treatment with stevioside after the administration of alloxan did not have a significant effect on blood glucose on Day 10. Islet cell regeneration was observed in pancreata of mice killed 10 days after alloxan administration but there were no significant differences between control and stevioside-treated mice in volume density of

endocrine versus exocrine pancreas, or the number of α and β cells per islet. The authors concluded that stevioside may have some therapeutic application of stevioside in diabetes mellitus.

This study was not designed as a toxicology study, and only one dose of stevioside was used. The lack of adverse effects of 10 days of repeated dosing with 20 mg/kg bw stevioside is noted.

Study of Reb A in the drinking water of C57BL/6 mice (Reynolds et al. 2017). Regulatory status: non-GLP

Male C57BL/6 mice, six months old at study start, were used to investigate the effects of Reb A in drinking water on circadian rhythms, insulin action and susceptibility to obesity. The source and purity of the test article are not specified. The housing and husbandry are not fully described, other than that the mice were kept in 'standard mouse cages', fed a standard rodent diet *ad libitum* unless noted otherwise, and acclimatised for 5 months before study start. Mice, 10/group, were randomly assigned to either a control group supplied with plain drinking water, or a treatment group supplied with drinking water containing 0.1% Reb A. At the same time, all mice were provided with running wheels. After seven days, wheel-running activity was monitored for 14 days (Study Days 8-22) during the 12 hour light/dark cycle. They were then maintained for a further ten days (Study Days 23-32) in constant darkness. At the end of this period they were returned to standard cages and maintained for a further 3 months on the same plain or supplemented water as they had been supplied during the wheel-running phase. At the end of the three-month period, the mice were given an intraperitoneal glucose tolerance test (IPGGT). After overnight fasting, 1.0 g/kg bw glucose was administered by intraperitoneal (IP) injection and blood glucose was measured in blood from the tail vein at 0, 15, 30, 45, 60 and 90 min. Mice were allowed to recover from the IPGGT for 7 to 10 days before pyruvate tolerance testing. Mice were administered 1.0 g/kg bw pyruvate by IP injection and blood glucose was measured in tail vein blood at 0, 15, 30, 45, 60 and 90 min. The mice were given a further 7 to 10 days to recover before insulin tolerance testing. Mice were administered 0.5 U/kg bw insulin and blood glucose was measured in tail vein blood at 0, 15, 30, 45, 60 and 90 min. After assessment of in vivo insulin action, mice were placed on a high fat diet for eight weeks. Caloric intake was measured by measuring food intake over seven days, and water intake was also measured over seven days. Bodyweights of the mice were recorded prior to the introduction of the high fat diet and at 2, 4 and 8 weeks after introduction of the high fat diet. At the end of the study, mice were killed by IP injection of sodium pentobarbital. Overall, the total time of treatment appears to have been six months.

Administration of 0.1% Reb A in drinking water resulted in a mean intake of 5.9 mg/day. Consumption of Reb A in water had no effect on circadian wheel running activity, bodyweight changes during the wheel-running phase, or group mean blood glucose results on glucose tolerance, pyruvate tolerance or insulin tolerance tests. Reb A did not alter caloric intake or the susceptibility to obesity when the mice were fed a high fat diet. It was concluded from this study that Reb A does not promote obesity or alter the action of insulin, and that it does not disrupt circadian rhythms.

This study is of limited value to safety assessment because the dosage in mg/kg bw/day cannot be calculated, because there is a lack of information about the purity of the test article or the body weights of the mice. Furthermore the mice were not subject to necropsy or to detailed clinical pathology prior to termination. However, the safety of chronic intake of Reb A in mice is noted.

Six-week repeat-dose oral gavage study of stevioside in male Wistar rats (Noosud et al. 2017) Regulatory status: non-GLP

This study was conducted to determine whether subchronic repeat dosing with stevioside had any effect on selected pro-inflammatory markers. The test article had a purity of 96-98%. The test subjects were ale Wistar rats, weighing 170-220 at the start of acclimatization. Rats were individually housed under standard controlled laboratory conditions, fed a commercial rat chow and supplied with water *ad libitum*. After one week of acclimatization, rats were assigned to three groups comprising 6 rats/group, and gavaged daily for six weeks with 0, 500 or 1000 mg/kg bw/day of stevioside in water. At the end of the experiment 4 mL blood was collected from each rat. The location of the blood collection and the disposal of the rats is not specified, but the volume removed implies that the blood collection was terminal, and most likely from the abdominal vena cava or aorta. Plasma was separated from 1 mL of the blood and peripheral blood mononuclear cells were separated from 3 mL of the blood. Mononuclear cells were assessed for viability using Trypan Blue exclusion assay, and incubated for 24 h at 37°C, with and without lipopolysaccharide (LPS). At the end of incubation, the supernatant was collected. Determination of Tumor Necrosis Factor α (TNF-α) and interleukin 1β (IL-1β) by commercial ELISA kits was performed on both the plasma and the supernatant collected after incubation of the mononuclear cells.

Six weeks of treatment with up to 1000 mg stevioside/kg bw/day had no effect on viability of peripheral blood mononuclear cells. TNF-α and IL-1β were not detected in plasma collected when rats were killed, irrespective of dose group. Supernatant collected from mononuclear cells incubated with LPS showed a dose-related decrease in TNF-α, with group mean levels of 248.6 (±21.4), 186.8 (±18.6) and 151.4 (±15.4) pg/mL from cells of rats in 0, 500 and 1000 mg/kg bw/day groups respectively. A similar dose-related decrease in IL-1β was observed in supernatant from mononuclear cells incubated with LPS, with group mean levels of 294.4 (±16.1), 220.0 (±12.1) and 158.1 (±22.6) pg/mL from cells of 0, 500 and 1000 mg/kg bw/day groups respectively.

This study was not designed as a toxicology study and detailed measurements of endpoints such as clinical pathology parameters and pathology findings were not made. The apparent lack of significant effects on survival, clinical findings or gross findings at termination after 10 days' exposure to 1000 mg/kg bw/day are noted.

4.5.7 Human tolerance studies

Two-week study of rebaudioside A and erythritol in subjects with glucose intolerance (Shin et al. 2016) Regulatory status: non-GLP

Subjects for this study were patients between 18 and 74 years who had a fasting plasma glucose of 100 to 125 mg/dL) Patients with overt diabetes were excluded, as were patients who had taken oral hypoglycaemic agents in the previous three months. Pregnancy and an extensive list of diseases were also grounds for exclusion. Twenty-five subjects, comprising seven men and 18 women, were enrolled in the study, and stopped using any sweetening agents similar to rebaudioside A or erthritol at least one week before the study commenced. The test articles were dispensed in packs, each containing 16 mg Reb A and 986 mg erythritol. Subjects consumed two packs/day by dissolving each pack in water and consuming one pack after lunch and the other after dinner. Subjects were instructed to maintain their normal diet during the study, and dietary stability was confirmed by diet records at baseline and at the end of the study. At the start and end of the study, body weight, serum fructosamine, fasting and 2-hour plasma glucose, serum insulin and serum hexokinase were determined for each subject. Insulin resistance and pancreatic β cell function were calculated.

Consumption of 32 mg/d Reb A and 1972 mg erythritol/d had no effect on group mean values for serum fructosamine, fasting and 2-hour plasma glucose, serum insulin, serum hexokinase, calculated insulin resistance or calculated pancreatic β cell function. There were

also no significant changes in blood pressure, body weight or fasting total cholesterol. Two subjects experienced abdominal discomfort that resolved spontaneously, and one of these subjects stopped taking the sweetener. It was concluded that Reb A and erythritol did not alter glucose homeostasis in people with glucose intolerance.

4.5.8 Potential for allergenicity

The few reports of allergic reactions to steviol glycosides all precede 2008, when highly purified steviol glycosides were introduced to the market. A comprehensive literature search did not support the conclusion that highly purified steviol glycosides are likely to be allergenic (Urban et al. 2015).

Potential allergenicity of the enzymes was investigated using the AllergenOnline Database version 18B, as updated March 23, 2018, maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Sequence homology searches using full-length FASTA alignment were conducted for UGT-A, UGT-B1 and UGT-B2. Two homologies were found for UGT-A. One was with an allergen precursor of the German cockroach (*Blattella germanica*), and the other was to pollen of timothy grass (*Phleum pratense*). However the E-values were 0.76 and 0.81 respectively, and E-values larger than 1×10^{-7} are considered unlikely to represent proteins that may share immunologic or allergic cross-reactivity (Hileman *et al*., 2002). Shared identities to the allergens were only 23.1% and 22.6% respectively. Eleven homologies were found for UGT-B1, of which ten were tropomysins and one was an allergen precursor of the German cockroach (*Blattella germanica*), for which the E value was 1.0. The tropomyosins were those of various members of the phylum Mollusca, and E values ranged from 0.028 to 0.72. Only one sequence homology was found for UGT-B2, to an allergen precursor of the German cockroach (*Blattella germanica*), and the E value was 0.96. On the basis of these results, cross-reactivity to known allergens is unlikely for UGT-A, UGT-B1 or UGT-B2.

4.5.9 Other studies

Sixty-day study of Stevia leaf powder in subjects with type 2 diabetes mellitus (Ritu and Nandini 2014) Regulatory status: non-GLP

Deficiencies in the reporting of this study mean that the methods and results are difficult to interpret. The test article was a commercial preparation of *Stevia* leaf powder. Only a proximate analysis (i.e. moisture, protein, fat, fibre, carbohydrate etc.) of the test article is provided. Subjects were eight men and 12 women with type 2 diabetes mellitus. Although the subjects were screened for psychosocial and socioeconomic information, medical history, anthropometric parameters, and plasma levels of glucose, triglycerides, total cholesterol, LDL cholesterol, VLDL cholesterol and HDL cholesterol, results to indicate whether the two study groups were approximately matched in these parameters are not presented. Prior to the start of treatment, diet was recorded by recall for three consecutive days, and an oral questionnaire was also administered. Subjects were divided into two groups, each group comprising four men and six women. The first group served as the control group while the second group was given 1 g Stevia leaf powder, presumably daily, although there is a lack of information on compliance or how it was measured. Blood was collected prior to intervention and on days 30 and 60 for determination of fasting blood glucose, post-prandial blood glucose, total cholesterol, triglyceride, HDL-C, LDL-C, VLDL-C, LDL/HDL ratio and atherogenic index.

Energy intake was similar between the two groups. The subjects in the treatment group had a higher carbohydrate intake and higher protein intake than subjects in the control group. Men in both groups had a fat intake well in excess of the recommended daily allowance, and all subjects had low fibre intake.

Group mean fasting and postprandial blood glucose levels in treated subjects were lower than those of control subjects after 30 days of treatment, but the differences did not reach statistical significance. However after 60 days, both group mean values were significantly lower than those of the control group. In addition, significantly lower group mean serum triglyceride, compared to that of controls, was found in treated subjects on Days 30 and 60. It was concluded that Stevia can safely be used as a herb in subjects with diabetes and may help to prevent cardiovascular diseases in such patients.

The lack of critical details in this study, including but not limited to the stevioside content of the extract, and the compliance with treatment, mean that this study is of limited value to this hazard assessment.

4.6 Assessments by other Regulatory Agencies

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

JECFA reviewed the safety of steviol glycosides at their 51st, 63rd, 68th, 69th and 82nd meetings. A temporary ADI of 0 to 2 mg/kg bw was established at the 63rd meeting, which was subsequently replaced with a full ADI of 0 to 4 mg/kg bw, expressed as steviol, for steviol glycosides at the 69th meeting. A re-evaluation of steviol glycosides at the 82nd meeting, in 2016, confirmed this ADI (FAO/WHO 2016a). The 82nd meeting included review of a manufacturing process for Reb A using a genetically modified strain of a yeast, *Yarrowia lipolytica*. In 2016 JECFA issued 'tentative' specifications for steviol glycosides from *Stevia rebaudiana* Bertoni (FAO/WHO 2016b) that define steviol glycosides as "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 and deoxyglucose". The purity of steviol glycosides must be no less than 95% on a dry weight basis.

4.6.2 Health Canada

Health Canada expanded their definition of steviol glycosides to include Reb M in 2016, in recognition of the common metabolic fate of steviol glycosides; that is, hydrolysis to steviol, conjugation by glucuronidation and urinary excretion (Health Canada 2016). More recently Health Canada further expanded their definition of steviol glycosides to include all steviol glycosides found in *Stevia rebaudiana* Bertoni (Health Canada 2017).

4.6.3 United States Food and Drug Administration (FDA)

Since the beginning of 2016, 13 GRAS notices for steviol glycosides have been submitted to the US FDA. These notices include a GRAS notice for the same product that is the subject of the current application. The FDA has responded with no questions concerning the GRAS status of 11 of these products, and two responses are currently pending.

4.7 Hazard assessment discussion and conclusion

Recent assessments by FSANZ and JECFA have confirmed that all steviol glycosides undergo the same metabolic pathway to steviol, which is then glucuronidated and excreted in the urine. A single ADI, expressed as steviol, is therefore appropriate to all steviol glycosides.

A small number of genotoxicity studies, studies in laboratory rodents and studies in human volunteers of steviol glycosides have been published since the last assessment by FSANZ, but there is no new information that gives reason to change the existing ADI of 0-4 mg/kg bw/day steviol.

The Reb M that is the subject of this application is chemically the same as Reb M extracted

directly from *Stevia rebaudiana* Bertoni and would therefore follow the same metabolic pathway. This Reb M complies with purity specifications of JECFA (FAO/WHO 2016b) and no evidence of contamination with pesticide residues has been found. No major allergens are used to culture the yeast or at any other stage of the production process.

There is no evidence that purified steviol glycosides are allergens. Sufficient information has been provided concerning potential homology between the novel enzymes and known allergens. There is no evidence of homology sufficient to give rise to concern.

In conclusion, the existing ADI of 0-4 mg/kg bw/day as steviol is appropriate for the Reb M produced using enzymes from genetically modified *Pichia pastoris* as described in this application.

5.0 References

Ahmad M, Hirz M, Pichler H and Schwab H (2014). Protein expression in *Pichia pastoris*: recent achievements and perspective for heterologous protein production. Applied Microbiology and Biotechnology 98: 5301-5317

Aranda-González I, Moguel-Ordóñez Y, Chel-Geurrero L, Segura-Campos M and Betancur-Ancona D (2016). Evaluation of the antihyperglycemic effect of minor steviol glycosides in normoglycaemic and induced-diabetic Wistar rats. Journal of Medicinal Food 19(9): 844-852

EFSA BIOHAZ Panel, Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Fernandez Escamez PS, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Cocconcelli PS, Peixe L, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Correia S and Herman L (2017). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 6: suitability of taxonomic units notified to EFSA until March 2017. EFSA Journal 2017;15(7):4884 Accessed 13 Jun 2018

EU (2012). Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. Off J Eur Union 55(L83):1-295. Accessed 20 April 2018

FAO/WHO (2016a) Evaluation of certain food additives. Eighty-second report of the Joint FAO/WHO Expert Committee on Food Additives. Accessed 18 June 2018

FAO/WHO (2016b) Compendium of food additive specifications. Joint FAO/WHO Expert Committee on Food Additives. 82nd meeting 2016. Accessed 18 June 2018

Health Canada (2016) Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Reb M as a Sweetener in Various Unstandardized Foods - Reference Number: NOM/ADM-0065. Accessed 18 June 2018

Health Canada (2017) Notice of Modification to the List of Permitted Sweeteners to Enable the Use of Steviol Glycosides from Stevia rebaudiana Bertoni as a Sweetener. Accessed 5 July 2018

Hileman RE, Silvanovich A, Goodman RE, Rice EA, Holleschak G, Astwood JD and Hefle SL (2002). Bioinformative methods for allergenicity assessment using a comprehensive allergen database. International Archives of Allergy and Immunology 128: 280-291

Humphrey TV, Richman AS, Menassa R, Brandle JE (2006). Spatial organisation of four enzymes from *Stevia rebaudiana* that are Involved in steviol glycoside synthesis. Plant Molecular Biology 61(1/2):47–62. DOI:10.1007/s11103-005-5966-9. Accessed 20 April 2018

Ilić V, Vukmirović S, Stilinović N, Čapo I and Arsenović M (2017). Insight into anti-diabetic effect of low dose of stevioside. Biomedicine and Pharmacotherapy 90: 216-221

JECFA (2007). 3.1.9 Steviol glycosides. *Evaluation of Certain Food Additives and Contaminants*. Sixty eighth Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), June 19-28, 2007, Geneva, Switz. (WHO Technical Report Series, no 947). Geneva, Switz.: World Health Organization (WHO), pp. 50-54, 206, 215. Accessed 18 April 2018

JECFA (2016). Steviol glycosides from Stevia *rebaudiana Bertoni* (tentative) [New specifications prepared at the 82nd JECFA, 2016), Superseding specifications prepared at the 73rd JECFA (2010)]. In: *Compendium of Food Additive Specifications*. 82nd Meeting, June 7-16, Geneva, Switz. (FAO JECFA Monographs 19). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), pp. 106-111. Accessed 20 April 2018

Jiang XP, Yin ML, Chen P and Yang Q (2012) Constitutive expression, purification and characterization of bovine prochymosin in *Pichia pastoris* GS115. World Journal of Microbiology and Biotechnology 28(5): 2087–2093

Mao G, Luo Y, Lin Y, Yu X, inventors; Bedford (MA): Conagen Inc., applicant (2016a). *Non-Caloric Sweeteners and Methods for Synthesizing*. US Patent Publication US 2016/0097070 A1 [Apr. 7, 2016]. Accessed 20 April 2018

Mao G, Luo Y, Lin Y, Yu X, inventors; Bedford (MA): Conagen Inc., applicant (2016b). *Non-caloric Sweeteners and Methods for Synthesizing*. US Patent Publication US 2016/0097071 A1 [Apr. 7, 2016]. Accessed 20 April 2018

Noosud J, Lailerd N, Kayan A and Boonkaewwan C (2017). *In vitro* and *in vivo* assessment of inhibitory effect of stevioside on pro-inflammatory cytokines. Avicenna Journal of Phytomedicine 7(2): 101-106

Reynolds IV TH, Soriano RA, Obadi OA, Murkland S, Possidente B (2017). Long term rebaudioside A treatment does not alter circadian activity rhythms, adiposity, or insulin action in male mice. PLOS One https://doi.org/10.1371/journal.pone.0177138

Richman A, Swanson A, Humphrey T, Chapman R, McGarvey B, Pocs R, et al. (2005). Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of *Stevia rebaudiana*. *the* Plant Journal 41(1):56-67. DOI:10.1111/j.1365-313X.2004.02275.x. Accessed 20 April 2018

Ritu M and Nandini J (2016) Nutritional composition of Stevia rebaudiana, a sweet herb, and its hypoglycaemic and hypolipidaemic effect on patients with non-insulin dependent diabetes mellitus. Journal of the Science of Food and Agriculture 96: 4231-4234

Rumelhard M, Hosako H, Eurlings IMJ, Westerink WMA, Staska LM, van de Wiel JAG and La Marta J (2016). Safety evaluation of rebaudioside A produced by fermentation. Food and Chemical Toxicology 89: 71-84

Sharif R, Chan KM, Ooi TC and Mohammad NF (2017) Cytotoxicity and genotoxicity evaluation of stevioside on CCD18Co and HCT 116 cell lines. International Food Research Journal 24(1): 341-345

Shin DH, Lee JH, Kang MS, Kim TH, Jeong SJ, Kim CH, Kim SS and Kim IJ (2015). Glycemic effects of Rebaudioside A and Erythritol in People with Glucose Intolerance. Diabetes and Metabolism Journal 40: 283-289

Sørensen HP (2010). Towards universal systems for recombinant gene expression. Microbial Cell Factories 9:27 Accessed 14 Jun 2018

Tice R, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J and Sasaki Y (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environmental and Molecular Mutagenesis 35(3): 206-221

Uçar A, Yilmaz S, Yilmaz S and Kiliç MS (2018). A research on the genotoxicity of stevia in human lymphocytes. Drug and Chemical Toxicology 41(2): 221-224

Urban JD, Carakostas MC and Taylor SL (2015). Steviol glycoside safety: Are highly purified steviol glycoside sweeteners food allergens? Food and Chemical Toxicology 75:71-78